IN VITRO REGENERATION OF PAPAYA (*Carica papaya*)

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IN VITRO REGENERATION OF PAPAYA (*Carica papaya*)

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CERTIFICATE

*This is to certify that the thesis entitled "**IN VITRO REGENERATION OF PAPAYA (Carica papaya)**" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY,** embodies the result of a piece of bonafide research work carried out by **NAHIDA HASAN, Registration No. 11-04355** under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.*

I further certify that any help or sources of information as has been availed of during the course of this work has been duly acknowledged & style of the thesis have been approved and recommended for submission.

Dated : JUNE, 2018

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Dedicated To

My

Beloved Parents

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IN VITRO REGENERATION OF PAPAYA (*Carica papaya*)

ABSTRACT

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of September 2017 to June 2018 to study on *in vitro* regeneration of Papaya (*Carica papaya*). Shoot tip of young shoot was used as the main suitable explant, which was sterilized using freshly prepared 0.1% $HgCl_2$ mixing with few drops Tween-20. Explants were inoculated in MS media treated with both singly or in combination of the Benzyladenine (BA), Kinetin (KIN), Indole-3-butyric acid (IBA), Naphthalene acetic acid (NAA). The minimum days to shoot induction were achieved on MS medium containing 0.5 mg L^{-1} BA. The highest shoots, leaves, length of shoot observed in treatment 1.0 mg L^{-1} BA. The combine treatment 1.0 mg L^{-1} BAP+0.75 mg L^{-1} KIN gave the highest number of shoots and number of leaf plantlet. The minimum days to root induction was reported in media treated with 2.0 mg L^{-1} IBA along with maximum roots per plantlet. The highest length of root was observed in 2.0 mg L^{-1} IBA. The minimum days to root induction were noticed on same culture media supplemented with 2.00 mg L^{-1} NAA. In regenerated plantlets, 80% survival during growth chamber conditions and 75% during shade house stage and 75% in open atmosphere were achieved. *In vitro* regeneration was found to be very effective and promising method in the proliferation of papaya and this experiment can be a useful tool for proliferation of papaya.

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AEZ	=	Agro-Ecological Zone
BARI	=	Bangladesh Agricultural Research Institute
BBS	=	Bangladesh Bureau of Statistics
LAI	=	Leaf area index
ppm	=	Parts per million
et al.	=	And others
N	=	Nitrogen
TSP	=	Triple Super Phosphate
MP	=	Muriate of Potash
RCBD	=	Randomized complete block design
DAS	=	Days after sowing
ha^{-1}	=	Per hectare
G	=	gram (s)
Kg	=	Kilogram
µg	=	Micro gram
SAU	=	Sher-e-Bangla Agricultural University
SRDI	=	Soil Resources and Development Institute
HI	=	Harvest Index
No.	=	Number
Wt.	=	Weight
LSD	=	Least Significant Difference
^0C	=	Degree Celsius
mm	=	millimeter
Max	=	Maximum
Min	=	Minimum
%	=	Percent
cv.	=	Cultivar
NPK	=	Nitrogen, Phosphorus and Potassium
CV%	=	Percentage of coefficient of variance
Hr	=	Hour
T	=	Ton
viz.	=	Videlicet (namely)

CHAPTER I
INTRODUCTION

Papaya, (*Carica papaya* L.) is an important tropical fruit crop. Ripe fruits are consumed fresh while unripened fruits can be used in salads or as a vegetable and as a source of papain. Papaya fruits are a rich source of vitamins and minerals, low in sodium, fat and calories, and lack starch (Hewajulige and Dhekney, 2016). Papain, a proteolytic enzyme obtained from immature fruits is used in the pharmaceuticals, leather, wool and rayon industries (Seelig, 1970). Papaya is grown on 0.43 million ha with an annual production of 11 million tons. A majority of the production occurs in Asia and the Americas (FAOSTAT, 2013).

Papaya is a member of the Caricaceae family, which consists of herbaceous plants (Badillo, 1993). The crop is believed to have originated in Central America in regions ranging from Mexico to Panama. The Caricaceae was originally comprised of 31 species in the Carica, Jacartia and Jarilla genera from Central America and the African Cylicomorpha genus (Nakasone and Paull, 1998). Taxonomic revisions resulted in some species being transferred from Carica to the Vasconcella genus (Badillo *et al*., 2000). Consequently, the Vasconcella genus now consists of 21 species followed by Jacartia with 7 species, while papaya is now the sole species in the Carica genus (Badillo, 1993).

Major goals of papaya improvement programs include increased yield and productivity, resistance to biotic and abiotic stress and improved quality characteristics. Papaya cultivars with improved traits such as high yield and quality have been successfully developed through intensive breeding programs worldwide (Chan, 2002; Nakasone and Paull, 1998). Minisatellite and microsatellite markers have been explored to accelerate papaya genetic improvement, analyze phenotypic variation for traits of interest, and understand genetic relationships for efficient management and conservation of genetic resources. Papaya improvement for stress tolerance (abiotic and biotic) via hybridization with species from other genera of the Caricaceae family has been marginally successful. Limitations for transfer of useful

traits are attributed to several post-zygotic incongruities including embryo abortion, poor seed viability and sterility in progeny obtained following hybridization between two genera (Horovitz and Jimenez, 1967; Manshardt and Wenslaff, 1989). The limitations encountered in papaya improvement via conventional breeding can be overcome by biotechnological approaches such as tissue culture, embryo rescue and genetic engineering. Genetic engineering of papaya allows incorporation of specific traits into elite cultivars without potentially altering the existing phenotype.

In papaya, micropropagation another biotechnological tool has been attempted for a number of cultivar to solve some papaya production constraints such as producing plants of known gender (George, 1996, Chan Teo, 2002, Fitch 2005). A number of advantages reported in micropropagation of papaya that includes reduced time to produce new varieties, ease of maintaining genetic uniformity and production of plants of the same gender (Hansen, 2005).

Tissue culture techniques have four decades played a great role in the *in vitro* regeneration of horticultural and ornamental plants. In fact, the first ever successful plant tissue culture was achieved in horticultural plants (Altman and Ziv, 1997). These techniques have been widely used in disease elimination and vegetative propagation (Husseyg, 1979). Nowadays, it is very common practice all over the world to explore different aspects about papaya using this technology.

Sexual propagation is the commercial method of propagation for papaya. Being heterozygous and a cross pollinated crop, sexual propagation has resulted in immense variation among populations for yield, size, shape, quality of fruit and disease susceptibility leading to production of non-true-to-type plants (Panjaitan *et al.*, 2007). The significance of vegetative propagation in the maintenance of genetic uniformity and preservation of identity of elite clone or cultivar is well recognized in horticultural crops. To develop true-to-type plants, an effective propagation technique is required. Conventional vegetative propagation methods in papaya such as grafting (Allan *et al*., 2010) and rooted cuttings (Rajan and Markose, 2007) exist, but they are often not carried out on a large scale. Hence, the alternative method is *in vitro* regeneration techniques for mass multiplication of elite materials.

Development of an efficient *in vitro* regeneration system would be a remarkable progress for mass propagation and uniform plants for both commercial and research purposes.

Papaya is most commonly propagated *in vitro* by shoot tip or axillary bud culture (Teixeira da Silva *et al*., 2007). As such several protocols for *in vitro* plantlet regeneration from shoot tips of papaya have been developed in other regions of the world (Kabir *et al*., 2007; Panjaitan *et al*., 2007). Very limited work has been done in our country regarding tissue culture in papaya.

Keeping in view of above facts, **"***in vitro* regeneration of papaya (*Carica papaya*)**"**was conducted during 2017-18 to fulfill the following objectives:

1. To study the role of phytohormone on *in vitro* regeneration in papaya.
2. Identification of appropriate hormonal dose for *in vitro* regeneration of papaya.
3. To obtain true-to-true type *in vitro* plantlet papaya.
4. Establishment of *in vitro* regeneration protocol of Papaya

CHAPTER II
REVIEW OF LITERATURE

Plant tissue culture forms the backbone of plant biotechnology, which is comprised of micropropagation, induction of somaclones, somatic hybridization, cryopreservation and regeneration of transgenic plants. Plant tissue culture is a technique through which any plant part is cultured on a sterile nutrient medium in controlled light and temperature with the purpose of obtaining growth. The idea of plant tissue culture originated from the cell theory that was formulated by Schwann in 1839. But unfortunately, it is very limited in Bangladesh. However, some .related works already performed by different institutes home and abroad have been reviewed and some of the most relevant literatures are cited below

2.1. Embryogenic cell culture system

The papaya embryogenic culture system involves the production of somatic embryos from a wide array of explant material. Embryogenic cultures are produced on induction medium via an indirect embryogenic pathway that involves a callus phase or a direct pathway where embryos are produced without an intervening callus phase. Somatic embryo development and maturation is observed when cultures are transferred to medium devoid of growth regulators. Embryogenic cultures are used as target tissues for inserting desired genes of interest and developing cultivars with improved traits (Fitch, 2005).

