MICROPROPAGATION OF *Nicotiana tabacum*

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MICROPROPAGATION OF *Nicotiana tabacum*

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*This is to certify that the thesis entitled "**MICROPROPAGATION OF Nicotiana tabacum**" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY,** embodies the result of a piece of bona fide research work carried out by **Abdullah Al Rafi,** **Registration No. 11-04282**, 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.

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LIST OF ACRONYMS

AEZ	**=**	**Agro-Ecological Zone**
BARI	**=**	**Bangladesh Agricultural Research Institute**
BBS	**=**	**Bangladesh Bureau of Statistics**
LAI	**=**	**Leaf area index**
ppm	**=**	**Parts per million**
et al.	**=**	**And others**
N	**=**	**Nitrogen**
TSP	**=**	**Triple Super Phosphate**
MP	**=**	**Muriate of Potash**
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)**

MICROPROPAGATION OF *Nicotiana tabacum*

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 for *in vitro* renegaration of tobacco. The healthy, disease free shoot tips of 0.25-0.50 cm length were used as explants, It was sterilized by 0.1% $HgCl_2$ mixing with few drops Tween-20. The explant was inoculated in MS media supplemented with different combination of the BA and IBA. The minimum days to shoot induction were achieved on BA 3.00 mg/L. The highest shoots and leaves per explant also observed in BA 3.00 mg/L. The combine effect of auxin and cytokinine hormone was also investigated. It was noticed that the minimum days to shoot induction were recorded in 3.00 mg/L BA+1.00 mg/L IBA. The highest number of shoot, number of leaf per explants and length of shoot and length of leaf were recorded in 3.00 mg/L BA+ 1.00 mg/L IBA. The highest number of root and length of root was observed in 3.00 mg/L BA+ 1.00 mg/L IBA. Micropropagation was found to be very effective and promising method in the proliferation of tobacco and this experiment can be a useful tool for tissue culture of tobacco

CHAPTER I

INTRODUCTION

Tobacco *(Nicotiana* species) is an ancient and the most important and widely grown commercial non-food crop in the world. This is also a major tropical cash crop of Bangladesh with considerable economic significance. The plant tobacco belongs to the genus *Nicotiana* under the large family Solanaceae (Garner, 1951). There are only two cultivated species under this genus *viz., Nicotiana tabacum* and *Nicotiana rustica* which was established by Carolas Linnaeus in 1753. There are few mentionable tobacco varieties of Bangladesh, such as Virginia, Jati, Motihari, Bengal, Sumatra, etc. Motihari belongs to *Nicotiana rustica* and the others belong to *Nicotiana tabacum.* Virginia and Sumatra are two recognized cigarette varieties, while others are used for Hokka, Bidi, and Zarda purpose.

In Bangladesh, tobacco has a prestigious and significant position in terms of economy where about 2-3 million people are employed for its production, processing and marketing. Tobacco earns foreign currency which occupies 4th position after jute, sugarcane, and tea. The land under tobacco cultivation is 114786 acres and production is 87628 tons (BBS, 2016).

Plant research often involves growing new plants in a controlled environment. These may be plants that we have genetically altered in some way or may be plants of which we need many copies all exactly alike. These things can be accomplished through tissue culture of small tissue pieces from the plant of interest. These small pieces may come from a single mother plant or they may be the result of genetic transformation of single plant cells which are then encouraged to grow and to ultimately develop into a whole plant. Tissue culture techniques are often used for commercial production of plants as well as for plant research.

Tissue culture involves the use of small pieces of plant tissue (explants) which are cultured in a nutrient medium under sterile conditions. Using the appropriate growing conditions for each explant type, plants can be induced to rapidly produce new shoots with the addition of suitable hormones and can be induced new roots. These plantlets can also be divided usually at the shoot stage to produce large numbers of new plantlets. The new plants can then be placed in soil and grown in the normal manner.

Tissue Culture or *in vitro* culture of cells, tissues or organs, one of most important content of cell engineering is based on theory of cellular totipotency, whereby Multicellular eukaryotic organisms can be distinguished by the ability of individual cells to regenerate into an entire organism (Buss, 1987, Kaplan and Hagemann, 1991), also allows one type of tissue or organ to be initiated from another type. The regeneration of whole organisms depends upon the concept that all plant cells can given the correct stimuli, express the total genetic potential of the parent plant, so this practice involves the culture environment, culture media, and growth regulators and so on.

Tobacco has been used as a model crop plant for *in vitro* studies on regeneration, since the classical studies of Skoog and Miller (1957). Totipotency was first demonstrated with *Nicotiana tabacum* by regeneration of mature plants from single cells (Vasil and Hildebrandt, 1965). Variability of regenerates has been obtained from tobacco tissue culture (Nikova and Zagorzka, 1984). Advanced quality hybrids and asymmetrical hybrids through protoplast fusion (Kortash and Kanevsku, 1987), resistance to herbicide (Freyssinet, 1986), obtaining TMV mosaic free plantlets through tobacco callus culture (Sanger *et al.,* 1986), overcoming cross incompatibility and obtaining male sterile forms (Nikova *et al.,* 1988), demonstration of gene transformation (Zhang *et al.,* 1998) and determining effect of antibodies on the *in vitro* growth response (Silva *et al.,* 2003) have been successfully obtained from tobacco tissue culture.

Majority of the discoveries in the field of plant cell, tissue culture and molecular biology have originated from the experimentation with tobacco plants'". As a result, tobacco has become a model system as the 'Cinderella of Plant Biotechnology'. This plant has been found to be an extremely versatile system for all aspects of cell and tissue culture research. Working with tobacco tissue cultures, Murashige and Skoogs devised an *in vitro* culture medium that has become the widely used nutrient formulation for an ever-growing range of plant species. In vitro studies with tobacco tissue culture, using different physical and chemical factors, have provided insight into the control of growth and differentiation. Induction of haploids and selection of mutant cell lines, owing to the experiments with tobacco tissue cultures have become useful tools**.**

So, there is no doubt that *in vitro* regeneration in tobacco has the great potentiality for its improvement. Although tissue culture techniques in tobacco have been successful long ago but in Bangladesh its application is limited. Considering the above mentioned information, the present study was undertaken to tissue culture of tobacco.

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

1. Establishment of tissue culture protocol of tobacco.
2. Assessment of combined effect of phytohormone for *in vitro* response tobacco.
3. To regenerate the plants those are genetically identical to the source material.
4. Acclimatization and cultivation of regenerated plantlets of tobacco.

CHAPTER II
REVIEW OF LITERATURE

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

2.1 Concept of tobacco tissue culture

The science of *in vitro* culture of plants takes its root from the path breaking research in plant science by the discovery of plant cells and proposal of cell theory. It was later proposed that, if proper environmental conditions and essential nutrients are given, a single cell should develop into a whole fertile plant. Based on this theory, Haberlandt tried to separate cells from different tissues from different plants and cultured in *in vitro* conditions in Knoop‘s salt solution using glucose as the carbon source. Due to the contributions, he is regarded as the Father of Plant Tissue Culture. The importance of vitamins and plant growth regulators were proposed by White (1937) also accelerated the tissue culture studies.

One of the most important developments was the formulation of a complete nutrient media for the culture of plant cells, tissues and organs. Murashige & Skoog (1962), made a very significant contribution in the formulation of defined Murashige and Skoog growth medium (MS) suitable for the wide range of species.

The MS salt formulation with various modification and additives now forms the basis of most of the tissue culture medium (Thorpe 2007).

Tobacco is a member of the Solanaceae family with more than 70 species reported worldwide. *Nicotiana tabacum* L. is one of the important species of the genera *Nicotiana* and is an allotetraploid (4X = 48) formed by the hybridisation of two different diploid parental species *N. sylvestris* and *N. tomentosiforms* 6 million years ago (Okamura & Goldberg 1985). Since the beginning of the *in vitro* culture along with carrot, tobacco was also used as a model crop for various studies (Skoog & Miller 1957). The ability of plant cell to develop into a complete plant (later termed as totipotency) was first demonstrated in tobacco. Since then, *Nicotiana tabacum* has become a model system for tissue culture and genetic engineering studies over the past several decades and continues to remain the *Cinderella of Plant Biotechnology'*.

It was when using tobacco, Murashige and Skoog in 1962, revised the culture media for the propagation of plants and this nutrient formulation was later used for the *in vitro* culture of other crops. Isolation, culture and regeneration of protoplast into a complete plant and somatic hybridization were studied successfully in tobacco. Burow *et al*., (1990) developed a simple genetic transformation method to tobacco based on leaf disc method, which has become standard for producing transgenic plants. Later employing tobacco as a model system, several genes have been transferred for insect resistance, herbicide resistance, and stress tolerance for producing recombinant proteins and recently for even expressing antibodies.

2.2 Explants

Leaf explants obtained from *N. tabacum*'Samsun' were cultivated on solidified MS medium supplemented with 3% (w/v) sucrose and enriched with different doses of extracts obtained from seedlings of the same plant cultivar or from *N. tabacum* 'Havana'. The aim of experiments was to verify the effectiveness of such medium additives on callus tissue vitality, proliferation and its organogenetic response. It was ascertained a significant positive influence of all kinds of added extracts on increase of callus fresh weight and the organogenetic capability of tobacco cultures. However, the impact of seedlings devoid of roots *Nicotiana* extracts was significantly lower in both stimulating of cell proliferation and shoot formation than extracts obtained from whole

seedlings. The biological activity was the most effective in the case of genotype compatibility between the plant material from which the extract was derived with the object of biotest (Fajerska and Ciarkowska, 2012).

Tobacco leaves were used as explants in this experiment. Large amount calli were formed after leaves cultured in basic MS medium with intermediate ratio of auxin to cytokinin, and the callus induction rate was up to 100%. Some differences were found between the treatments of lightness and darkness, the callus cultured in lightness represent more tight structure and regular shape with the color of Kelly, while that in darkness show itself less compact and irregular with the color of straw yellow. Subsequently, shoot regeneration was observed with the low ratio of auxin to cytokinin, which suggests that light wasn't the necessary element since shoot formed both in darkness and lightness and the No. of shoots had no significant difference. At the same time, slight differences were found in surface color of callus and the state of shoots (Chao yanjie, 2011).

The aim of the study was to estimate the potential for the production of larger numbers of uniform, well-rooted sweet potato plants by means of *in vitro* culturing. The study covered two cultivars – 'Carmen Rubin' and 'White Triumph'. The node explants were placed on two growth media containing the basic components of the MS medium as well as growth regulators. The first medium was supplemented with 1.0 mg dm^{-3} gibberellins and 0.1 mg dm^{-3} kinetin, while the second one with 0.5 mg dm^{-3} IAA. The induction of organogenesis and regeneration of the plants took place on the same medium, with no passage. Within 9 weeks, 4 plants were produced from each primary explants in two multiplication cycles. The properties of the plantlets depended on the cultivar, weight of the explants and composition of the medium. The average weight of the 'Carmen Rubin' plants was higher than that of the 'White Triumph' ones. Moreover, the 'Carmen Rubin' plants produced longer shoots and more developed root systems. The sweet potato micro-plants displayed an ability to acclimatize quickly (Doliński and Olek, 2013).

