STUDY ON POTATO (*Solanum tuberosum* L.) REGENERATION CAPABILITY IN AMMONIUM NITRATE FREE MEDIUM COMPOSITION

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CERTIFICATE

*This is to Certify That The Thesis Entitled "***Study on *Potato (Solanum Tuberosum L.) Regeneration Capability in Ammonium Nitrate Free Medium Composition"*** submitted to The Department Of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, In Partial Fulfillment Of The Requirements For The Degree Of Master Of Science In Biotechnology Under Agriculture Faculty, Embodies The Results Of A Piece Of Bonafide Research Work Carried Out By **Hasna Hena**, Registration No.**11-04595** Under My Supervision And Guidance . No Part Of The Thesis Has Been For Any Other Degree Or Diploma.*

I Further Certify That Any Help Or Source Of Information, Received During The Course Of This Investigation Has Been Duly Acknowledged.

Dated: June, 2017
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Dedicated to my beloved and sweet family

ABBREVIATIONS AND ACRONYMS

Acad.	:	Academia
Agril.	:	Agriculture
Am.	:	American
Annu.	:	Annual
Appl.	:	Applied
Biochem.	:	Biochemistry
Biosci.	:	Bioscience
Biotechnol.	:	Biotechnology
Biol.	:	Biological
Bot.	:	Botany
Cm	:	Centimeter
CRD	:	Completely Randomized Design
Cult.	:	Culture
Curr.	:	Current
CV	:	Co-efficient
DAI	:	Days After Inoculation
et al	:	And others
BAP	:	6-BenzylAmino Purine
KIN	:	Kinetin
IAA	:	Indoleacetic acid
NAA	:	*a*- Napthalene acetic acid
2,4-D	:	2,4- Dichloro phenoxy acetic acid
Int.	:	International
J.	:	Journal
mg/L	:	Miligram per litre
Microbiol.	:	Microbiology
MS	:	Murashige and Skoog
Org.	:	Organ
Physiol.	:	Physiology
Rep.	:	Report
Res.	:	Research
Rev.	:	Review
Sci.	:	Science
Tiss.	:	Tissue
^{o}C	:	Degree Celsius
etc.	:	Etcectera

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The Author

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STUDY ON POTATO (*Solanum tuberosum* L.) REGENERATION CAPABILITY IN AMMONIUM NITRATE FREE MEDIUM COMPOSITION

ABSTRACT

The experiment was conducted at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh during the period of January, 2016 to June, 2017 to evaluate the performance of ammonium nitrate free medium for *in vitro* regeneration of a potato (*Solanum tuberosum* L.) variety, Asterix. Sprouts and nodal segments were used as explants. A new chemical which is indicated as β chemical had been used in this research. Five different compositions of the stock solution-01 of the MS medium were used which were marked as Treatment-01 (standard MS dose, 1962), Treatment-02 (NH_4NO_3 free stock solution), Treatment-03 (NH_4NO_3 free stock solution with double dose of other ingredients), Treatment-04 (stock solution-01, developed in the Department of Biotechnology, SAU, Dhaka) and Treatment-05 (readymade MS powder). *In vitro* regeneration of a potato was successfully achieved in those media. The parameters like shoot length, shoot number, shoot diameter, node number per plantlet, leaf number per plantlet were highest in Treatment-04 at 14, 21 and 28 days of regeneration. But in case of root number and root length (cm) per plantlet, Treatment-05 showed the highest result and in some cases it was statistically similar with Treatment-04. Treatment-02 showed lowest result in each of the said parameter. It might be due to the low concentration of nitrogen and it indicated that NH_4NO_3 is essential for *in vitro* regeneration of potato. Treatment-03 performed moderately in most of the parameters. Asterix variety showed significant variation in case of Treatment-01 and Treatment-05 on most of the parameters under studied. Therefore, a relliable medium composition for *in vitro* potato regeneration has been established. So, the stock solution-01 which was developed from the Department of Biotechnology, SAU, Dhaka can be the best alternative to NH_4NO_3 in tissue culture study of plants.