Papaya embryogenic cultures are obtained from hypocotyl, axillary bud, stem, ovule, zygotic embryo and root explants (Anandan *et al*., 2012; Abreu *et al*., 2014; Razali and Drew, 2014). In most cases, cultures develop via indirect embryogenesis. Highly reproducible protocols for obtaining embryogenic cultures were reported by Fitch and Manshardt (1990) and Fitch (1993). Hypocotyl explants obtained from germinated seedlings produce embryogenic cultures on induction medium containing ½ strength MS salts and vitamins with 9.0 mM 2,4-D, 400 mg L^{-1} glutamine, 60 g L^{-1} sucrose and 7 g L^{-1} TC agar.

Repeated transfer of embryogenic cultures on induction medium results in the development and proliferation of proembryonic masses or PEMs. Embryogenic cultures can be maintained by repeated transfer of PEMs to induction medium. Alternatively, immature zygotic embryos excised from fruits obtained 90–113 days after pollination also produce embryogenic cultures on induction medium. Cultures appear 4 weeks after induction and the production of somatic embryos (SE) occurs exclusively from the embryo axis. Such cultures proliferate on induction medium and can be maintained up to 12 weeks. Somatic embryogenesis appears to be genotype-dependent with some genotypes exhibiting greater production of somatic embryos (Fitch, 1993). Embryogenic cultures have also been induced from ovule explants cultured on White's medium modified with the addition of 60 g L^{-1} sucrose, 400 mg L^{-1} glutamine, and 20% (v/v) filter-sterilized coconut milk (Litz and Conover, 1982). Other studies have indicated successful production of embryogenic cultures using 2,4-D in the induction medium (Bukhori *et al*., 2013).

Papaya embryogenic cultures can be maintained by transfer to semi-solid or liquid induction medium at 3 weeks intervals (Fitch, 1993; Litz and Conover, 1983; Mahon *et al*., 1996). Embryogenic cultures in liquid induction medium proliferate by repetitive budding of PEMs. Cultures growing in liquid medium can be synchronized by sieving to retain the smallest fraction of PEM, which is subsequently transferred to fresh medium for further proliferation. Such PEM effectively produce somatic embryos when transferred to growth regulator-free medium. The proliferation rate of PEMs is higher in suspension cultures after culture synchrony is attained (Von Arnold *et al*., 2002). Suspension cultures exhibit greater proliferation potential and higher plant regeneration rates compared to solid medium grown cultures and may be better suited as targets for gene insertion and the production of transgenic plants (Castillo *et al*., 1998; Ying. *et al*., 1999; Lines *et al*., 2002; Carlos-Hilario and Christopher, 2015).

Somatic embryo maturation occurs on induction medium devoid of growth regulators (Fitch, 1993; Litz and Conover, 1983). Development follows the classical globular, heart, torpedo and cotyledonary (early to late) stages. A synchronous development is frequently observed and can be attributed to differing access to nutrients following subculture to fresh medium (Conger *et al*., 1989). Abnormal embryo development, including embryos with fused cotyledons, multiple cotyledons, misshaped and fused embryos are frequently observed. Such abnormalities occur when cell divisions in meristematic areas occur prior to differentiation of the shoot apex (Litz and Gray, 1992). The presence of plant growth regulators in the culture medium often contributes to abnormal embryo development.

2.2. *In vitro* regeneration and micropropagation of papaya

Carica papaya L. is considered one of the most important fruit, especially in tropical and subtropical regions. It generates flowers continuously throughout the year and has been conventionally cultivated by seeds. This review summarizes the morphological and evolutionary descriptions of *Carica papaya* L., the protocols used to achieve *in vitro* micropropagation of *Carica papaya* using different plant tissue culture techniques, as well as the problems faced by papaya micropropagation. While there are many problems encountered during *in vitro* propagation of papaya through somatic embryogenesis, this review focuses specifically on contamination, rooting processes and acclimatization. Solving these problems will enable a reproducible protocol to be established.

Raising cultures of proliferating shoots of female and male plants of *Carica papaya* through shoot apices taken from mature plants was difficult because of high incidence of endogenous bacterial contamination. When they were easily raised through culture of young inflorescences tips of female and male plants. There was no difference in the requirement of nutrients and growth regulators for proliferation of shoots raised from shoot tips or inflorescence tips, but at the initial stage, the different explants differed in their requirement of growth

regulators for induction of shoot bud differentiation. BM1, a modification of MS medium was used as the basal medium in all the cases. There was a pronounced callusing tendency in tender shoots initially used for rooting, for which well-developed shoots (< 4 cm long) were found suitable. Rooting was achieved in developed shoots through a 4-step procedure: Step 1-An initial pulse treatment with a high concentration of IBA (10 mg L^{-1}) using BM_3 medium, a modified Knop medium with trace elements and disodium-ferric-ethylene-diaminetetraacetate (Na-Fe-EDTA) of MS medium and 2% sucrose for 24 h; Step 2-Their subculture in medium BM_2 differing from BM_1 in having 50 mg L^{-1} m-inositol and supplemented with IAA (0.25 mg L^{-1}) along with AdS (15 mg L^{-1}) and 2% sucrose for 7 days; Step 3- Roots at the cut end of about 95% shoots were visible, after about 10 days, in the same medium as used in Step 1, but having supplements of 3 vitamins, 2 amino acids and 0.25 mg L^{-1} IAA, while sucrose was removed; and Step 4- The just rooted shoots when finally transferred to the semi-sterilized moist Soilrite contained in culture tubes, formed healthy roots without intervening callusing, while the shoots also remained healthy. Such plantlets when ultimately transplanted along with Soilrite plugs to the potted soil showed about 80% transplant success. *In vitro*-raised plants appeared normal and fruited under field conditions after about 6 months of ex vitro growth (Agnihotri, *et al.* 2004)

Bindu (2015) carried out an experiment by enhanced release of axillary buds in Papaya variety CO-5. Apical buds and lateral buds from seedlings and mature plants were used as explant for *in vitro* propagation. Explants from papaya varieties and hybrids were subjected to different treatments of plant growth substances for culture establishment and shoot proliferation. The present study revealed that full strength MS medium supplemented with sucrose 30.00 g L^{-1} and agar 6.50 g L^{-1} under light condition produced highest shoot number and longest shoot in papaya variety CO-5. Application of BA 0.50 mg L^{-1} along with NAA 0.10 mg L^{-1} was found to be better for initial culture establishment and proliferation of papaya variety CO-5. *In vitro* rooting was best in full strength MS medium supplemented with IBA 3.00 mg L^{-1}, sucrose 30.00 g L^{-1} and activated

charcoal 0.05 percent.

Conventional vegetative propagation methods are not carried out; hence, there is need for micropropagation for mass multiplication of selected lines. An assessment for the effect of 6-benzylaminopurine (BAP) at 0.1, 0.5, 1.0 and 2.0 mg L^{-1} combined with α-naphthalene acetic acid (NAA) at 0.05, 0.1, 0.5 and 1.0 mg L^{-1} and a control on shoot multiplication and elongations, and indole-3-butyric acid (IBA) at 0, 0.1, 0.5, 1.0, 2.0, 2.5 and 3.0 mg L^{-1} on root induction were evaluated. Number of shoots and their length were recorded every three weeks for 12 weeks. Number of roots, root length and percentage rooting induction were recorded after eight weeks. The highest number of shoots was recorded in 0.5 mg L^{-1} BAP combined with 0.1 mg L^{-1} NAA and the longest shoots were recorded in 0.1 mg L^{-1} BAP combined with 0.05 mg L^{-1} NAA across the three lines. IBA at 2.5 mg L^{-1} produced the highest number of roots, root length and highest percentage of rooting induction. An *in vitro* regeneration of selected papaya lines through shoot tip culture was established (Mumo *et al.* 2013).

The technique of *in vitro* culture has made clonal propagation possibile in papaya. While standardizing the method of micropropagation of papaya, the factors influencing *in vitro* establishment and growth of papaya were examined namely by sources of explants, surface sterilants, pH of the medium, sucrose and adenine sulphate concentration in the medium. In multiplication study, the maximum shoot multiplication was observed in alternate sub culturing in basal medium and MS medium + 0.5 mg L^{-1} BAP + 0.1 mg L^{-1} NAA and gave highest number of shoots per explants. Sucrose 30 g L^{-1} in medium was found to be more favourable for maximum number of shoot and length of longest shoots. Out of various pH level tested, pH 5.7 recorded maximum numbers of shoots (3) and maximum length of longest shoots (2.75 cm). In proliferation medium, maximum length of shoot, numbers of shoots and growth rate was observed in MS medium fortified with 160 mg L^{-1} adenine sulphate. *In vitro* rooting occurred on shoot regeneration medium; however, it was a slow process. Rooting treatment consisting of half MS medium supplemented with 1.0 mg L^{-1}

IBA was found to be the best for early induction of roots (28 days), maximum number of roots/shoot (5.00) and length of root (6.00 cm) also. Among all potting mixtures tested, the soil: sand: FYM (1/1/1: V/V/V) was found to be suitable for hardening *in vitro* raised papaya plantlets (Patel *et al*. 2013).