Stem cuttings from 3 month old plants, young buds, rhizome cuttings with shoot bud primordial and juvenile shoots were cultured on ½ MS medium supplemented with different combinations of growth hormones. Whereas callus could not be induced on the stem explants, callus was induced on the juvenile shoots which on subculturing to

medium with varying concentrations of 2,4-D and BAP developed bud primordia. These buds on further subculturing grew into small plantlets. Young shoot buds along with a portion of rhizome when inoculated on MS medium containing 2,4-D and BAP each @ 0.5 mg/L ruptured and some callus was produced. The callus on further subculturing on media containing 0.1mg/L 2,4-D and 0.5 mg/L BAP and 0.1mg/L both of 2,4-D and BAP produced lateral buds and multiple shoots. Excised single shoot when transferred to a medium supplemented with 0.1 mg/L each of 2,4-D and BAP started rooting within 2-3 days. Young shoots inoculated on MS medium supplemented with 0.5 mg/L both of 2,4-D and BAP developed greenish white hard callus within 4 weeks. These calli when subcultured on a media containing 0.1 mg/L of 2,4-D and 0.5 mg/L BAP produced good callus growth within a week. Shoot buds were observed after 4 weeks which ultimately grew into small plantlets (10-15 plantlets in each culture). Good callus growth was observed within 4 weeks when 2 week old shoot buds were inoculated on a medium supplemented with BAP and 2,4-D each @ 0.5mg/L. Callus growth stopped on subculturing to plain basal medium and roots initiated from it, whereas the callus subcultured on a medium containing BAP and 2,4-D @ 0.5 mg/L developed into greenish white compact callus masses (Ilahi and Jabeen, 1987).

2.3 Callus induction and plantlet regeneration

Nicotiana tabacum and *N. benthamiana* have served as model species for the plant sciences for many years. For the studies involving transformation analysis using *Agrobacterium, Nicotiana benthamiana* has been gaining importance in recent years due to its ability to express auto fluorescent protein for detection of transformed genes and its efficiency in gene expression compared to other plants. This study aims at optimizing the culture media procedures for the *in vitro* culturing of this model plant. BAP (1 mg/L), IAA (0.1 mg/L) was suitable for callus induction in 20 days. This result was in accordance with previous studies. BAP (2 mg/L), IAA (1 mg/L), Kinetin (0.5 mg/L) was suitable for shoot regeneration in 25 days. This result differed from that of previous studies where BAP (1 mg/L) and NAA (0.1 mg/L) was used for both callus induction and shoot regeneration. Thus, this study forms the basis for *in vitro* rising of *Nicotiana benthamiana* for transformation and genetic engineering studies (Geethalakshmi *et al*, 2016).

Frequency of shoot proliferation was maximum at 2.5mg/L BAP and 0.5mg/L Kn and the number of shoot was 22-25 per explant. It took 26 days for shoot induction and 30 days for root induction. Multiplication rate in the treatment with BAP 0.5mg/L which showed 2 plantlets were lowest. Among the BAP-2,4 D formulations, maximum multiplication was observed at BAP 1.0 and 0.5 mg/L 2,4 D where the number of shoots was 15. Numerous adventitious shoot primordia were observed near the basal portion of the shoot cluster (Khatun *et al*., 2003). Augmentation of MS-medium with 4.5 mg/L BAP recorded the highest number of shoots and leaves (8.0 and 15.50 respectively). Shoot lets were highly rooted on half strength of B5 medium supplemented with 1.0 mg/L NAA. The maximum percentage of acclimatization, hardening and rhizomes production of *in vitro* derived plants in greenhouse was 80–100% (Mohammed *et al*., 2011).

2.4 Effect of Growth Regulators

Manipulation of the composition and ratio of plant growth regulators (PGRs) is often the primary empirical approach used for optimization of *in vitro* micropropagation methods (Shukla *et al*. 2012).

Ficus religiosa is known as a long-lived multipurpose forest tree. The tree plays an important role for religious, medicinal, and ornamental purposes. However, the propagation rate of *Ficus religiosa* is low in natural habitat so the plant tissue culture techniques are an applicable method for multiplication of this valuable medicinal plants. Thus, the aim of this study is to understand the effect of different auxin/cytokinin ratios on indirect shoot organogenesis of this plant. According to our results, the maximum callus induction frequency (100%) was obtained on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) plus 0.05 mg/L 6-benzylaminopurine (BAP) from petiole segments. For shoot induction purpose, the yellow-brownish, friable, organogenic calli were inoculated on shoot induction medium. On MS medium supplemented with 1.5 mg/L BAP and 0.15 mg/L Indole-3-butyric acid (IBA), 96.66% of the petiole-derived calli responded with an average number of 3.56 shoots per culture. The highest root formation frequency (96.66%), root number (5.5), and root length (4.83 cm) were achieved on MS medium containing 2.0 mg/L IBA plus 0.1 mg/L Naphthaleneacetic acid (NAA). The

rooted shoots were successfully transferred to field condition and the substrate with the mixture of cocopeat and perlite (1:1) had the highest survival rate (96.66%). This is the first report of an effective *in vitro* organogenesis protocol for *F. religiosa* by indirect shoot organogenesis through axenic seedling derived petiole explants, which can be efficiently employed for conservation of this important medicinal plant species as well as the utilization of active biomolecules (Hesami *et al*. 2018).

This *in vitro* propagation study investigated by Ling *et al* (2013) the effects of different plant growth regulators (PGRs) on *in vitro* leaf and stem explants of *L. pumila*. Methods: The capabilities of callus, shoot, and root formation were evaluated by culturing both explants on Murashige and Skoog (MS) medium supplemented with various PGRs at the concentrations of 0, 1, 3, 5, and 7 mg/L. Results: Medium supplemented with 3 mg/L indole-3-butyric acid (IBA) showed the optimal callogenesis from both leaf and stem explants with (72.34±19.55)% and (70.40±14.14)% efficacy, respectively. IBA was also found to be the most efficient PGR for root induction. A total of (50.00±7.07)% and (77.78±16.47)% of root formation were obtained from the *in vitro* stem and leaf explants after being cultured for (26.5±5.0) and (30.0±8.5) d in the medium supplemented with 1 and 3 mg/L of IBA, respectively. Shoot formation was only observed in stem explant, with the maximum percentage of formation ((100.00±0.00)%) that was obtained in 1 mg/L zeatin after (11.0±2.8) d of culture. Conclusions: Callus, roots, and shoots can be induced from *in vitro* leaf and stem explants of *L. pumila* through the manipulation of types and concentrations of PGRs.

Plant Micropropagation has also been used as a tool for the propagation of genetically manipulated superior clones. Our attempt was made to develop micropropagation as suitable condition for cloning of *Catharanthus roseus*. After optimizing the culture media of explant culture, the repeated subculturing was performed at regular intervals for 5 weeks. The roots were developed within 10 days. IBA concentration and the period of pulse treatment had significant effects on the average number of roots produced per shoot. Rooted plants were successfully acclimatized at room temperature in soil contained in pots. All plants flowered and set seeds in the greenhouse after 3 months. It is an attempt to highlight some of the important landmarks of tissue culture of medicinal plants and it is also as important recent development *in vitro* technology (Rajora, *et al*. 2013).

The genetic engineering of Lettuce (*Lactuca sativa* L.) requires a reliable and efficient tissue culture method. Callus induction and direct shoot regeneration of lettuce using cotyledon explants was investigated by studying the effects of genotype, explant age and different combinations of plant growth regulators. The landrace genotypes were collected from central (Yazd) and southwest (Ahvaz) of Iran. Cotyledon explants of Lettuce with different explant age and genotypes were cultured on MS medium supplemented with different concentrations of NAA and BA. The highest frequency of callus induction was obtained on 2.7 µM NAA and 4.4 µM BA from 3-day-old explants of Yazd genotype. In addition, the effect of explant age for callus induction was a genotype-dependent characteristic. The highest number of direct shoot regenerations was obtained at low BA concentrations (<1 µM). Pearson's correlation coefficient identified that callus induction correlated significantly and positively (0.408) with direct shoot regeneration. It was also shown that the region near the petiole of cotyledon was the best for direct shoot regeneration from cotyledon explants of lettuce. Direct shoot regeneration from the region near to petiole of cotyledons may aid the use of genetic engineering to improve important characteristics of lettuce (Mohebodini *et al*, 2011).

Plant growth regulator is indispensable material in culture media, and is important to the plant tissue induction, organ differentiation and growth. Therefore, the effects of seven plant growth regulators such as BA, TDZ, KT, NAA, IBA, IAA and GA_3 with different concentrations on the leaf regeneration, subculture of shoots and rooting were studied in *Ziziphus jujuba* cv. 'Dongzao'. The results indicated that the efficiency of TDZ was significantly higher than that of BA in the induction of adventitious bud from leaf. Leaves should be first induced on MS medium supplemented with TDZ (1.0 mg/L) and IBA (0.1 mg/L) for 28 days, and then transferred to medium MS+IBA 0.1 mg/L+GA_3 0.05 mg/L. In this way, the regeneration rate reached 92.45%. MS+BA 1.0 mg/L+KT 0.5 mg/L+IBA 0.1 mg/L was suitable for subculture of shoots, with the multiplication coefficient of 3.64. Supplements of GA_3 at 0.5 mg/L in the medium could significantly increase the elongation of shoots. The efficiency of IAA was best among that of IAA, IBA and NAA in the induction of rooting. The regenerated plantlets rooted well in 1/2 MS medium plus IAA (1.5 mg/L), with rooting percentage of 95.3% (Zhou and Liu, 2009).

The effects of auxins and cytokinin on callus formation, growth and regeneration of *Gracilaria tenuistipitata Chang* et Xia and *G. perplexa* Byrne et Zuccarello (Gracilariales, Rhodophyta) are reported. Plant growth regulators (PGR) in concentrations ranging from 0.1 to 100.0 μmol of indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid (2,4-D), and kinetin (K) were added to the ASP 12-NTA solid medium (0.7% agar), and apical and intercalary segments (5 mm long) were inoculated as initial explants. K stimulated growth rates of intercalary segments of *G. tenuistipitata* in a linear relation, and 2,4-D (1.0 μmol) and K (10.0 μmol) stimulated growth rates of apical and intercalary segments of *G. perplexa*, respectively. The simultaneous formation of apical, basal, and intermediate calluses is reported for the first time in axenic tissue cultures of red algae. With intercalary segments of *G. tenuistipitata*, basal callus induction rates were higher than those of apical and intermediate calluses in the majority of treatments, and auxins had stimulatory effects on the formation of all callus types. In apical segments of *G. perplexa*, intermediate callus formation was stimulated only by treatment with 1.0 μmol of K, while apical callus formation was stimulated by indole-3-acetic acid (1.0–10.0 μmol), 2,4-D (10.0–100.0 μmol), or K (0.1 μmol). Intercalary segments of *G. perplexa* developed only intermediate calluses, and the majority of treatments with PGR stimulated higher rates than those presented by apical segments. Potential for regeneration (development of adventitious plantlets originated from callus cells) was higher in apical calluses than in basal and intermediate calluses developed in intercalary segments of *G. tenuistipitata*. Moreover, auxins and cytokinin were essential to the induction of regeneration in intermediate calluses, while specific concentrations stimulated regeneration from basal and apical calluses. Plant regeneration in *G. perplexa* was observed only after transferring calluses from solid to liquid medium, and the majority of treatments with PGR had stimulatory effects. Regenerating plants of *G. perplexa* developed tetrasporangia, and released tetraspores giving rise to adult gametophytes. Our results indicate that auxins and cytokinin have a regulatory role in the growth and morphogenesis in *G. tenuistipitata* and *G. perplexa*, and diversity of responses presented by both species is related to specific developmental systems (Yokoya *et. al.* 2006).

2.5 In vitro Sterilization

For obtaining contamination free cultures the most important step is sterilization of explants. In the present study the sterilization procedure was standardize for potato cultivar Kufri Himalini. Comparison was done between two important sterilant sodium hypochlorite and mercuric chloride with three time duration 2, 5 and 8 minutes. After sprouting the sprouts of 0.5 to 1 cm. were taken for the study and treated by chemicals of surface sterilization with three selected timings i.e. 2, 5 and 8 minutes. Sterilized explants were inoculated on without hormones MS medium to evaluate the response of different chemicals. The observations were recorded regularly till to 30 days for the non-growing cultures, infected cultures and healthy cultures. Result showed that amongst the two sterilants i.e. NaOCl and HgCl2, NaOCl was found better for controlling the infection and it had not any adverse effect on explants even in long duration. Sodium hypochlorite (NaOCl) for 8 minute (T3) was selected for suitable sterilization chemical after 5 minute of savlon wash, 30-second dip in ethanol and at last washed with double distilled water (Badoni and Chauhan, 2010).

2.6 *In vitro* Regeneration

Regeneration of plants by micropropagation can be achieved from organ primordia existing in shoot tips and axillary bud explants. Alternatively, plants can be regenerated from unorganized callus tissues derived from different explants by dedifferentiation induced by exogenous growth regulators. Plant regeneration from calli is possible by *de novo* organogenesis or somatic embryogenesis. Callus cultures also facilitate the amplification of limiting plant material. In addition, plant regeneration from calli permits the isolation of rare somaclonal variants which result either from an existing genetic variability in somatic cells or from the induction of mutations, chromosome aberrations, and epigenetic changes by the *in vitro* applied environmental stimuli, including growth factors added to the cultured cells (Flick, *et al*. 1983).

Regeneration ability of five *Nicotiana* varieties viz., Virginia, Jati, Motihari, CC Bengal and Sumatra were investigated via callus induction using leaf discs. Explants were cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators. Callus formation frequency was 67.20%.

Among the varieties used, Motihari induced the highest percentage (97.50%) of callus followed by Jati (92.50%) in 2.0 rng/L Kinetin and 2.0 mg/L IAA. Shoots were induced from calli cultured on the same medium. Maximum shoot formation from leaf discs was 82.50% on medium supplemented with 2.0 mg/L Kinetin and 2.0 mg/L IAA. It was also revealed from this study that Motihari was the best variety for callus formation and subsequent plantlet regeneration which is a pre-requisite for vector mediated transformation for varietal improvement of *Nicotiana* species. The rooting response of regenerated shoots was observed by using $^{1}/_{2}$ MS medium with IBA (0.0, 0.5, and 1.0 mg/L). The highest root formation was found in Motihari (90%) with ½ MS medium supplemented with 0.5 mg/L IBA. After that regenerated plantlets with plenty of roots were transferred successfully to pots and subsequently to the field (Rahman *et al*. 2010).

CHAPTER III
MATERIALS AND METHODS

3.1 Time and location of the experiment:

The present research was carried out in Biotecnnology Laboratory in 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 Tobacco (*Nicotiana tabacum* L) were collected from Aditmari Upzilla, Lalmonirhat District.

3.2.2 Explant Collection

Seed was shown in agronomic field of Sher-e-Bangla Agricultural University and also shown in *in vitro* condition at Biotecnnology Laboratory in the Department of Biotechnology, Sher-e-Bangla Agricultural University. After seedling establishment the healthy, disease free shoot tips of 0.50 cm length were used as explants for the study of *in vitro* regeneration.

3.2.3 Instruments

Metal instruments *viz*., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121^0C for 30 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) Erlesn meyer flasks, culture bottles, flat bottom flasks, pipettes, petridishes, beaker and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) were used for media preparation**.** The glassware's were first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water, until the detergent was removed completely. Finally they were rinsed with distilled water and sterilized in oven at 160-180^0C for 3-4 hours.

3.2.5 Culture medium

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

1. BA (0, 1.00, 2.00, 3.00, and 4.00 mg/L) alone or in combination with IBA (0, 0.50, 1.00, 1.50 and 2.00 mg/L), were used for shoot proliferation.
2. IBA (0, 0.50, 1.00, 1.50 and 2.00 mg/L) alone or in combination with, BA (0, 1.00, 2.00, 3.00, and 4.00 mg/L) were used for root formation.
3. Sucrose (3%) was used as carbon source and media were solidified with agar (0.8%).
4. The pH was adjusted to pH 5.8 prior to autoclaving at a temperature of 121^0C for 15 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.3 The preparation of the stock solution of hormones:

To prepare these hormonal supplements, they were dissolved in proper solvent as shown against each of them below.

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

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

3.4 The preparation of culture media:

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

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

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

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

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

Step-5. The pH was adjusted at 5.8.

Step-6. Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting 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 15 minutes at 1.06 kg/cm^2 (15 PSI) pressure. After autoclaving the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explants.

3.6 Preparation of explants

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

3.7 Inoculation of culture

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

For inoculation, explants were transferred to large sterile glass petridish 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 (0.25-0.50 cm), explants are transferred to culture bottles containing MS medium with plant growth regulator (Plate 1). After vertically inoculating the explants in culture bottle, the mouth of bottle is quickly flamed and capped tightly. After proper labeling, mentioning media code, date of inoculation etc. the bottles was transferred to growth room.

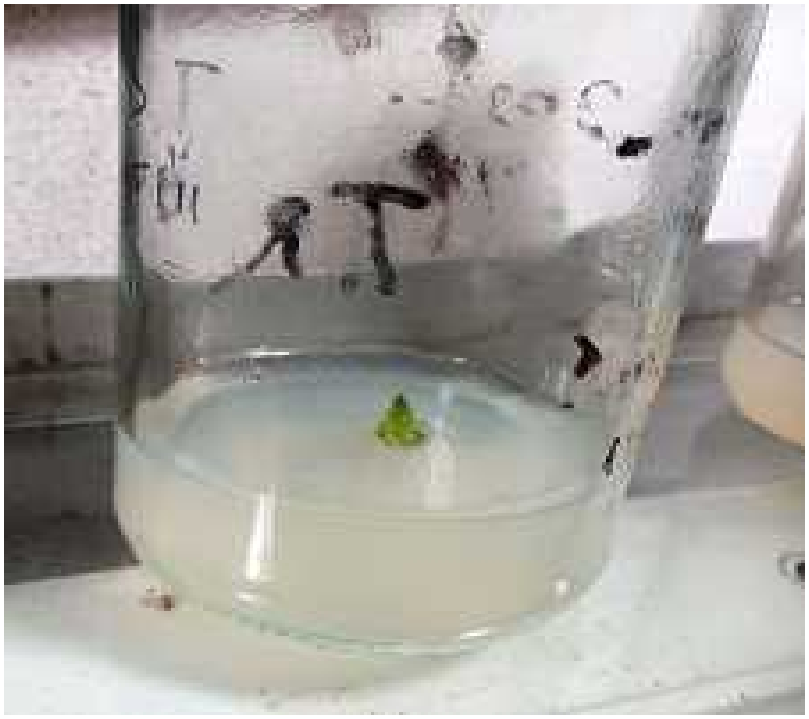

Plate 1. Inoculation of explants in the culture vial.

3.8 Incubation

The bottles were kept to the culture racks and allowed to grow in controlled environment. The cultures were maintained at 21±1°C with light intensity varied from 2000–3000 lux (23 W white bulbs). White fluorescent lamps were used for growth of the culture. The photoperiod was generally 14 hours light and 10 hours dark having 70% relative humidity (RH).

3.9 Shoot proliferation

The explants were cultured on MS nutrient medium supplemented with different concentration of BA alone or in combination of IBA. Percentage of explants showing shoot proliferation, days for shoot induction, number shoots per explants, average length of shoots, and number of leaves per explants were considered as parameter for evaluating this experiment. After successful shoot proliferation, subculture was done with newly form shoots. Shoots ware excised in aseptic condition with help of sterile scalpel blade and sterile forceps and transferred to new MS media which was supplemented with same concentration of growth hormones in order to increase budding frequency. The observations on development pattern of shoots were made

throughout the entire culture period. Data recording was started after 7, 14, 21, 28 days from inoculation.

3.9 Rooting of multiple shoots

Newly formed shoots with adequate length were excised individually from the culture vial and transferred to rooting media. Different concentration of BA alone or in combination of IBA was used with MS media. The observations on development pattern of roots were made throughout the entire culture period. Data were recorded from 7, 14, 21, 28 days of inoculation.

3.11 Acclimatization

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

Step-1: After 30 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 23±1 °C with light intensity varied from 3000–5000 lux. The photoperiod was generally 14 hours light and 10 hours dark and 70% RH for 7 days with consecutive irrigation.

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

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

3.12 Data recording

The observations on development pattern of shoots and roots were made throughout the entire culture period. Five replicates each of them containing 4 bottles (single shoot per culture bottle) were used per treatment. Data were recorded after 7, 14, 21 and 28 days of culture, starting from day of inoculation on culture media in case of shoot proliferation. In event of root formation, it was done every day starting from 7 days to 28 days of culture. The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

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

3.12.1 Calculation of days to 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.

3.12.2 Calculation of number of shoots and roots per explant

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

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

3.12.3 Calculation of number of leaf

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

3.12.4 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.

3.12.5 Length of leaf

Length of leaf of each sample plant was recorded and sum total of them was divided by the total number of leaves of the sample plant.

3.13 Statistical analysis

The experiment was one factorial set up in a completely randomized design (CRD) with five replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique and differences among treatment means were compared by using Duncan's multiple range test (DMRT) at 5% probability level using MSTAT-C (1990) program.

CHAPTER IV
RESULTS AND DISCUSSION

Three separate experiments were performed for *in vitro* regeneration of tobacco. The overall objective of the present study was to develop a system for the tissue culture of tobacco. The results of these experiments were presented and discussed in this chapter with Figure (1-8) and Tables (1-7).

4.1 Sub-experiment 1. *In vitro* regeneration and multiple shoot proliferation

This experiment was conducted under laboratory condition to tissue culture of tobacco. Manipulating the relative ratio of BA to IBA has been successfully used in the current investigation. The response of explants in different plant growth regulators were varied significantly. The results are presented separately under different headings below.

4.1.1 Effect of BA on shoot proliferation

The effect of different concentration of BA singly has been presented under following headings with Figure 1-4, Table1 and Plate 2& 3.

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 (18.6) to shoot induction were recorded in control treatment and the treatment 3.00 mg/L required minimum 9.00 days (Figure 1).

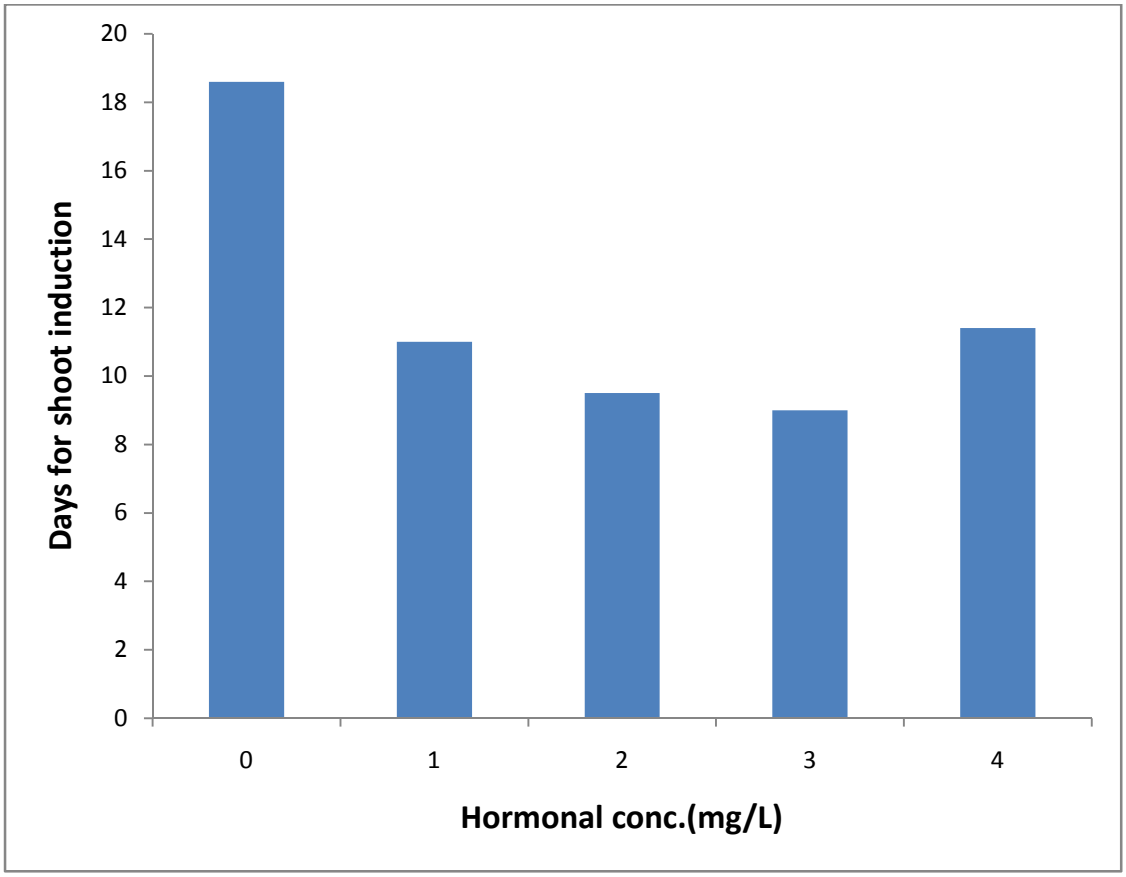

Figure 1: Effect of BA on days to shoot induction in tobacco.