A revised protocol has been developed by Patil *et al.* (2007) for *in vitro* propogation of papaya using explants from field-grown trees. Successful establishment of papaya *in vitro* using lateral buds could be obtained by treating the buds with Carbendazim (0.2%) and Streptomycin (0.1%) for 24 h, followed by surface sterilization with mercuric chloride (0.1%) for 3 minutes and culturing on MS medium supplemented with BAP (0.3 mg L^{-1}) and NAA (0.1 mg L^{-1}). Established buds were proliferated on modified MS medium supplemented with BAP (0.3 mg L^{-1}) and NAA (0.1 mg L^{-1}). Modified MS medium supplemented with BAP (0.3 mg L^{-1}), NAA (0.1 mg L^{-1}) and GA_3 (1 mg L^{-1}) caused extensive elongation of shoots. Elongated shootlets were rooted on half-strength MS medium supplemented with BAP (0.1mg L^{-1}), NAA (0.1 mg L^{-1}) and IBA (2 mg L^{-1}). Rooted plantlets were initially hardened on a potting mixture consisting of soilrite and later on a mixture of sand, soil and FYM (1:1:1).

Micropropagation enables rapid production of uniform, disease-free planting material of elite papaya cultivars (Fitch, 2005). MS macro and micronutrients combined with BA and NAA at various concentrations are most frequently used for shoot proliferation while auxins are included in the culture medium to induce rooting (Fitch *et al*., 2003; Drew, 1988; Mumo *et al*., 2013;Tetsushi *et al*., 2008;Roy *et al*., 2013;Veena *et al*., 2015).

Limitations encountered during papaya micropropagation include the presence of endophytic bacteria in cultures, poor response of mature explant tissues and loss of regeneration potential following long-term culture (Drew, 1988; Drew and Smith,1986; Litz and Conover, 1982; Thomas *et al*., 2007). Presence of endophytic bacteria affects shoot proliferation and rooting. Frequent indexing of cultures stock assists in the identification of endophytes, which can be eliminated to improve plant regeneration (Thomas *et al*., 2007). Other

techniques including alternating culture regimes in liquid and solid medium, and eliminating sucrose in the medium following shoot proliferation to produce clean plants (Drew, 1988; Fitch *et al*., 2003). Modifications in culture environment such as providing ventilation to improve gas exchange in culture vessels, CO_2 enrichment of culture vessels, and providing illumination using red light during micropropagation may stimulate photo autotrophic conditions to improve plant growth and recovery (Lai *et al*., 1998;Yu *et al*., 2000;Perez *et al*.,2015; Schmildt *et al*., 2015). Improved regeneration rates are attributed lower ethylene accumulation in culture vessels, which decreases shoot epinasty and leaf senescence, symptoms that are frequently associated with the presence of ethylene in culture vessels. Alternatively, addition of ethylene biosynthesis inhibitors such as amino ethoxy vinyl glycine (AVG) and silver thiosulfate (STS) during culture also improves shoot quality and plant regeneration (Magdalita *et al*., 2002). Plants obtained by micropropagation exhibit uniform growth and vigor, precocious bearing and higher yields compared to seedling-derived plants (Fitch *et al*., 2005a, 2005b). Papaya plants obtained through *in vitro* culture exhibit higher levels of antioxidants compared to seedling-derived plants (Tiwari *et al*., 2014).

2.3 Effect of Growth Regulators

Setargie *et al*. (2015) conducted an experiment to develop a protocol for *in vitro* propagation of hermaphroditic papaya (*Carica papaya* L.) from shoot buds. In this study, MS media modified with varying concentration of auxin and cytokinins were used to evaluate the effect of cytokinin and auxin for establishment of the appropriate medium in regeneration of carica papaya under *in vitro* condition. Best initiated and proliferated shoots were obtained on MS medium with 1 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. The highest mean number of shoots (16), highest shoot length (1.7 cm) and mean number of leaves (21) were recorded on MS medium with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} of NAA. Likewise, the minimum and maximum number of days for shoot emergence was counted on BAP at 0.5 mg L^{-1}+0.5mg L^{-1} NAA and BAP at 2.0 mg L^{-1} 0.5 mg L^{-1}

NAA respectively. The lowest number of shoots, number of leaves and shoot length was recorded as BAP was increased from 0.5 mg L^{-1} to 2.0 mg L^{-1} and at 0.5 mg L^{-1} BAP. On the other hand, the maximum number of roots (16.25) and root length (3.92 cm) were measured on shoots pretreated on MS media supplemented with 1.5 mg L^{-1} IBA. Unlike this, minimum root length and number were obtained from 3 mg L^{-1} of IBA. Lowest survival capacity (40%) of seedlings was recorded during acclimatizationon mixture of garden soil, sand and cow dung at the rate of 2:1:1. Overall, the study showed that BAP and NAA combination at medium level concentrations for shoot initiation and multiplication; and IBA at lower and medium level concentrations for rooting were the best basal medium for *in vitro* propagation of papaya taken from shoot buds; nevertheless, further investigations are needed to minimize low survival rate of the seedlings for mass and commercial propagation of the *in vitro* developed protocol.

Samanmalie *et al*. (2017) conducted an experiment to develop a procedure for the rapid tissue culture propagation of papaya hybrid plantings at commercial scale. Shoot tips were surface sterilized with 20% NaOCl for 20 minutes before establishing on hormone free Murashige and Skoog (MS) medium. MS medium supplemented with 3 mg L^{-1} Benzyl Amino Purine (BAP), 0.3 mg L^{-1} Naphthalene Acetic Acid (NAA) and 500 mg L^{-1} Casein was the most suitable medium for formation of shoots. Well grown roots were obtained in MS medium containing 2 mg L^{-1} IBA and 500 mg L^{-1} Casein. Successful hardening (100% survival rate) was achieved when the plantlets were transplanted in pots containing moist mixture of sand: top soil: compost to a 1:1:1: ratio at sterile condition together with a weekly application of Albert solution. Flowering was observed at 7 weeks after the field planting. Micro propagated plants produced fruits with average fruit weight of 1.405 kg and average Brix value of 14.5, which were similar to fruits obtained from plants grown from seedlings.

An efficient *in vitro* regeneration system was developed by Kunlin *et al* (2012) for rapid clonal propagation of hermaphroditic *Carica papaya* cv. Meizhonghong using shoot buds from seedlings and axillary buds from greenhouse and field fruit-bearing plants. Explants from seedlings were more easily disinfected and initiated, and had higher multiplication rates and rooting percentages than explants from mature plants. The highest percentage of contamination-free explants from seedlings was 81.7% using $AgNO_3$ treatment after conventional disinfection, which was higher than the antibiotic treatments. Shoot initiation was achieved in Murashige and Skoog (MS) medium with 0.5 mg L^{-1} 6 benzyladenine (BA) and 40 g L^{-1} sucrose. An average of 6.7 fold proliferation rate per subculture was obtained on MS medium containing 0.25 mg L^{-1} BA and 40 g L^{-1} sucrose after five subcultures. Shoot elongation was induced in MS medium supplemented with 0.25 mg L^{-1} BA and 1.0 mg L^{-1} GA_3 and 40 g L^{-1} sucrose. Cultures were maintained for 20 months (22 subcultures) without any loss in multiplication rate or change in growth habit. The 3/2 MS medium (i.e. with 1.5 times the macro-elements of MS) with 500 mg L^{-1} activated charcoal and 5 g L^{-1} sucrose was optimum for root development. In the greenhouse, bags with sand (1/3 v) over a mixture of sieved peat and vermiculite (1:1 v/v) were used and 87% of plants survived. About 100,000 plantlets were produced successfully for field growth within three years. Compared to mother plants, the *in vitro* raised plants were all hermaphrodites and showed no distinct phenotypic variation. This regeneration system is suitable for mass multiplication of cv. Meizhonghong.

in vitro culture of lateral buds of field grown mature plants were tested in MS medium supplemented with different concentrations of BAP and NAA. Seasonal endophytic contamination was suppressed by shaking propagules for 2 h in 300 mg L^{-1} rifampicin before surface sterilization. Maximum survival rate was found in MS medium supplement with 1.0 mg L^{-}

^{1} BAP plus 0.20 mg L^{-1} NAA. The highest number of shoots was produced in MS medium containing 0.50 mg L^{-1} BAP and 0.20 mg L^{-1} NAA. Both the highest multiplication rate and the longest shoot were found in MS medium

supplemented with 0.50 mg L^{-1} BAP plus 0.20 mg L^{-1} NAA. On the same treatment an increasing trend was observed in multiplication rate of shoot upto 5th subculture which decreased thereafter. First subculture produced the highest shoot length which decreased with the increase in number of subculture. Half strength MS medium supplemented with 1.0 mg L^{-1} IBA was found as the best treatment to produce root in the culture media. Rooted plantlets were transplanted successfully (Rohman *et al.,* 2007).