4.1.1.2 Number of shoots per explants

There was significant influence of different concentration of BA on the number of shoots per explants. Data were recorded after 14, 21 and 28 days of culture on MS media. The results have been presented in Figure 2.At 14 DAI, the highest number of shoots (3.60) was found in the treatment 3.00 mg/L BA, whereas the lowest number of shoots (1.20) was found with hormone free media, which was statistically similar with the treatment 1.00, 2.00 and 4.00 mg/L BA. The highest number of shoots (5.20) was found in the treatment 3.00 mg/L BA at 21 DAI, whereas the lowest number of shoots (1.40) was found with hormone free media. At 28 DAI, the highest number of shoots (7.00) was found in the treatment 3.00 mg/L BA. The lowest number of shoots (1.40) was found with hormone free media variations of BAP affecting shoot proliferation of tobacco were also reported by Geethalakshmi *et al*., (2016) noticed 4.2 shoot per explant in media supplemented with 2.00 mg/L BAP.

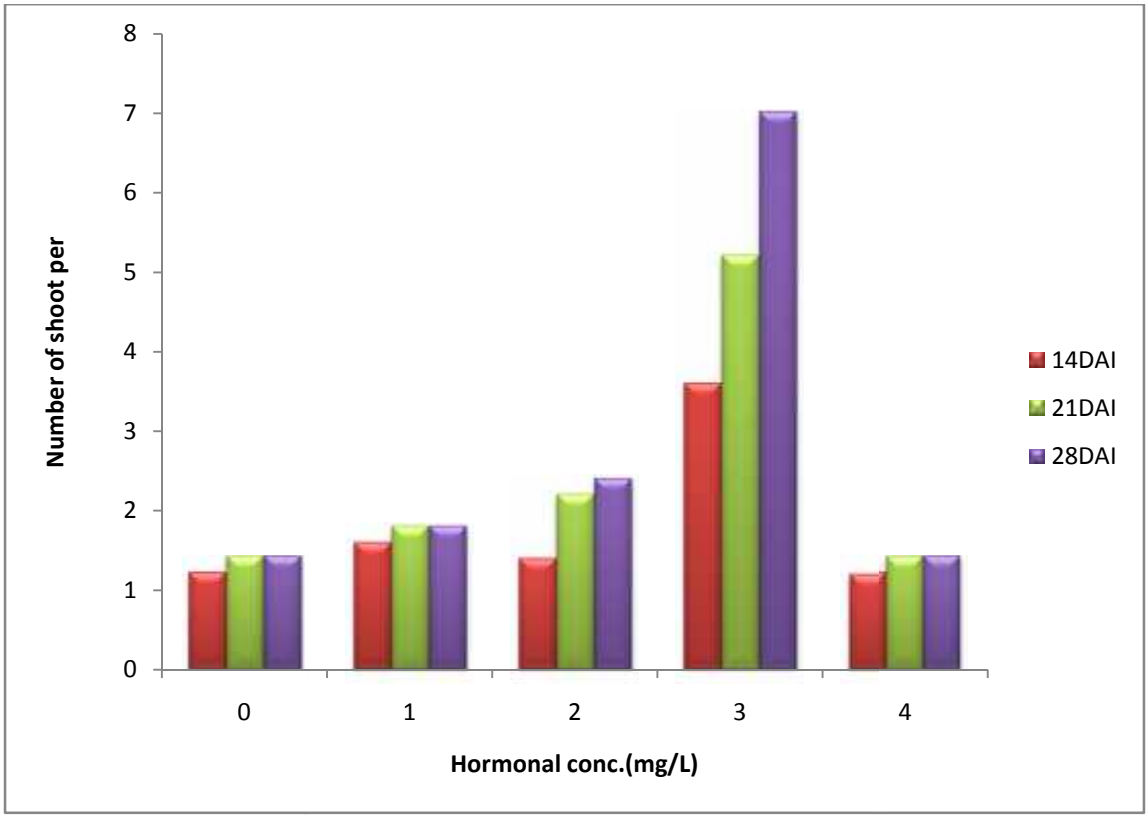

Figure 2: Effect of BA on the number of shoots per explants in tobacco.

2A: Number of shoots at 14 DAI. **2B: Number of shootsat 21 DAI.**

2C: Number of shoots 28 at 21 DAI.

Plate 2: Number of shoots per explants in the treatment 3.00 mg/L of BA.

4.1.1.3 Length of shoot (cm)

The length of shoots was significantly influenced by different concentration of BA at 14, 21 and 28 DAI. The results of length of shoots have been presented in Figure 3. The maximum length of shoot (2.62, 3.98 and 4.92 cm at 14, 21 and 28 DAI respectively) was noticed from the treatment 3.00 mg/L BA which was statistically identical with other concentration of BA and whereas the minimum (1.54, 1.86, and 2.86 cm at 14, 21 and 28 DAI respectively) in control treatment (Figure 3). Geethalakshmi, *et al.* (2016) noticed 3.2 cm length of shoot of tobacco in 2.0 mg/L BAP.

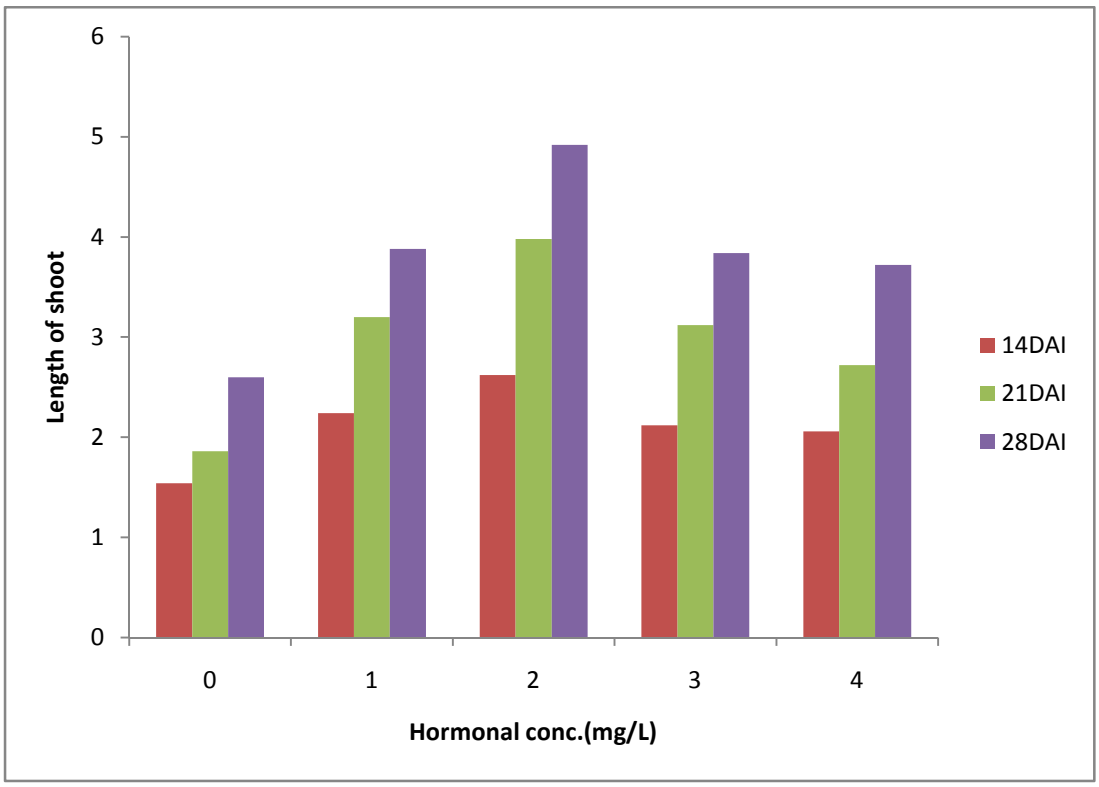

Figure 3: Effect of BA on length of shoot (cm) in tobacco.

4.1.1.4 Number of leaf per explants

With different concentration of BA, significant influence was found on the number of leaf at 14, 21 and 28 DAI. The maximum number of leaves (3.00, 4.40 and 5.00 at 14, 21 and 28 DAI respectively) was recorded with the treatment 2.00 mg/L BA and the minimum number of leaves (1.4, 1.4 and 2.4 at 14, 21 and 28 DAI respectively) in case of lack of BA (Figure 4).

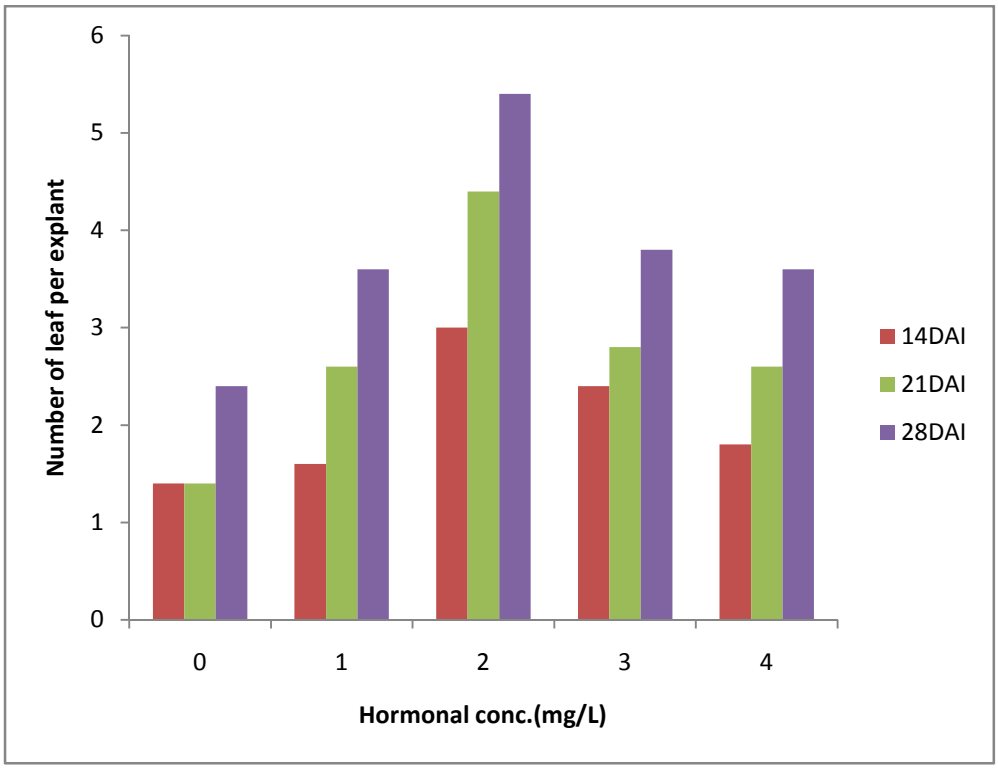

Figure 4: Effect of BA on number of leaf per explants in tobacco.

4.1.1.5Length of leaf (cm)

Length of leaf was significantly influenced by different concentration of BA at 14, 21 and 28 DAI. The maximum length of leaf (1, 1.2, and 1.58 cm at 14 and 21 DAI respectively) was obtained from the treatment 2.00 mg/L BA which was statistically different from rest of others whereas the minimum length of leaf (0.48 cm, 0.60 cm at 14, 21 DAI respectively) in control treatment (Table1 and plate 3). At 28 DAI, The highest length of leaf (2.14 cm) was obtained from the treatment 1.00 mg/L BA which was statistically similar with the treatment 2.00 mg/L BA whereas the minimum length of leaf (0.78 cm) in control treatment.

Table 1: Effect of BA on length of leaf (cm) in tobacco.

Hormones concentration of BA (mg/L)	Length of leaf (cm)		
	14 DAI	21 DAI	28 DAI
0	0.48 d	0.6 c	0.78 c
1	0.88 b	1.36 b	2.14 a
2	1.12 a	1.58 a	2.08 a
3	0.78 bc	1.28 b	1.62 b
4	0.66 cd	1.16 b	1.66 b
LSD (0.05)	0.18	0.20	0.25
CV (%)	5.68	7.85	6.94

*DAI=Days 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 3. Length of leaf in tobacco in the treatment 2.00 mg/L of BA.

4.2 Sub experiment 2. Combined effect of phytohormone on rapid multiplication in tobacco.

4.2.1 The combine effect of BA and IBA on multiple shoot proliferation

The result of the combined effect of different concentration of BA + IBA have been presented under following headings with Table 2-5 and Plate 4 & 5.

4.2.1.1 Days for shoot induction

Variation was observed among different concentration of BA+IBA on days to shoot induction. The maximum days to shoot induction were recorded in control (15.80 days) and 3.00 mg/L BA+1.00 mg/L IBA required minimum 6.40 days (Table 2).

4.2.1.2 Number of shoots per explants

Data were recorded after 14, 21 and 28 days of culture on MS media. There was significant influence of different concentration of BA+IBA on the number of shoots per explants after 14, 21 and 28 Days of inoculation. The results have been presented in Table 2. The treatment 3.00 mg/L BA+1.00 mg/L IBA gave the highest number of shoots (4.6, and 6.20 at 14 and 21 DAI) respectively whereas the lowest number of shoots (1.00 and 1.60 at 14 and 21 DAI) respectively was found with hormone free media (Table 2). At 28 DAI, the treatment 3.00 mg/L BA+0.50 mg/L IBA gave the highest number of shoots (7.60) whereas the lowest number of shoots (1.80) was found with hormone free media. Aggarwal and Barna (2004), observed 4.08 shoots of tobacco in media containing BA 1.00 mg/L and IBA 0.20 mg/L on 4 weeks of culture. Chukwujekwu, (2001) reported 11 shoots in tobacco from the combinations of 1.0 mg/L BA + 1.0 mg/L IBA. Bhandari, A.K. *et al.* (2010) reported 3.3 shoots per explants in combination with BAP 1.00 mg/L +IBA 0.20 mg/L after four weeks of culture.

Table 2. Combined effect of BA and IBA on shoot induction potentiality.

Name of the Hormones	Hormones concentration (mg/L)	Days for shoot induction	Number of shoots per explants		
			14 DAI	21 DAI	28 DAI
MS 0 (Control)	0	15.80 a	1.00 d	1.60 e	1.80 f
BA+IBA	1+0.5	10.80 de	1.80 cd	2.40 de	3.00 ce
	1+1.0	11.00 d	1.40 cd	2.00 de	2.80 cf
	1+1.5	11.60 d	1.60 cd	2.20 de	3.20 cd
	1+2.0	11.20 d	1.40 cd	2.20 de	3.20 cd
	2+0.5	7.60 gh	2.20 c	2.80 d	3.40 c
	2+1.0	9.40 f	1.60 cd	2.40 de	3.00 cde
	2+1.5	9.80 ef	1.40 cd	2.20 de	2.60 cf
	2+2.0	11.20 d	1.40 d	1.60 e	2.20 def
	3+0.5	7.80 g	3.60 b	5.16 bc	7.60 a
	3+1.0	6.40 h	4.60 a	6.20 a	6.60 b
	3+1.5	7.00 gh	3.60 b	5.60 ab	6.60 b
	3+2.0	7.40 gh	3.40 b	4.60 c	6.40 b
	4+0.5	12.00 cd	1.40 cd	1.60 e	2.20 def
	4+1.0	13.00 bc	1.20 d	1.40 e	2.20 def
	4+1.5	13.60 b	1.40 cd	1.80 e	2.20 def
	4+2.0	14.80 a	1.40 cd	2.00 de	2.00 ef
LSD (0.05)		1.13	0.72	0.85	0.92
CV (%)		8.44	8.06	5.95	10.33

*DAI=Days 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.

3A:Number of shoots 14 DAI. **3B: Number of shoots after 21 DAI.**

3C: Number of shoots after 28 DAI.

Plate 4: Number of shoots per explants in tobacco in the treatment of 3.00 mg/L BA and 1.00 mg/L IBA.

4.2.1.3 Length of shoot (cm)

With different concentration of BA+IBA, significant influence was found on the length of shoot (cm) and the results have been presented in Table 3 and Plate 5. The maximum length of shoot (3.48 cm) was found from the 3.00 mg/L BA+0.50 mg/L, whereas the minimum (1.40 cm) in control treatment at 14 DAI. At 21 DAI, the Length of shoot (4.12 cm) was obtained from the treatment 3.00 mg/L BA+1.00 mg/L IBA, whereas the minimum (1.66 cm) in control treatment. At 28 DAI, the length of shoot (4.92 cm) was noticed from the treatment 3.00 mg/L BA+2.00 mg/L IBA, which was statistically similar with 3.00 mg/L BA +0.50 mg/L IBA, 3.00 mg/L BA +1.50 mg/L IBA ad 3.00 mg/L BA +2.00 mg/L IBA whereas the minimum (2.08 cm) in control treatment.

Table 3. Combined effect of BA and IBA on length of shoots per explants of tobacco.

Name of the Hormones	Hormones concentration (mg/L)	Length of shoots (cm)		
		14 DAI	21 DAI	28DAI
MS 0(Control)	0	1.40 J	1.66 e	2.08 d
BA+IBA	1+0.5	2.84 cd	3.24 bc	3.48 c
	1+1.0	2.70 def	3.16 bc	3.32 c
	1+1.5	2.58 ef	3.08 bc	3.20 c
	1+2.0	2.50 f	3.02 c	3.12 c
	2+0.5	2.62 ef	3.84 a	4.66 a
	2+1.0	3.10 b	3.36 b	4.02 b
	2+1.5	3.02 bc	3.28 bc	3.54 c
	2+2.0	2.92 bc	3.20 bc	3.44 c
	3+0.5	3.48 a	4.00 a	4.90 a
	3+1.0	2.72 de	4.12 a	4.92 a
	3+1.5	2.62 ef	3.94 a	4.88 a
	3+2.0	2.64 def	3.96 a	4.90 a
	4+0.5	1.72 hi	2.06 d	2.32 d
	4+1.0	1.60 I	2.04 d	2.22d
	4+1.5	1.84 gh	2.16 d	2.40 d
	4+2.0	1.92 g	2.34 d	2.50 d
LSD (0.05)		0.19	0.29	0.41
CV (%)		10.03	11.07	12.98

*DAI=Days 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 5: Length of shoot in tobacco in the treatment 3.00 mg/L BA+1.00 mg/L IBA.

4.2.1.4 Number of leaf per explants

The number of leaf per explants was significantly different according to the various concentrations of BA+IBA supplemented. The results have been presented in Table 4. The maximum number of leaf per explants (6.20) was noticed from the treatment 3.00 mg/L BA+1.00 mg/L at 14 DAI. The minimum number of leaf per explants **(**1.2) was found in hormone free media. At 21 DAI, the maximum number of leaf per explants (8.20) was noticed from the treatment 3.00 mg/L BA+1.00 mg/L. The minimum number of leaf per explants **(**1.6) was found in hormone free media. At 28 DAI, the maximum number of leaf per explants (10.6) was noticed from the treatment 3.00 mg/L BA+1.00 mg/L. The minimum number of leaf per explants **(**2.20at 28 day) was found in hormone free media.

Table 4. Combined effect of BA and IBA on number of leaf per plantlet potentiality.

Name of the Hormones	Hormones concentration (mg/L)	Number of leaf per explants		
		14 DAI	21 DAI	28 DAI
MS 0(Control)	0	1.20 h	1.60 g	2.20 f
BA+IBA	1+0.5	4.00 bc	5.80 b	7.00 c
	1+1.0	3.60 bcd	5.20 bc	6.40 cd
	1+1.5	3.60 bcd	4.40 cd	6.20 cd
	1+2.0	3.20 de	5.20 bc	6.40 cd
	2+0.5	3.00 de	4.40 cd	5.80 cd
	2+1.0	3.00 de	4.40 cd	5.80 cd
	2+1.5	3.00 de	4.60 cd	6.00 cd
	2+2.0	3.00 de	4.60 cd	5.80 cd
	3+0.5	4.20 b	5.40 bc	8.40 b
	3+1.0	6.20 a	8.20 a	10.60 a
	3+1.5	3.40 cde	4.60 cd	6.40 cd
	3+2.0	2.80 ef	3.80 de	5.60 d
	4+0.5	2.20 fg	2.80 f	3.40 ef
	4+1.0	2.20 fg	2.60 f	3.40 ef
	4+1.5	1.80 gh	3.00 ef	4.00 e
	4+2.0	1.80 gh	2.80 f	3.40 ef
LSD (0.05)		0.68	0.88	1.13
CV (%)		7.57	6.22	5.57

*DAI=Days 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.

4.2.1.5 Length of leaf (cm)

With different concentration of BA+IBA, significant influence was found on the length of leaf (cm) the results have been presented in Table 5. The maximum length of leaf(1.64 cm) was noticed from the treatment the 3.00 mg/L BA+1.00 mg/L IBA, which was statistically similar with 2.00 mg/L BA+0.50 mg/L IBA at 14 DAI. The minimum length of leaf (00.38) was found in control. At 21 DAI, the maximum length of leaf (1.82 cm) was noticed from the treatment 2.00 mg/L BA+0.50 mg/L IBA, which was statistically similar with 3.00 mg/L BA+1.00 mg/L IBA at 21 DAI. The minimum length of leaf (0.38) was found in control. At 28 DAI, the maximum length of leaf (2.60) was noticed from the 3.00 mg/L BA+1.00 mg/L IBA. The minimum length of leaf (0.40 cm) was found in control.

Table 5. Combined effect of BA and IBA on the Length of leaf in tobacco.