Gatambia *et al*. (2015) study was an attempt to determine rapid *in vitro* methods for regeneration of papaya plantlets using liquid and semisolid/liquid double layer media and different concentration of growth regulators. Shoot meristems were harvested from hermaphrodite 30 cm tall papaya seedlings raised in greenhouse and cultured in Murashige and Skoog basal media supplemented with 30 g L^{-1} sucrose, and different concentrations of hormones which were treatments in liquid medium. The retreatments comprised five levels (0, 0.05, 0.1, 0.15, and 0.2) of mg L^{-1} 2 Chloro 4 Pyridy -
N Phenyl-urea (CPPU) in liquid and semi-solid/liquid double layer combination for shoot induction. For root initiation, four levels of Indole-3-butyric acid (IBA) (0, 2.0, 2.5 and 3.0 mg L^{-1}) were used. The experiments were laid out in a completely randomized design with 3 replicates. Number of shoots, shoot length and leaf number were recorded every 2 weeks for 8 weeks. The percentage number of shoots that produced roots was also recorded. There was significant differences between the liquid and semi-solid /liquid media and the level of hormones used on shoot induction. The highest number of shoots (10), shoot length and number of leaves was recorded in the semi solid/ liquid double layer combination with CPPU at 0.15 mg L^{-1}. The highest root initiation (73.33%) was at 3.0 mg L^{-1} of IBA. Results indicated that semi-solid/liquid double layer combination could be used for rapid regeneration of papaya plantlets of known gender a completely randomized design with 3 replicates. Number of shoots, shoot length and leaf number were recorded every 2 weeks for 8 weeks. The percentage number of shoots that produced roots was also recorded. There was

significant differences between the liquid and semi-solid /liquid media and the level of hormones used on shoot induction. The highest number of shoots (10), shoot length and number of leaves was recorded in the semisolid/ liquid double layer combination with CPPU at 0.15 mg L^{-1}. The highest root initiation (73.33%) was at 3.0 mg L^{-1} of IBA. Results indicated that semi-solid/liquid double layer combination could be used for rapid regeneration of papaya plantlets of known gender.

A large number of shoots regenerated from lateral buds and young leaves of *Carica papaya* L. cv. Shahi on MS supplemented with 1.0 mg L^{-1} zeatin and 0.2 mg L^{-1} NAA. Addition of 200 mg L^{-1} case in hydrolysate (CH) to the medium increased the number of shoots per culture and incorporation of 2.0 g L^{-1} activated charcoal (AC) to the medium resulted effective shoot growth with healthy leaf. While addition of 100 mg L^{-1} urea and 2.0 g L^{-1} activated charcoal to the medium showed proper shoot elongation. Best rooting was obtained from shoots cultured on half-strength of MS fortified with 4.0 mg L^{-1} IBA. Within four weeks of transfer to the rooting medium, 90% microcuttings produced 12-14 roots. The regenerated plant lets were successfully transferred to potted soil. About 84% plantlets survived in the experimental field (ROY *et al*., 2012).

CHAPTER III
MATERIALS AND METHODS

3.1 Time and location of the experiment:

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of September 2017 to June 2018.

3.2 Experimental materials:

3.2.1 Source of material

The planting materials of papaya (*Carica papaya* L.) 'Red Lady' variety were collected from Agargaon nursery, Sher-e-Bangla Nager, Dhaka-1207.

3.2.2 Plant materials

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

3.2.3 Instruments

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

3.2.4 Glass ware

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

3.2.5 Culture medium

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

1. Sucrose (3%) was used as carbon source and media were solidified with agar-agar (0.8%).
2. Charcoal (2 g L^{-1}) was used in media to avoid browning.
3. The pH was adjusted to pH 5.8 prior to autoclaving at a temperature of 121^{0}C for 20 minutes at 1.06 kg/cm^{2} (15 PSI) pressure.

3.3 Hormone dose and preparation of the stock solution of hormones:

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

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

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

The following hormonal dose and combinations were used in regeneration of papaya.

i. Hormones used in *in vitro* shoot induction in papaya

Name of hormone	Conc. (mg L^{-1})
BA	0 mg L^{-1}
	0.5 mg L^{-1}
	1.0 mg L^{-1}
	1.5 mg L^{-1}
	2.0 mg L^{-1}

ii. Combined dose of BA and KIN on shoot induction potentiality

Name of the Phytohormones	Phytohormones concentration (mg L^{-1})
MS_0(Control)	0
BA+KIN	0.5+0.25
	0.5+0.5
	0.5+0.75
	0.5+1
	1+0.25
	1+0.5
	1+0.75
	1.0+1
	1.5+0.25
	1.5+0.5
	1.5+0.75
	1.5+1
	2+0.25
	2+0.50
	2+0.75
	2+1.00

iii. Hormone used for i*n vitro* root formation in papaya

Name of hormone	Conc. (mg L^{-1})	Name of hormone	Conc. (mg L^{-1})
IBA	0	NAA	0
	0.5		0.5
	1		1
	1.5		1.5
	2		2
	2.5		2.5

3.4 The preparation of culture media:

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

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

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

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

Step-4. Then other ingredients (activated charcoal) are used as required.

Step-5. The volume was made up to 1000 ml with addition of sterile distilled water.

Step-6. The pH was adjusted at 5.8.

Step-7. Finally, 8 g agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

3.5 Steam heat sterilization of media (Autoclaving)

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

3.6 Preparation of explants

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

3.7 Inoculation of culture

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

For inoculation, explants were transferred to large sterile glass petri dish or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed with sterile scalpel blade to make suitable size. After cutting explants into suitable size (1.5-2 cm), explants are transferred to culture bottles containing MS medium with plant growth regulator (Plate 1). After vertically inoculating the explants singly

in culture bottle, the mouth of bottle is quickly flamed and capped tightly. After proper labeling, mentioning media code, date of inoculation etc. the bottles was transferred to growth room.

Plate 1. Explant (Nodal segment) was inoculated in the culture medium

3.8 Incubation

The bottles were kept to the culture racks and allowed to grow in controlled environment. The cultures were maintained at 21±2 ℃ with light intensity varied from 3000–5000 lux (23 W white bulbs). White fluorescent lamps were used for growth of the culture. The photoperiod was generally 14 hours light and 10 hours dark having 70% relative humidity (RH).

3.9 Shoot and root proliferation

The explants were cultured on MS nutrient medium supplemented with different concentration of BAP alone or in combination of KIN and NAA. After successful shoot proliferation, subculture was done with newly form shoots. Shoots were excised in aseptic condition with help of sterile scalpel blade and sterile forceps and transferred to new MS media which was supplemented with same concentration of growth hormones in order to increase budding frequency. The observations on development pattern of shoots were made throughout the entire culture period. Newly formed shoots with adequate length were excised individually from the culture vial and transferred to rooting media. one type of growth regulators (IBA) was used in different concentration (0, 0.5, 1.5, and 2.5 mg L^{-1}) along with MS media. The observations on development pattern of roots were made throughout the entire culture period. Data were recorded from 3^{rd} week of inoculation.

3.10 Acclimatization

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

Step-1: After 35 days of culture on rooting media, the plantlets were taken out from culture vial with the help of forceps with utmost care to prevent any damage to newly formed roots and dipped in gentle warm water to remove any traces of solidified agar media for acclimatization. Plastic pots (6×6 cm) were kept ready filled with garden soil and compost in the proportion of 1:1 respectively. Immediately after removing solidified agar media from newly formed roots, the plantlets were then transplanted in to the pots with special care. **Step-2:** After planting, the plantlets were thoroughly watered and were kept at 25±2 °C with light intensity varied from 2000–3000 lux. The photoperiod was generally 14 hours light and 10 hours dark and 70% RH for 7 days with consecutive irrigation.

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

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

3.11 Data recording:

The observations on development pattern of shoots and roots were made throughout the entire culture period. Five replicates each of them containing 4 bottles (single shoot per culture bottle) were used per treatment. Data were recorded after 3, 6 and 9 weeks of culture, starting from day of inoculation on culture media in case of shoot proliferation. In event of root formation, it was done every week starting from third week to fifth week of culture. The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

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

3.11.1 Calculation of days for shoots and roots induction

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

3.11.2 Calculation of number of shoots and roots per explant

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

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

3.11.3 Calculation of number of leaf

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

3.11.4 Calculation of shoots and root length (cm)

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

3.13 Statistical analysis

The experiment was one factorial set up in a completely randomized design (CRD) with five replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique and differences among treatment means were compared by using Duncan's multiple range test (DMRT) at 5% probability level using MSTAT-C program.

CHAPTER IV
RESULTS AND DISCUSSION

Three separate experiments were performed for the *in vitro* regeneration of papaya. The overall objective of the present study has developed a system for the mass propagation of papaya. The results of these experiments are presented and discussed in this chapter with several figures, tables and Plates.

4.1. Sub-experiment 1. *In vitro* shoot induction in Papaya

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

4.1.1 Effect of BA on shoot induction

The result of the effect of different concentration of BA individually has been presented under following headings with Figure 1-4 and Plate 2.

4.1.1.1 Days for shoot induction

Significant variation was observed among different concentration of BA on days to shoot induction. The maximum days to shoot induction were recorded in control treatment (25.75 days) and 0.5 mg L^{-1} of BA required minimum 10.25 days (Figure 1).