Name of the Hormones	Hormones concentration (mg/L)	Length of leaf (cm)		
		14 DAI	21 DAI	28 DAI
MS 0(Control)	0	0.38 f	0.38 h	0.40 h
BA+IBA	1+0.5	1.16 bcd	1.42 cd	1.60 de
	1+1.0	1.08 cde	1.40 cd	1.54 de
	1+1.5	1.00 de	1.16 f	1.24 f
	1+2.0	0.92 e	1.12 f	1.22 f
	2+0.5	1.54 a	1.82 a	2.16 b
	2+1.0	1.30 b	1.52 bc	1.92 c
	2+1.5	1.16 bcd	1.34 de	1.64 d
	2+2.0	0.96 e	1.22 ef	1.46 e
	3+0.5	1.14 bcd	1.50 bcd	2.28 b
	3+1.0	1.64 a	1.80 a	2.60 a
	3+1.5	1.18 bc	1.60 b	2.20 b
	3+2.0	1.08 cde	1.54 bc	2.12 b
	4+0.5	0.48 f	0.62 g	0.84 g
	4+1.0	0.42 f	0.52 gh	0.68 g
	4+1.5	0.40 f	0.50 gh	0.68 g
	4+2.0	0.46 f	0.58 g	0.72 g
LSD (0.05)		0.15	0.15	0.15
CV (%)		12.21	10.42	8.14

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.

4.3 Sub-experiment 3. Root formation in tobacco.

To develop root in the regenerated shoots, they were excised and transferred to rooting media supplemented with IBA. The results of experiment have been presented under different heading utilizing Figure 5-7 and plate 6.

4.3.1 Days for root induction

Hormonal concentration has significant level of variation on days for root induction. The maximum 26.6 days to root induction was required in media lack of growth regulator. Minimum 9.00 days was required by 1.00 mg/L IBA (Figure 5).

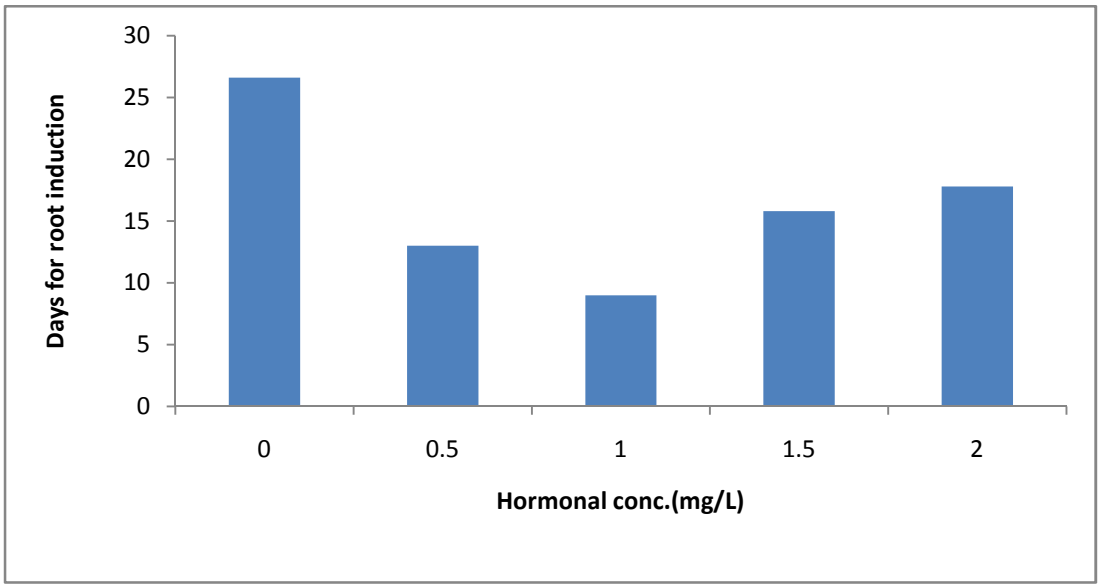

Figure 5: Effects of IBA on days for root induction in tobacco.

4.3.2 Number of roots per explants

To check the response and effectiveness of IBA on the number of roots a range of treatment 0.50 mg/L, 1.00 mg/L, 1.50 mg/L and 2.00 mg/L were applied and significant variation were observed during data recording at 14, 21 and 28 DAI. Roots regenerated in the medium containing IBA were thin and long even during initial stage of development. In At 14 DAI, the highest number of roots (3.2) was found with 1.00 mg/L IBA. The highest number of roots (4.60) was found with 1.00 mg/L IBA at 21 DAI. At 28 DAI, the maximum number of roots (6.40) was found with 1.00 mg/L IBA. The minimum number of roots (1.20, 1.6 and 1.8 at 14, 21 and 28 DAI, respectively) was obtained in control (Figure 6).

Geethalakshmi, *et al.* (2016) found 10 roots in tobacco in medium with IBA (0.50 mg/ L) in 8 weeks of time.

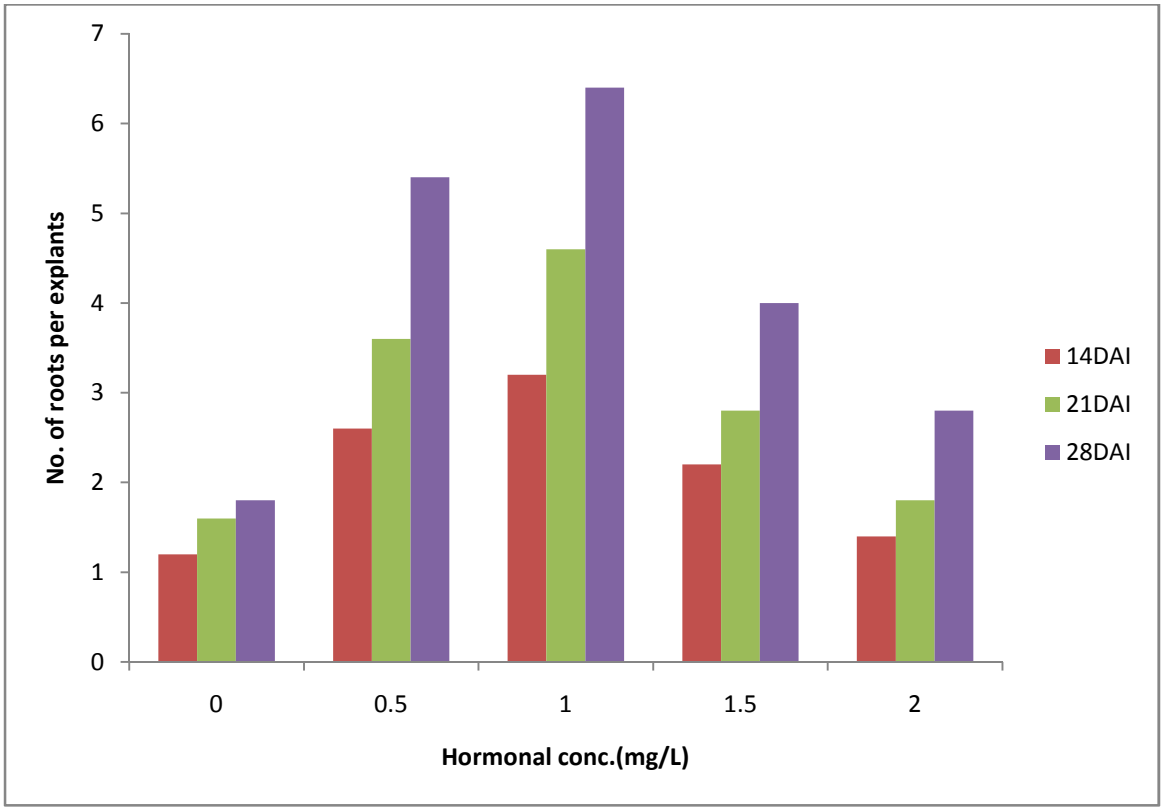

Figure 6. Effect of IBA on number of roots in tobacco.

4A:Number of root at 14 DAI. **4B: Number of root at 21 DAI.**

4C: Number of root at 28 DAI.

Plate 6: Number of root in tobacco in the treatment 1.00 mg/L IBA.

4.3.3 Length of roots per explants (cm)

Length of roots per explants (cm) was greatly regulated by the different concentration of IBA. The maximum root length (1.00, 4.12 and 5.10 cm at 14, 21 and 28 DAI respectively) was obtained from 1.00 mg/L IBA (Figure 7). The minimum length of roots per explants (1.40, 1.62 and 2.00 cm at 14, 21 and 28 DAI respectively) was in hormone free treatment.

Hashembadi, and Kaviani, (2010) obtained the maximum (8.75 cm) roots in tobacco were achieved on medium supplemented with 1.00 mg/L IBA + 1.00 mg/L NAA. Baksha, R. *et al.* (2005) noticed the highest average length of 3.50 cm with 0.50 mg/L NAA and 2.2 cm in IBA 1.50 mg/L.

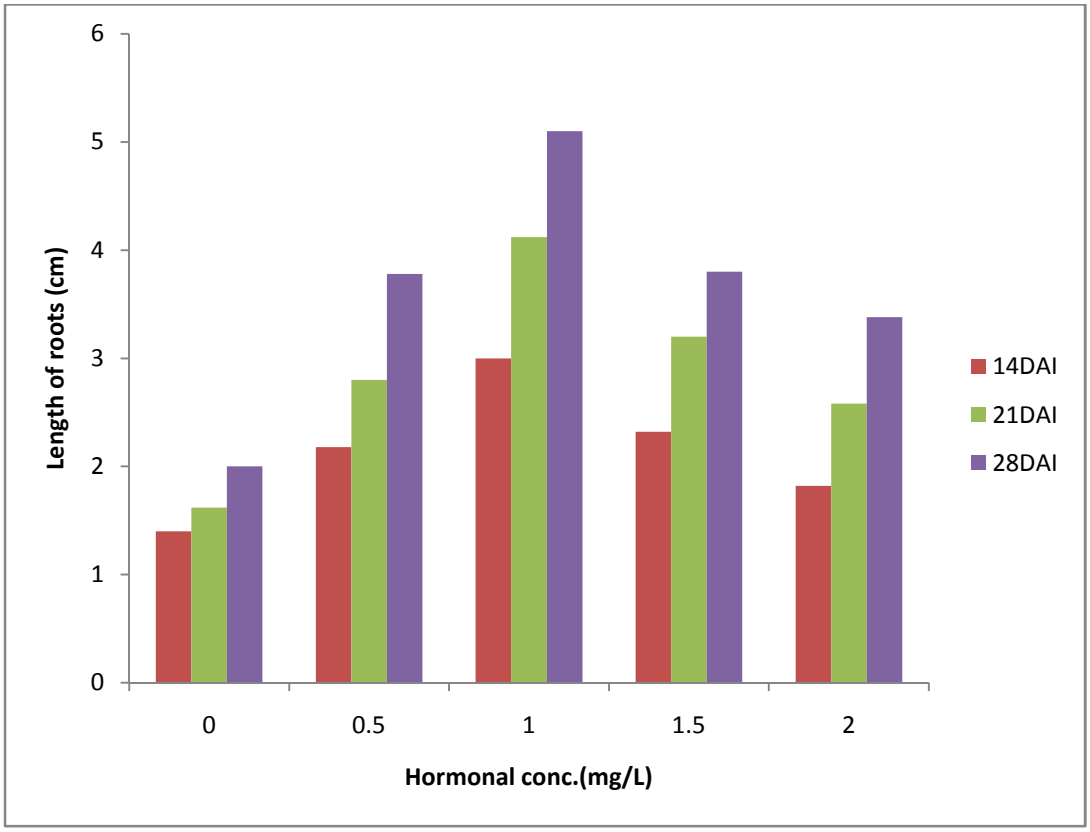

Figure 7. Effect of IBA on length of roots per explants (cm) in tobacco.

4.3.4 The combine effect of BA + IBA on multiple root formation

The result of the combined effect of different concentration of BA + IBA have been presented under following headings with Table 6-7.

4.3.4.1 Days for root induction

Variation was observed among different concentration of BA+IBA on days to root induction. The maximum days to root induction were recorded in control (29.28 days) and 3.00 mg/L BA+1.00 mg/L IBA required minimum 8.80 days, which was statistically similar with 1.00 mg/L BA+1.00 mg/L IBA, 2.00 mg/L BA+1.00 mg/L IBA and 4.00 mg/L BA+1.00 mg/L IBA (Table 6).

4.3.4.2 Number of roots per explants

Data were recorded after 14, 21 and 28 days of culture on MS media. There was significant influence of different concentration of BA+IBA on the number of roots per explants after 14, 21 and 28 Days of inoculation. The results have been presented in Table 6. The highest number of roots (3.33 and 4.8, at 14, 21 DAI respectively) was found in the treatment 3.00 mg/L BA+1.00 mg/L IBA, which was statistically similar with 1.00 mg/L BA+1.00 mg/L IBA, 2.00 mg/L BA+1.00 mg/L IBA, 4.00 mg/L BA+1.00 mg/L IBA at 14, 21 DAI, respectively. At 28 DAI, the highest number of roots (6.66) was found in 3.00 mg/L BA+1.00 mg/L IBA, which was statistically similar with 1.00 mg/L BA+1.00 mg/L IBA, 2.00 mg/L BA+1.00 mg/L IBA, 4.00 mg/L BA+1.00 mg/L IBA at 14, 21 and 28 DAI, respectively. the lowest number of roots (1.06) respectively was found with hormone free media.

Table 6. Combined effect of BA and IBA on root induction potentiality.

Name of the Hormones	Hormones concentration (mg/L)	Days for root induction	Number of roots per explants		
			14 DAI	21 DAI	28 DAI
MS 0(Control)	0	29.80 a	1.20 d	1.60 de	1.6 f
BA+IBA	1+0.5	18.00 ef	2.20 bc	2.60 bc	3.40 c
	1+1.0	9.00 i	3.10 a	4.60 a	6.66 a
	1+1.5	17.40 fg	2.20 bc	2.40 cd	2.80 cd
	1+2.0	18.20 def	1.60 cd	2.20 cde	2.80 cd
	2+0.5	12.20 h	2.40 b	3.20 b	4.20 b
	2+1.0	9.40 i	3.20 a	4.60 a	5.80 a
	2+1.5	16.40 g	1.40 d	1.60 de	2.60 cde
	2+2.0	17.00 fg	1.60 cd	2.20 cde	2.20 def
	3+0.5	19.60 cd	1.40 d	2.00 cde	2.40 def
	3+1.0	8.80 i	3.30 a	4.80 a	6.60 a
	3+1.5	19.40 cde	1.40 d	1.60 de	2.20 def
	3+2.0	19.60 cd	1.40 d	1.60 de	2.20 def
	4+0.5	20.00 c	1.40 d	1.40 e	2.00 def
	4+1.0	9.00 i	3.20 a	4.60 a	6.20 a
	4+1.5	22.80 b	1.20 d	1.40 e	1.60 f
	4+2.0	24.00 b	1.40 d	1.60 de	1.80 ef
LSD (0.05)		1.41	0.64	0.75	0.75
CV (%)		5.55	5.05	7.72	5.25

*DAI=Days 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.

4.3.4.3 Length of root (cm)

With different concentration of BA+IBA, significant influence was found on the length of root (cm) the results have been presented in Table 7. The maximum length of root (1.50, 4.60, 5.70 cm at 14, 21 and 28 DAI) was noticed from the 3.00 mg/L BA+1.00 mg/L IBA, whereas the minimum (1.38, 1.62, 2.04 cm at 14, 21 and 28 DAI) in control.

Table 7. Combined effect of BA and IBA on root induction potentiality.

Name of the Hormones	Hormones concentration (mg/L)	Length of roots (cm)		
		14 DAI	21 DAI	28 DAI
MS 0(Control)	0	1.38 h	1.62 i	2.04 f
BA+IBA	1+0.5	2.74 cd	4.14 bc	4.26 d
	1+1.0	3.00 bc	4.00 bcd	5.00 b
	1+1.5	2.66 cd	3.94 cd	4.08 d
	1+2.0	2.52 de	3.80 cd	4.00 d
	2+0.5	3.00 bc	4.20 abc	5.40 ab
	2+1.0	3.00 bc	4.00 bcd	5.40 ab
	2+1.5	2.98 bc	3.90 cd	4.74 c
	2+2.0	2.64 cd	3.64 de	4.18 d
	3+0.5	2.22 ef	3.28 ef	3.50 e
	3+1.0	3.50 a	4.60 a	5.70 a
	3+1.5	1.88 fg	3.02 fg	3.44 e
	3+2.0	2.00 fg	2.88 fgh	3.22 e
	4+0.5	1.90 fg	2.52 h	3.06 e
	4+1.0	3.20 ab	4.40 ab	5.60 a
	4+1.5	1.72 gh	2.70 gh	3.14 e
	4+2.0	1.68 gh	2.56 h	3.00 e
LSD (0.05)		1.39	1.39	1.45
CV (%)		12.3	9.9	11.4

*DAI=Days 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 7: Length of root in tobacco in the treatment 3.00 mg/L BA+1.00 mg/L IBA at 28 days after inoculation.

4.4 Sub experiment 4. Acclimatization of plantlets

The results of acclimatization or "hardening' have been presented in Table 8 and Plate 5. After 35 days of culture on rooting media, the plantlets were taken for acclimatization.

Table 8. Survival rate of *in vitro* regenerated plants of tobacco.

Acclimatization	No. of plants transplanted	Duration of observation	No. of plants survived	Survival rate (%)
In growth chamber	25	7days	24	96
In shade house	24	14 days	22	91.67
In field condition	22	30 days	18	81.81

The results of acclimatization showed that the 96% of plantlets were survived to growth chamber (Table 8). 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 91.67% of the plantlets showed survival (Table 8). After 3 weeks, the plantlets were transferred to the soil following depoting and poting into different pots of bigger size. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary (Plate 8). In open atmosphere, survival rate was 81.81% (Table 8). 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 transferred to the polyhouse, then 85% of the plantlets survived. After keeping them for initial 10 days in polyhouse, plants were transferred to shade house under less humidity. In shade house, 82% plantlets were 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, R. *et*

al.(2005) noticed well-developed rooted plantlets were successfully transferred to the soil with 70% survival.

Plate 8. After 14 days of transplanting.

Plate 9. Acclimatization of regenerated planted in field condition after 30 days.

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 evaluate the tissue culture of tobacco.

Shoot tip of young lateral shoot was used as an explant for *in vitro* regeneration of tobacco. The major findings have been presented below.

Significance variation was observed among the hormone on all parameter. The highest number of shoots (3.60, 5.20 and 7.00 at 14, 21 and 28 DAI respectively) and length of shoot (2.62, 3.98 and 4.92 cm at 14, 21 and 28 DAI respectively) was found in treatment 3.00 mg/L BA. The maximum number of leaves (3.00, 4.40 and 5.00 at 14, 21 and 28 DAI respectively) and length of leaf (1.12, and 1.58 cm at 14 and 21 DAI respectively) was recorded in treatment 2.00 mg/L BA. The highest length of leaf (2.14 cm) was obtained in treatment 1.00 mg/L BA at 28 DAI.

Variation was observed among different concentration of BA+IBA on days to shoot induction. The maximum days to shoot induction were recorded in control treatment (15.80 days) and treatment 3.00 mg/L BA+1.00 mg/L IBA required minimum 6.40 days. There was significant influence of different concentration of BA+IBA on the all parameter after 14, 21 and 28 Days of inoculation. The treatment 3.00 mg/L BA+1.00 mg/L IBA gave the highest number of shoots (4.6, and 6.20, 7.8 at 14 21, and 28 DAI, respectively). The maximum length of shoot (3.48, 4.12, 4.92 cm at 14 21, and 28 DAI, respectively) and number of leaf per explants (6.20, 8.20, and 10.60 at 14 21, and 28 DAI, respectively) was noticed in treatment 3.00 mg/L BA+0.50 mg/L. The maximum length of leaf (1.64 cm) was noticed from the treatment the 3.00 mg/L BA+1.00 mg/L IBA. At 21 DAI, The maximum length of leaf (1.82 cm) was noticed in treatment 2.00 mg/L BA+0.50 mg/L IBA. At 28 DAI, the maximum length of leaf (2.60) was in treatment 3.00 mg/L BA+1.00 mg/L IBA.

Hormonal concentration has significant level of variation on days for root induction. The maximum 26.6 days to root induction was required in media lack of growth regulator. To check the response and effectiveness of IBA on the number of roots and root length range of treatment (0.5, 1.0, 1.5and 2.0) were applied and significant variation were observed during data recording at 14, 21 and 28 DAI. The highest number of roots (3.2, 4.6, 6.40 at 14, 21 and 28 DAI, respectively) per explants and root length (1.00, 4.12 and 5.10 cm at 14, 21 and 28 DAI, respectively) was recorded in treatment 1.00 mg/L IBA.

There was significant influence of different concentration of BA+IBA on the number of roots per explants after 14, 21 and 28 Days of inoculation. The highest number of roots (3.33, 4.8, and 6.6 at 14, 21, 28 DAI respectively) and length of root (1.50, 4.60, 5.70 cm at 14, 21 and 28 DAI) was found in treatment 3.00 mg/L BA+1.00 mg/L IBA. Regenerated plantlets showed 96% survival during in growth chamber conditions and 91.67% in shade house stage of hardening and 81.81 % in open atmosphere. Regenerated plants were found to be morphologically similar to the mother plant. Findings of the present study showed that micropropagation is effective method in the proliferation of Tobacco and this experiment can be a useful tool for tissue culture of Tobacco.

CHAPTER VI

RECOMMANDATIONS

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 and IBA other types of cytokinin and auxin be taken into trial.

iii. Except shoot tip culture, meristem and callus culture could be practiced.

iv. To uncover the influence of genotype if any, research should be carried on with different type of genotype of tobacco .

REFERENCES

Aggarwal, D. and Barna, K.S. 2004. Tissue culture propagation of elite plant of *Aloe vera* Linn. *J. Plant Biochem. Biotech*. **13**: 77-79.

Altman, A. and Ziv, M. 1997. Horticultural biotechnology: A historical perspectiveand future prospects. *Acta Horticulturae*. **447**: 31-35.

Badoni, A. and Chauhan, J. S. 2009. Effect of Growth Regulators on Meristem-tip Development and *in vitro* Multiplication of Potato Cultivar 'Kufri Himalini'. Nature and Science, **7**(9):31-34.

Baksha, R., Jahan, M.A.A., Khatun, R. and Munshi, J. L. 2005. Micropropagation of *Aloe barbadensis* Mill. Through *In vitro* Culture of Shoot tip Explants. Plant Tissue Cult. & Biotech. **15**(2): 121-126.

BBS. 2016. Statistical Yearbook of Bangladesh. Bangladesh Bureau of Statistics. Planning Division, Ministry of Planning, Govt. Peop. Repub. Bangladesh, Dhaka. p. 142.

Bhandari, A.K., J.S. Negi, V.K. Bisht and M.K. Bharti. 2010. *In vitro* propagation of *Aloe vera*-A Plant with Medicinal Properties. *Nature and Science*. **8**(8):174-176.

Chao yanjie, 2011. Callus induction and plant regeneration From leaf explants of tobacco. *Class 2 of Biotechnology, College of Life Science and Technology, Huazhong Agricultural University,* Wuhan 430070, China.

Chen, Z. N.; Xing, J. L. and Bian H. J. et al. 2001. Application of cell engineering technology to the tumour immunotherapeutic drug. *Cell biology International*.Vol 25, Issue**10**:1013-1015.

Choudhary, A.K., Ray, A.K., Jha, S. and Mishra, I.N. 2011. Callus formation, shoot initiation and in vitro culture of *Aloe vera*. *Biotechnol. Bioinf. Bioeng*.**1**(4): 551-553.