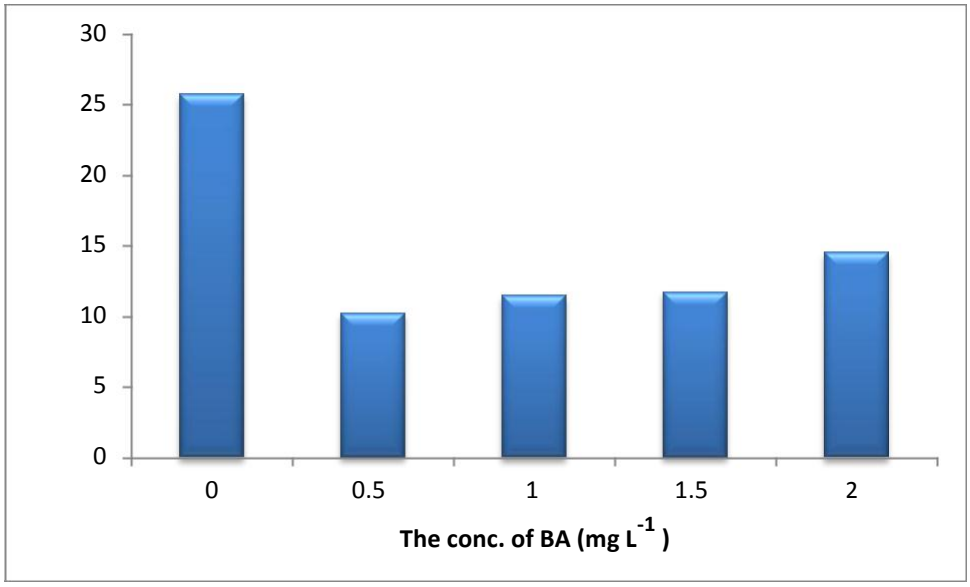

Figure 1: Effect of BA on days to shoot induction in papaya.

4.1.1.2 Number of shoots per explant

There was significant influence of different concentrations of BA on the number of shoots per explant. Data were recorded after 3, 6 and 9 weeks of culture on MS media. The results have been presented in Figure 2. The treatment 1.0 mg L^{-1} BA gave the highest number of shoots (1.25, 3.25 and 4.50 at 3 WAI, 6 WAI and 9 WAI, respectively) whereas the lowest number of shoots (1.0, 1.1 and 1.2 at 3 WAI, 6WAI and 9 WAI) respectively was found with hormone free media (Figure 2 and plate 2). BA variations affecting shoot proliferation were also reported by Bhandari *et al*., (2010) and Gantait *et al.,* (2010). Baksha *et al.* (2005) noticed 3.2 shoot per explant in media supplemented with 2.0 mg L^{-1} BA in papaya. As Qu *et al.* (2000) reported high cytokinin level present in the medium causing cytogenetic instability thus unsuitable for clonal propagation. Moreover, most of the shoots so formed were stunted.

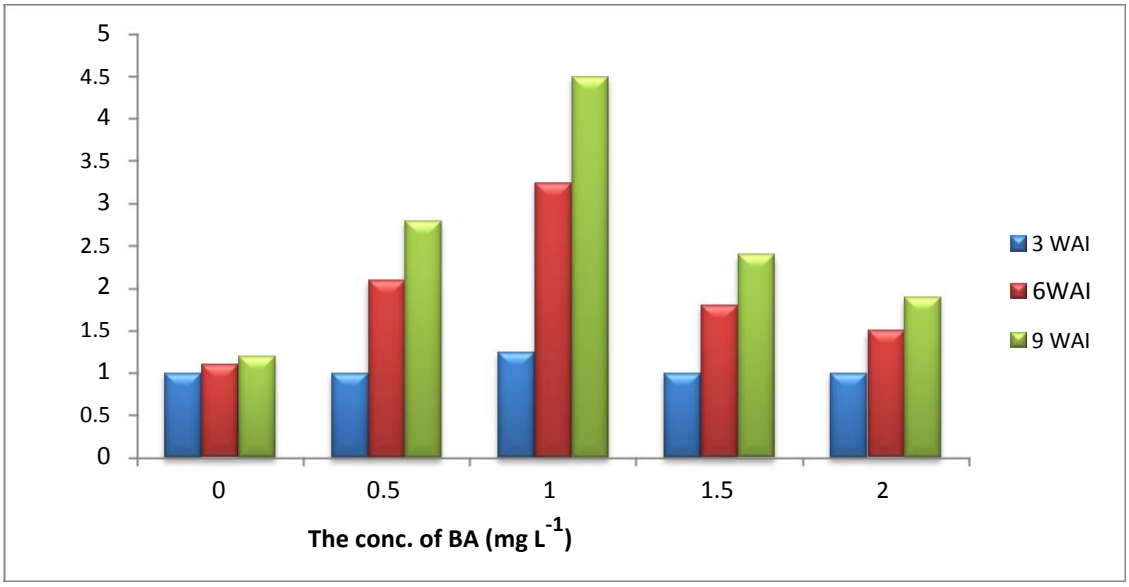

Figure 2: Effect of BA on the number of shoots per explants in Papaya.

2A: After 3 week **2B: After 9 week**

Plate 2: The number of shoots per explant in the treatment 1.00 mg L^{-1} (A) after 3 weeks, (B) after 9 weeks of inoculation

4.1.1.3 Length of shoot (cm)

The results of length of shoots have been presented in Figure 3. The maximum length of shoot 5.75 cm was noticed in the 1.0 mg L^{-1} BA which was statistically similar with 1.5 mg L^{-1} BA (4.40 cm) and statistically different from rest of others whereas the minimum length 2.00 cm was in control (Figure 3). Baksha *et al.* (2005) noticed 2.5 cm length of shoot in 4.0 mg L^{-1} BA. This decline in the shoot length of papaya might be due to the inhibitory effect of BA, which provoke a little suppression of plant growth and activity of some proteolytic enzymes (Petkova *et al.*, 2003).

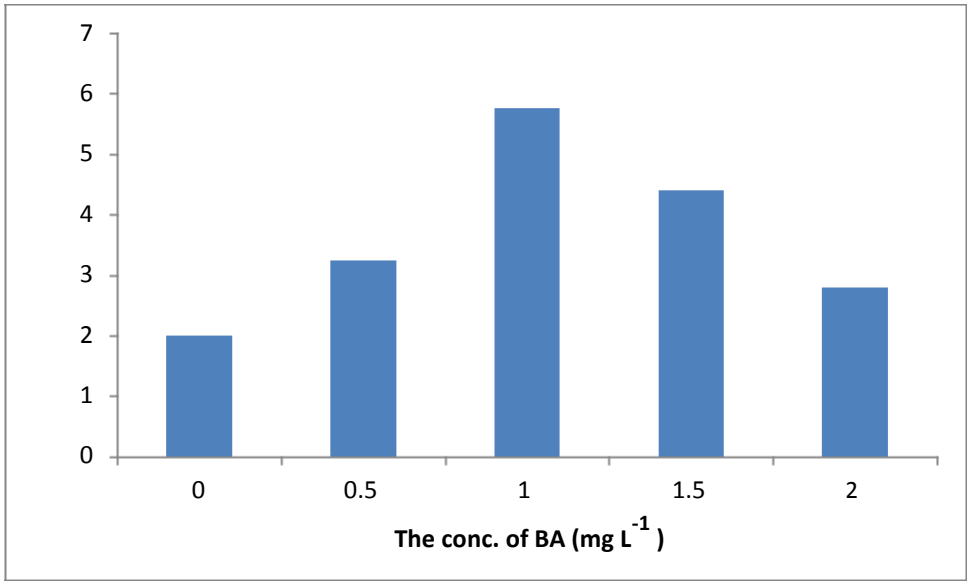

Figure 3: Effect of BA on length of shoot (cm) in papaya.

4.1.1.4 Number of leaf per explants

With different concentration of BA, significant influence was found on the number of leaf. The maximum 5.5 leaves were recorded with 1.0 mg L^{-1} BA and the minimum 1.5 in case of lack of hormone (Figure 4). Similar report was reported by Drew (1992) on leaf morphology of *in vitro* shoots of papaya.

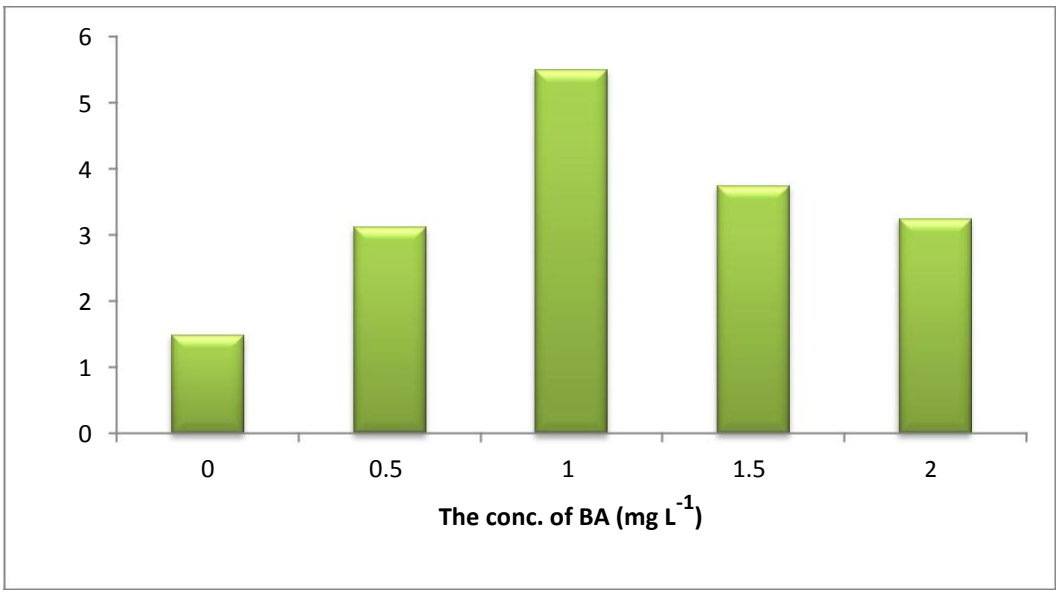

Figure 4: Effect of BA on number of leaf per explants in Papaya.