Chukwujekwu, J.C. 2001. Micropropagation acclimatization of *Aloe polyphylla* and *Platycerium bifurcatum*. M.Sc. Thesis, University of Natal, Pietermaritzburg.

Daneshvar, M.H., Moallemi, N. and Abdolahzadeh, N. 2013.The effect of different media on shoot proliferation from the shoot tip of *Aloe vera* L. *Jundishapur J Nat Pharm Prod*. **8**(2):93-7.
Daneshvar, M.H., Moallemi, N. and Abdolahzadeh, N. 2013.The effect of different media on shoot proliferation from the shoot tip of *Aloe vera* L. *Jundishapur J Nat Pharm Prod*. **8**(2):93-7.

Doliński, R. and Olek, A. Micropropagation of sweet potato (*ipomoea batatas* (l.) lam.) from node explants. *Acta Sci. Pol., Hortorum Cultus* **12**(4) : 117-127.

Dwivedi, N.K., Indiradevi, A., Asha, K.I., Asokan Nair, R. and Suma, A. 2014. A protocol for micropropagation of *Aloe vera* L. (Indian Aloe) – a miracle plant. Research in Biotechnology. **5**(1): 01-05.

Ebad, F. A. S., Marwa EL-Sebai Abd EL-sadek and EL-Kazzaz A.A. 2015. Micropropagation of four potato cultivars *in vitro. Academia J. Agril. Res*. **3**(9): 184-188.

Flick, C. E., Evans, D. A., and Sharp, W.R. 1983. Organogenesis, in Handbook of Plant Cell Culture, vol. I (Evans, D. A., Sharp, W. R., Ammitato, P. V., and Yamada, Y. eds.), MacMillan, New York, pp. 13-81.

Freyssinet, G. 1986. Tobacco and biotechnology; resistance to herbicides, Annales du Tabe., Numero special 115-119.

Gantait, S., Mandal, N., Bhattacharya, S. and Das, P. K. 2010. A Novel Strategy for *in vitro* conservation of *Aloe vera* L. through Long Term Shoot Culture. Biotechnology .

Garner, W. W. 1951. The production of tobacco. First Edn. McGraw-Hill Book Company, Inc. New York. **pp**. 3-4.
Garner, W. W. 1951. The production of tobacco. First Edn. McGraw-Hill Book Company, Inc. New York. **pp**. 3-4.

Geethalakshmi, S., Hemalatha, B and Saranya, N.2016. Optimization of Media Formulations for Callus Induction, Shoot Regeneration and Root Induction in *Nicotiana benthamiana*. *J Plant Sci Res*.**3**(1): 150.

Gonzales, R. A. and Widhlom, J. M. 1985. Selection of plant cells for desirable characteristics: Inhibitor resistance, in *Plant cell culture-A practical approach.* edited by R A Dixon (IRL Press, Oxford).67-78.

Guma T., B., Jane K., Justus O. and Kariuki P. N.1998. "Standardization of in vitro sterilization and callus induction protocol for leaf explants of anchote : coccinia abyssinica", Int. J. Res. Dev. Pharm. L. Sci., **4**(2), pp. 1427-1433.

Hanus-Fajerska, E. and Ciarkowska, K. 2012. The effect of *Nicotiana tabacum* L. extracts on cultures of tobacco callus. Acta Sci. Pol., Hortorum Cultus **11**(4): 59-66.

Haque, S.M. and Ghosh, B. 2013. High frequency microcloning of *Aloe vera* and their true-to-type conformity by molecular cytogenetic assessment of two years old field growing regenerated plants. **54**: 46.

Hashembadi, D, and Kaviani, B. 2008. Rapid micro-propagation of *Aloe vera* L. via shoot multiplication. *African J. Biotechnology*. **7**(12): 1899-1902.

Hashembadi, D, and Kaviani, B. 2010. *In vitro* proliferation of an important medicinal plant *Aloe*- A method for rapid Production. *Australian journal of crop science. AJCS.* **4**(4):216-222.

Hesami, M. Najafabadi, M. Y. Alizadeh, M. 2018. Effect of plant growth regulators on indirect shoot organogenesis of *Ficus religiosa* through seedling derived petiole segments. *J. Genetic Engineering and Biotechnology*. **16**(1): 175-180

Hossain, A., Hassan, L., Patwary, A.K., Sultan, M.M., Ahmad, S.D., Shah, A.H and Batool, F. 2010. Establishment of a suitable and reproducible protocol for in vitro regeneration of ginger. Pakistan Journal of Botany **42**(2): 1965-1074,

Husseyg. 1979. Tissue culture and its application to plant propagation. Plantsman. **1**: 133-145.

Ilahi, H and Jabeen, N. 1987. Micropropagation of Zingiber officinale. Pakistan Journal of Botany **19**: 61-65.

Jafari, N.A. and Hamidoghli, Y. 2009. Micropropagation of thornless trailing blackberry (*Rubus sp*). by axillary bud explants. *Aust. J. Crop Sci*. **3**(4): 191-104.

Kaplan, D.R. and Hagemann, W. 1991.The relationship between cell and organism in vascular plants: Are cells the building blocks of plant form. *BioScience,***41**: 693-703

Korstash, M. A. and I. F. Kanevsku. 1987. Asymmetrical hybrids of *Nicotiana* obtained by protoplast fusion. Gametraya Zigotnaya selektsiya rastenu. Respublikankaya Konferentsiya, 161-163.

Kumawat, N. 2013. *In vitro* Regeneration in Ghritkumari (*Aloe barbadensis* Mill.). M.Sc. Thesis, Swami Keshwanand Rajasthan Agricultural University, Bikaner.

Lee, Y.S., Yang, T.J., Park, S.U, Baek, J.H., Wu, S.Q. and Lim, K.B. 2011. Induction and proliferation of adventitious roots from *Aloe vera* leaf tissues for *in vitro* production of aloe-emodin. *Plant Omics Journal, POJ*. **4**(4):190-194..

Ling, A.P.K., Tan, K. P. and Hussein, S. 2013. Comparative effects of plant growth regulators on leaf and stem explants of *Labisia pumila* var. *alata* . J Zhejiang Univ Sci B. **14**(7): 621–631.

Lobine, D., Soulange, J. D., Sanmukhiya, M.R. and Lavergne, C. 2015. A Tissue Culture Strategy Towards The Rescue Of Endangered Mascarene Aloes .Arpn *Journal of Agricultural and Biological Science*. **10**(1): 1990-6145.

Minaei, H. Kahrizi, D. and Zebarjadi.A. 2013. Effect of Plant Growth Regulators and Explant Type upon Cell Dedifferentiation and Callus Induction in Chickpea (Cicer arietinum L.). *J. Applied Biotechnology Reports*, **2** (2):241-244.

Mohammed, A and Quraishi. 1999. Clonal propagation of ginger through shoot tissue culture. Pakistan Journal of Biological Sciences **2**: 145-147.

Mohebodini, M. Javaran, M. J. Mahboudi, F. and Alizadeh, H. 2011. Effects of genotype, explant age and growth regulators on callus induction and direct shoot regeneration of Lettuce (Lactuca sativa L.). AJCS **5**(1):92.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant,***15:** 473-479.

Nayanakantha, N.M.C., Singh, B.R. and Kumar, A. 2010. Improved culture medium for micropropagation of *Aloe vera* L. Tropical Agricultural Research & Extension. **13**(4): 2010.

Nikova, V. and N. Zagorzka. 1984. Variability of regeneration obtained in tissue cultures from the hybrid Nicotiana velutiana wheeler *Nicotiana tabacurn* h. 3-Nats-Konf.- tistogenet.- uchastie,-Plovdiv,-Okjit-8- 12,-1984-TI. 191-194.

Nikova, V., M. Petkova, and N. Zagorsk. 1988. Overcoming incompatibility between *Nicotiana qfricana* Marxi and *Nicotiana tabacum* by *in vitro* techniques. Genetika-i- selektsiya. **4**(4): 283-289.

Patel, D. and Sail, S.S. 2012. Studies seed tissue culture and organogensis of *Aloe vera*. *J. Cell and Tissue Res.* **12** (2): 3241-3244.

Rahman, M. A., Alam, M.A., Hossain, M.R., Hossain, A. and Afroz, R. 2010. *In vitro* regeneration of popular tobacco varieties of bangladesh from leaf disc. *Bangladesh J. Agril. Res*. **35**(1): 125-134.

Rajora, R. K. Sharma, N. K. and Sharma, V. 2013. Effect of plant growth regulators on micropropagation of *Catharanthus roseus.* Intel. J. Advanced Biotech. and Res. **4**(1) :123-130

Sanger, R. B. S., R. Chandr, and S. M. P. Khurance. 1986. Regeneration of TMV-free tobacco plantlets through callus. *Indian J. Plant Pathol*. **4**(1): 80-82.

Shahab-ud-din, M., Sultan, N., Kakar, M.A., Yousafzai, A. and Sattar, F.A. 2011. The Effects of Different Concentrations and Combinations of Growth Regulators on the Callus Formation of Potato *Solanum tubrosum* Explants. *Current Res. J. of Biol. Sci*., **3**(5): 499-503.

Shukla, M.R., Jones, A.M.P., Sullivan, J.A., Liu, C.Z., Gosling, S., Saxena, P.K. 2012. *In vitro* conservation of American elm (*Ulmus americana*): potential role of auxin metabolism in sustained plant proliferation. *Can J Forest Res*. **42**:686–697.

Silva, JAT Da., D. T. Nhut, M. Tanaka, S. Fukai, and J. A. T. Da. Silva. 2003. The effect of antibiotics on the *in vitro* growth response of chrysanthemum and tobacco stem transverse thin cell layers (TCLS). *Scientia Hort.* **49**(3-4): 397-4 10.

Skoog, F. and C. 0. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro. Symp. Soc. Exp. Biol.* ii: 118-130.

Sultana, A., Hassan, L., M.M., Ahmad, S.D., Shah, , A.H., Batool, F., Islam, M.A., Rahman, R and Moonmoon, S. 2009. In vitro regeneration of ginger using leaf, shoot tip and root explants. Pakistan Journal of Botany **41**(4): 1667-1676.

Thorpe, T.A. and Murashige, T. 1970. Some histochemical changesunderlying shoot initiation in tobacco callus culture. Canadian Journal of Botany. **48**:277-285.

Vasil, V. and A. C. Hildebrandt. 1965. Differentiation of tobacco plants from single isolated cells in micro cultures. *Sci*. **150:** 889-892.

Veluthambi, K, Gupta, A.K. and Sharma, A . 2003. Current status of plant transformation technologies. Current Science. **84**:368-380.

White, P.R. 1937. Survival of isolated tomato roots at sub-optimal and supra-optimal temperatures. Plant Physiology. **12**:771-776.

Yokoya, N. S., West, J.A. and Luchi, A. E. 2006. Effects of plant growth regulators on callus formation, growth and regeneration in axenic tissue cultures of *Gracilaria tenuistipitata* and *Gracilaria perplexa* (Gracilariales, Rhodophyta). Phycological research. 27.

Zhang, Z. Q., Y. Zhou, Y. H. Zhang, W. J. Zhong, J. J. Zhang and L. Q. Yin. 1998. Induction of adventitious buds and regeneration plants from cotyledons of Chinese cabbage *(Brassica campestris* L. spp.). *Acta Agric. Shanghai.* **14**(2): 25-28.

Zhou, R.J. and Liu, M.J. 2009. Effect of plant growth regulators on tissue culture in chinese jujube. Acta Hortic. **840**: 309-314.

APPENDICES

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

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

Total concentration of Micro and Macro elements including vitamins: 4405.19 mg/L
Manufacturing Company: Duchefa Biochem