4.1.2. The combine effect of BA + KIN on shoot proliferation

The result of the combined effect of different concentration of BA + KIN have been presented under following headings with Table 1-2, Figure 5 and Plate 3.

4.1.2.1 Days for shoot induction

Variation was observed among different concentrations of BA+KIN on days to shoot induction. The maximum days to shoot induction were recorded in control (28.00 days) and 1.0 mg L^{-1} BA+0.75 mg L^{-1} KIN required minimum 16.80 days (Table 1).

4.1.2.2 Number of shoots per explant

Data were recorded after 3, 6 and 9 weeks of culture on MS media. There was significant influence of different concentrations of BA+KIN on the number of shoots per explant after 3 week of inoculation. The results have been presented in Table 1. There was no significant variation at 3 WAI among different concentration of BA+KIN. But 1.0 mg L^{-1} BA+0.75 mg L^{-1} KIN gave the highest number of shoots (3.75 and 5.25 at 6 WAI and 9 WAI, respectively) whereas the lowest number of shoots (1.0 and 1.25 at 6 WAI and 9 WAI, respectively) was found with hormone free media (Table 1).

Table 1. Combined effect of BA and KIN on shoot induction in papaya

Name of the Phytohormones	Phytohormones concentration (mg L^{-1})	Shoot induction potentiality			
		Days for shoot induction	No. of shoots per explant (3 WAI)	No. of shoots per explant (6 WAI)	No. of shoots per explant (9 WAI)
MS_0(Control)	0	28.00 a	1.00	1.00 d	1.15 f
BA+KIN	0.5+0.25	22.00 de	1.00	1.17 cd	1.25 ef
	0.5+0.5	22.80 cd	1.00	1.50 bcd	1.35 d-f
	0.5+0.75	23.80 cd	1.00	1.80 bcd	1.80 b-f
	0.5+1	21.60 def	1.00	1.40 cd	2.10 b-f
	1+0.25	24.60 bc	1.00	1.67 bcd	1.70 c-f
	1+0.5	20.60 efg	1.00	2.17 bc	1.85 b-f
	1+0.75	16.80 h	1.00	3.75 a	3.17 b
	1.0+1	19.20 g	1.00	1.33 cd	5.25 a
	1.5+0.25	26.20 ab	1.00	2.00 bcd	2.33 b-f
	1.5+0.5	27.00 a	1.00	2.10 bc	2.65 b-d
	1.5+0.75	23.80 cd	1.00	2.50 b	2.53 b-e
	1.5+1	22.20 de	1.00	1.17 cd	2.75 bc
	2+0.25	23.40 cd	1.00	1.35 cd	1.89 b-f
	2+0.50	22.00 de	1.00	1.80 bcd	2.80 bc
	2+0.75	19.80 fg	1.00	1.98 bcd	1.95 b-f
	2+1.00	24.40 bc	1.00	1.75 bcd	2.35 b-f
LSD (0.05)		1.93		0.88	1.16
CV(%)		5.75		7.9	5.25

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

4.1.2.3 Length of shoot (cm)

With different concentrations of BA+KIN, significant influence was found on the length of shoot (cm).The results have been presented in Figure 5. The average **(**5.78 cm) length of shoot was noticed from the 1.0 mg L^{-1} BA+0.75 mg L^{-1} KIN followed by 1.5 mg L^{-1} BA+0.75 mg L^{-1} KIN, whereas the minimum 0.7 cm was in control treatment.

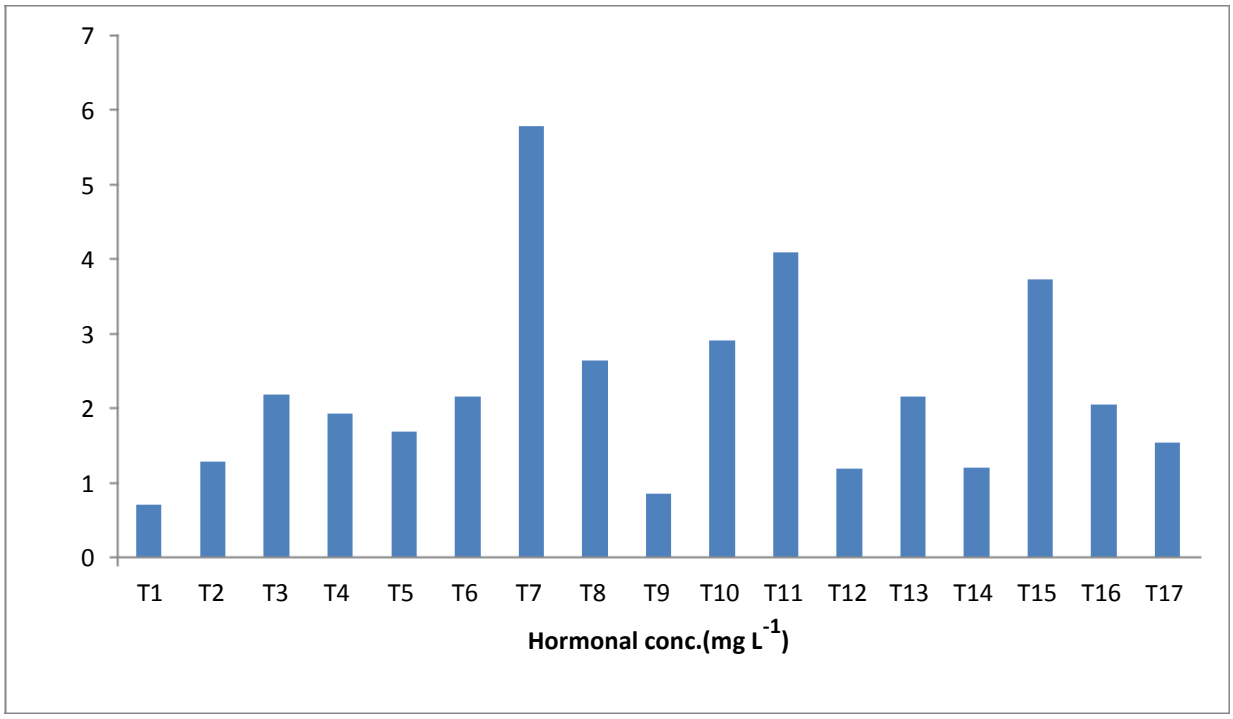

Fig 5. Combined effect of BA and KIN on the length of shoot in Papaya

Note:

T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}	T_{13}	T_{14}	T_{15}	T_{16}
0	0.5+ 0.25	0.5+ 0.5	0.5+ 0.75	0.5+ 1.0	1+ 0.25	1+ 0.5	1+ 0.75	1+ 1.0	1.5+ 0.25	1.5+ 0.5	1.5+ 0.75	1.5+ 1.0	2+ 0.25	2+ 0.5	2+ 0.75	2+ 1.0

4.1.2.4 Number of leaf per explants

The number of leaf per explants was significantly different according to the various concentration of BA+KIN supplemented. The results have been presented in Table 2. The maximum number of leaf per explants (8.2) was noticed from 1.0 mg L^{-1} BA+0.75 mg L^{-1} KIN which was followed by statistically similar with 1.5 mg L^{-1} BA+0. 5 mg L^{-1} KIN (7.15), whereas the minimum 1.5 in control.

Table 2.Combined effect of BA and KIN on the number of leaf in Papaya.

Name of the Phytohormones	**Phytohormones concentration (mg/L)**	**No. of leaf per explants**
MS_0(Control)	0	1.10 g
BA+Kin	0.5+0.25	2.40 def
	0.5+0.5	5.20 abcd
	0.5+0.75	3.60 cde
	0.5+1	3.40 cde
	1+0.25	5.00 bcd
	1+0.5	6.00 abc
	1+0.75	8.20 a
	1.0+1	2.00 def
	1.5+0.25	6.00 abc
	1.5+0.5	7.15 ab
	1.5+0.75	3.80 cde
	1.5+1	3.00 cde
	2+0.25	3.20 cde
	2+0.50	1.60 efg
	2+0.75	2.00 def
	2+1.00	1.50 fg
LSD (0.05)		0.82
CV(%)		6.85

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

Plate 3: The number of leaves per plantlet in papaya in the treatment 1.0 mg L^{-1} BA+0.75 mg L^{-1} Kin

4.2 Sub-experiment 2. *In vitro* root formation in papaya

To develop root in the regenerated shoots, they were excised and transferred to rooting media supplemented with IBA and NAA separately. The results of experiment have been presented under different heading utilizing Figure 6-9, Table 3-4 and Plate 4-5.

4.2.1 Days to root induction by NAA and IBA individually

Hormonal concentration has significant level of variation on days for root induction. The maximum 22 days to root induction was required in media lack of growth regulator. Minimum 8.5 days in case of 2.0 mg L^{-1} NAA and 9.1 days was required by 2.0 mg L^{-1} IBA (Figure 6). Baksha, *et al.* (2005) noticed that roots began to emerge from the tenth day in the medium with 0.5 mg L^{-1} of NAA.

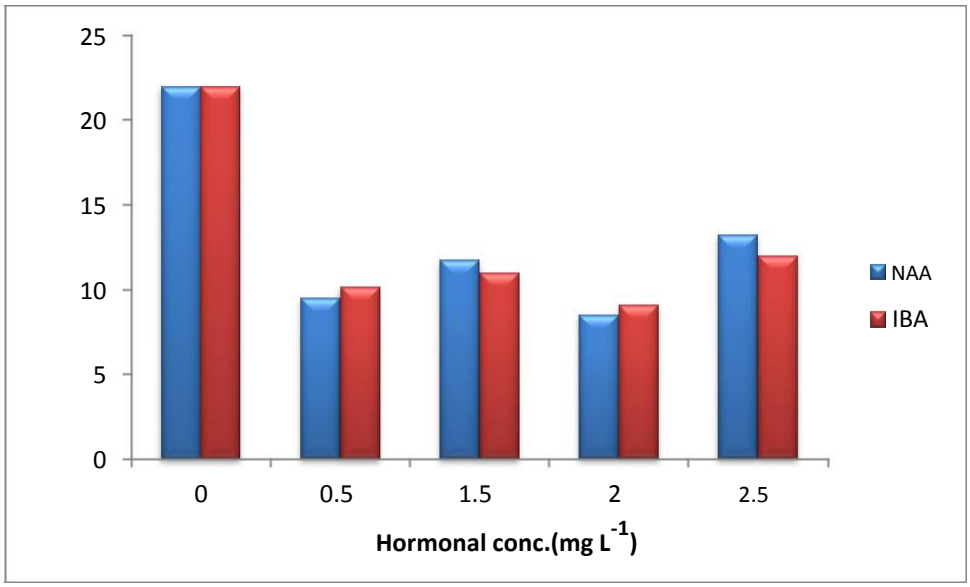

Figure 6: Effects of plant growth regulators (IBA and NAA) on days for root induction

4.2.2 Effect of NAA and IBA on root development

4.2.2 (a) In the case of IBA

To check the response and effectiveness of IBA and NAA on the number of roots a range of treatment (0, 0.5, 1.5,2.0,2.5) were applied and significant variation were observed during data recording at 3 WAI, 6 WAI and 9 WAI. Roots regenerated in the medium containing IBA were thin and long compared to roots from NAA which were relatively thick and strong.

In the case of IBA, the highest number of roots (3.2) per explant was recorded in 2.5 mg L^{-1} IBA at 3 WAI, which was statistically different with 2.5 mg L^{-1} IBA (3.5). But the highest number of roots (6.4 and 11.25 at 6 WAI and 9 WAI, respectively) were found only with 2.5 mg L^{-1} IBA. The minimum number of roots (0.1, 2.6 and 3.5 at 3 WAI, 6 WAI and 9 WAI, respectively) were obtained in control treatment (Table 3). Dwivedi, *et al.* (2014) found 10 roots in medium with IBA (0.5 mg L^{-1}) in 8 weeks of time. Bhandari, *et al.* (2010) reported 2.6 roots in IBA 0.2 mg L^{-1} after 15 days of culture. Baksha, *et al.* (2005) noticed 3.2 roots per explant in IBA 1.5 mg L^{-1}.

Table 3. Effect of IBA on number of roots in papaya

Name of the Phytohormones	Phytohormones concentration (mg/L)	No. of roots per explants (3 WAI)	No. of roots per explants (6 WAI)	No. of roots per explants (9 WAI)
IBA	0	1.00 e	2.60 d	3.50 e
	0.5	2.40 c	4.40 b	8.50 b
	1.5	3.00 b	4.60 b	6.50 c
	2.0	2.80 d	3.60 c	5.75 d
	2.5	3.20 a	6.40 a	11.25 a
	LSD (0.05)	0.08	0.21	0.63
	CV(%)	5.40	5.89	7.26

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

Plate 4: Number of root in papaya after 9 weeks of inoculation in the treatment 2.5 mg L^{-1}

4.2.2 (b) In the case of NAA

In the case of NAA, the highest number of roots (3.1, 4.8 and 8.25 at 3 WAI, 6 WAI and 9 WAI, respectively) were found with 2.0 mg L^{-1} NAA which was statistically different from other treatment. The minimum number of roots (1.0, 2.3 and 3.1 at 3 WAI, 6 WAI and 9 WAI, respectively) were obtained in control treatment (Figure 7). Biswas, *et al.* (2013) achieved highest average number of root per explants was 5.2 produced in 0.5 mg L^{-1} Napthaleneacetic acid concentration after 15 days of culture of micro shoots on rooting medium. Daneshvar, *et al.* (2013) noticed 6.8 roots with 0.5 mg L^{-1} NAA. Hashembadi, and Kaviani, (2010) obtained the largest number of roots (9.71) on medium supplemented with 0 mg L^{-1} IBA + 1 mg L^{-1} NAA. Baksha *et al.* (2005) noticed the highest number of roots per root was 4.8 with 0.5 mg L^{-1} NAA.

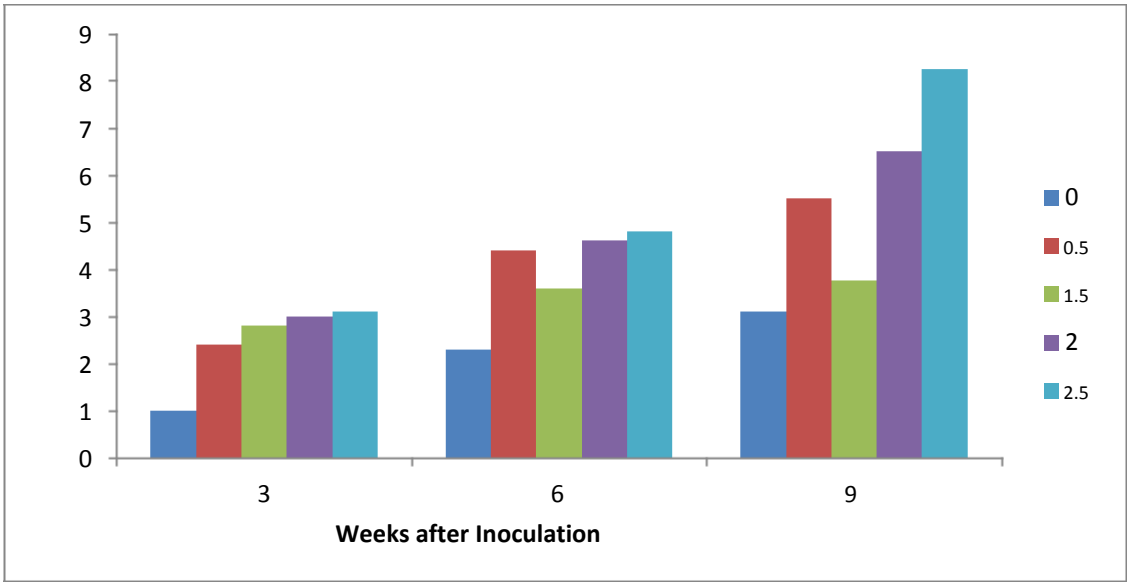

Figure 7: Effects of NAA on number of root at 3 WAI, 4 WAI and 5 WAI in Papaya.

4.2.4 Length of roots per explant (cm)

Length of roots per explants (cm) was greatly regulated by the different concentration of both IBA and NAA. The maximum average root length (3.8 cm) was obtained from 2 mg L^{-1} IBA (Figure 8) and 6.92 cm in case of 2.0 mg L^{-1} NAA (Figure 9). The minimum 1.00 cm average length of roots per explants (cm) was in control treatment. Jafari and Hamidoghli (2009) explained that concentration of 2 mg L^{-1} IBA has given a bigger number of roots and the maximum root length. Dwivedi *et al.* (2014) found length 6 cm were obtained in medium with IBA 0.5 mg L^{-1} in 8 weeks of time. Daneshvar *et al.* (2013) noticed 6.32 cm root length in 1.0 mg L^{-1} IBA. Hashembadi, and Kaviani, (2010) obtained the maximum (8.75 cm) roots on medium supplemented with 1 mg L^{-1} IBA + 1 mg L^{-1} NAA. Baksha *et al.* (2005) noticed the highest average length of 3.5 cm with 0.5 mg L^{-1} NAA and 2.2 cm in IBA 1.5 mg L^{-1}.

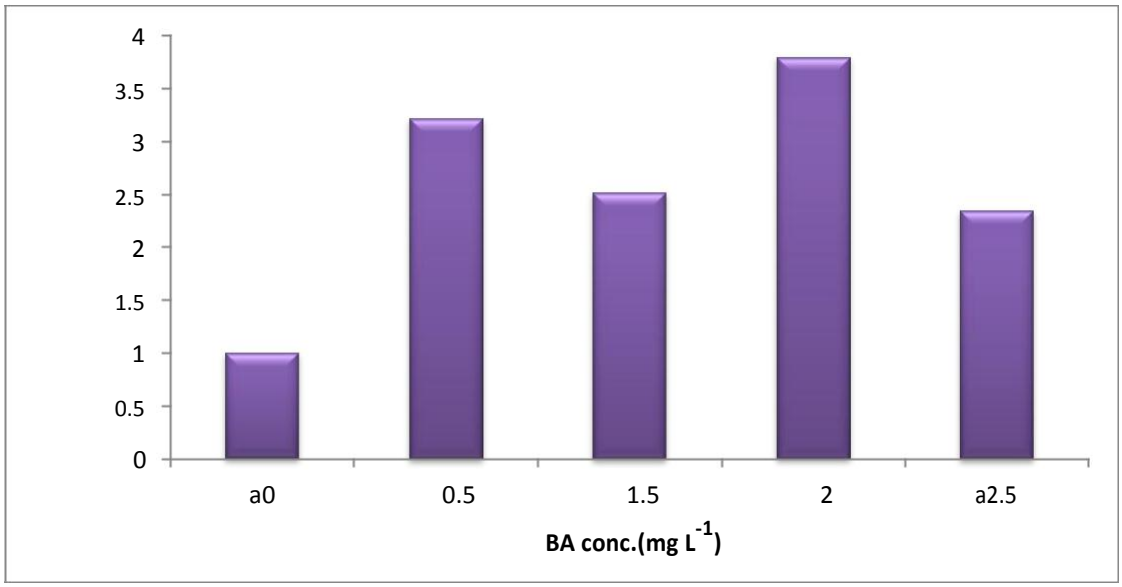

Fig8. Effect of IBA on length of roots per explants (cm) papaya

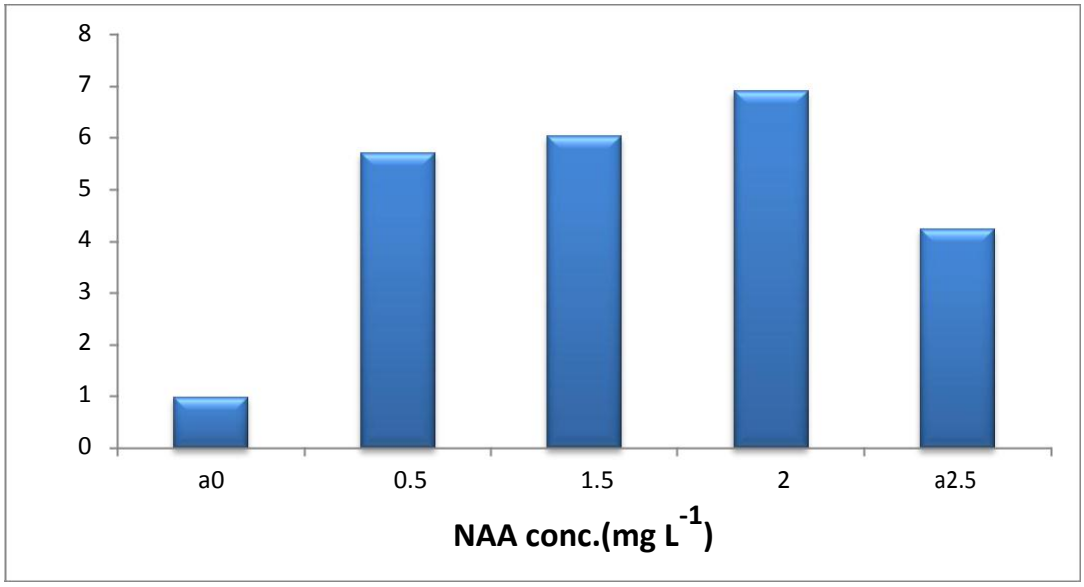

Figure 9: Effects of NAA on length of roots (cm) in papaya.

4.3 Experiment 3. Acclimatization of plantlets

The results of acclimatization or 'hardening' have been presented in Table 4 and Plate 5. After 35 days of culture on rooting media, the plantlets were taken for acclimatization.

Table 4. Survial rate of *in vitro* regenerated plants of Papaya.

Acclimatization	No. of plants transplanted	Duration of observation	No. of plants survived	Survival rate (%)
In growth chamber	20	15 days	16	80
In shade house	20	15 days	15	75
In field condition	20	30 days	15	75

The results of acclimatization showed that the 80% of plantlets were survived in growth chamber (Table 4). Then the plantlets were shifted to shade house with

less humidity (70% RH) and indirect sunlight. In the shade house, the top of the pots were covered with transparent plastic sheet and grew at room temperature for 14 days with periodic irrigation (2 days interval). In these conditions, the 75 % of the plantlets showed survival (Table 4). After 3 weeks, the plantlets were transferred to the soil following de-potting and potting into different pots of bigger size. The plants were watered periodically whenever necessary (Plate 5). In open atmosphere, survival rate was 75% (Table 4). It was also revealed that regenerated plants were morphologically similar to the mother plant.

Aggarwal and Barna (2004) used soil and farmyard manure (1:1) for hardening, and plantlets were transferred to the poly house, then 85% of the plantlets survived. After keeping them for initial 10 days in poly house, plants were transferred to shade house under less humidity. In shade house, 82% plantlets survived. Dwivedi *et al.* (2014) found 83% the survival rate and the plants established well in 4-6 weeks of growth. Bhandari *et al.* (2010) observed plantlets that were transferred to the plastic pots in poly house showed 90% survival and under shade house (50%) it was found 80%. Baksha *et al.* (2005) noticed well-developed rooted plantlets successfully transferred to the soil with 70% survival.

Plate 5. Acclimatization *in vitro* regenerated plantet of papaya in natural condition in pots.

CHAPTER V

SUMMARY AND CONCLUTION

The research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of September 2017 to June 2018 to study *in vitro* regeneration of Papaya (*Carica papaya*).

The highest number of shoot (1.25, 3.25 and 4.50 at 3 WAI, 6 WAI and 9 WAI, respectively) were recorded in 1.0 mg L^{-1} BA and the lowest number of shoot 1.0 and 1.1, 1.2 at 3 WAI, 6 WAI and 9 WAI, respectively) in only hormone free media.

Variation was observed among different concentration of BA+KIN on days to shoot induction. The maximum days to shoot induction were recorded in control (28.00 days) and 1.0 mg L^{-1} BA+0.75 mg L^{-1} KIN required minimum 16.80 days.

The 1.0 mg L^{-1} BA+0.75 mg L^{-1} KIN gave the highest number of shoots (3.75 and 5.25 at 6 WAI and 9 WAI, respectively) whereas the lowest number of shoots (1.0 and 1.25 at 6 WAI and 9 WAI, respectively) was found with hormone free media.

The number of leaf per explants was significantly different according to the various concentration of BA+KIN supplemented. The maximum number of leaf per explants (8.2) was noticed from 1.0 mg L^{-1} BA+0.75 mg L^{-1} KIN.

Hormonal concentration has significant level of variation on days for root induction. The maximum 22 days to root induction was required in media lack of growth regulator. Minimum 8.5 days in case of 2.0 mg L^{-1} NAA and 9.1 days was required by 2.0 mg L^{-1} IBA. The highest number of roots (3.2) per explant was recorded in 2.5 mg L^{-1} IBA at 3 WAI, which was statistically different with 2.5 mg L^{-1} IBA (3.5). But the highest number of roots (3.4 and 8.25 at 6 WAI and 9 WAI) respectively were found only with 2.5 mg L^{-1} IBA.

The minimum number of roots (0.1, 2.6 and 3.5 at 3 WAI, 6 WAI and 9 WAI, respectively) were obtained in control.

Length of roots per explants (cm) was greatly regulated by the different concentration of both IBA and NAA. The maximum root length (3.8 cm) was obtained from 2 mg L^{-1} IBA

Regenerated plantlets showed 80% survival during in growth chamber conditions and 75% in shade house stage of hardening and 75% in open atmosphere. Regenerated plants were found to be morphologically similar to the mother plant. Findings of the present study showed that *in vitro* regeneration is effective method in the proliferation of papaya.

The following conclusions were deduced:

1. Leaf and shoot formation was best performed in hormone concentration of 1.0 mg L^{-1} BA.
2. The highest leaf and shoot formation was obtained in 1.0 mg L^{-1} BA+0.75 mg L^{-1} KIN.
3. Root formation was significantly better in IBA.
4. IBA was better than NAA in improving the performance of papaya root formation.
5. Addition of 1 mg L^{-1} benzyl adenine improved shoot proliferation of cultures *in vitro*.

RECOMMENDATIONS

Following recommendations could be addressed based on the present experiment:

i. For future research more doses of hormone combination can be taken as treatments with fewer intervals which will give us specific result.
ii. At the same time, along with BA, KIN, NAA and IBA other types of cytokinin and auxin can be taken into trial.
iii. Except shoot tip culture, meristem and callus culture should be practiced.
iv. To uncover the influence of genotype if any, research should be carried on with other different types of genotypes of papaya.
v. Precise and details investigation on influence of other factors such as different elicitors, antioxidants should be considered.

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APPENDICES

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

Components	Concentrations ($mg\ L^{-1}$)	Concentrations
Micro Elements	**$mg\ L^{-1}$**	**µM**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91
Macro Elements	**$mg\ L^{-1}$**	**mM**
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
NH_4NO_3	1650.00	20.61
Vitamins	**$mg\ L^{-1}$**	**µM**
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	0.10	0.30

Total concentration of Micro and Macro elements including vitamins: 4405.19 $mg\ L^{-1}$
Manufacturing Company: Duchefa Biochem