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum L.)* is one of the world's most versatile vegetables which is a tuberous plant under the Solanaceae family. It is eaten as a starchy vegetable, particularly in the America and Europe. Potato is the fourth most consumed crop in the world, behind rice, wheat and corn, according to the U.S. Department of Agriculture (Moeinil *et al.*, 2011). It is known for high yield, high water content, valuable starch content, for a range of industrial purposes and also for remunerative income to the growers. This vegetable is native to the Americas, most likely in the Andes, Peru and Bolivia. The wild potato species can be found throughout the Americas from the United States to southern Chile (Hijmans and Spooner, 2001) The potato was originally believed to have been domesticated independently in multiple locations but later genetic testing of the wide variety of cultivars and wild species proved a single origin for potatoes in the area of present-day southern Peru and extreme Northwestern Bolivia, they were domesticated approximately 7,000–10,000 years ago (Spooner *et al.*, 2005 and Francis, 2005). Potato is foundational in a wide range of international and all-American cuisine. The total world potato production is estimated at 381,682,000 tonnes in 2014 (FAOSTAT, 2017). In the last fiscal year (FY'16), Bangladesh's potato production hit an all-time-high of 9.47 million tones on 4.75 million hectares in FY'16, against 9.254 million tonnes on 4.62 million hectares in FY'15 (BBS, 2016).

Potatoes are one of the most common and important food sources on the planet and they contain a wealth of health benefits that make them all the more essential as a staple dietary item for much of the world's population. These health benefits include their ability to improve digestion, reduce cholesterol levels, boost heart health, protect from polyps, prevent cancer and manage diabetes. They strengthen the immune system, reduce signs of aging, protect the skin, increase circulation, reduce blood pressure, maintain fluid balance, reduce insomnia and aid in eye care. Potatoes supply at least 12 essential vitamins and minerals including an extremely high content of vitamin C. Potatoes also provide significant amounts of protein and iron (Gray and Hughes, 1978). Our food ranking system qualified potatoes as a very good source of vitamin B6 and a good source of potassium, copper, manganese, phosphorus, niacin, dietary fiber and pantothenic acid. Potatoes also contain a variety of phytonutrients that have antioxidant activity. Among these important health-promoting compounds are carotenoids, flavonoids and caffeic acid as well as unique tuber storage proteins, such as patatin, which exhibit activity against free radicals.Potatoes are more energy-packed than any other popular vegetable and have even more potassium than a banana. Potatoes are naturally gluten-free and they're packed with nutritional benefits. As the potato is a root vegetable, it is rich in essential minerals that help you build strong bones and benefit your nerve and muscle function. UK scientists at the Institute for Food Research have identified blood pressure-lowering compounds called kukoamines in potatoes.

In spite of having all these extraordinary advantages, production of potato is yet to reach its maximum production potential. There are storage cost problem which is higher than the production cost. At present two-thirds of the total produce do not find even any space in the cold storage and a part of which is consumed shortly after harvest and the rest is kept in traditional storage at home under room temperature and humidity at farm level. Most cases the excess production goes to waste.

Potato being vegetatively propagated crop, is prone to accumulation and further spread of several diseases affecting its yield and quality. The bigger reason for its poor quality productivity i.e. viral diseases, the most common contributing factor (Wambugu, 1991) and the major cause of cultivar decline. Healthy planting material has essential role in potato production chain. Clonal selection (a conventional seed production method), used for decades, requiring intensive process control and seed programmes based on clonal selection could take 10 years and more. Other propagation techniques have been developed and involved in seed production systems, thus decreasing time needed for seed multiplication. These techniques include obtaining good quality seeds through virus eradication using meristem culture, rapid *in vitro* plants multiplication and increased number of individuals of the first year clones through minitubers production (Struik and Wiersema, 1999). This *in vitro* plants multiplication techniques or tissue culture techniques are used worldwide to produce pre-basic, virus-free seed potatoes known as micro tubers. In addition, *in vitro* methods can be used for conservation, storage and easy distribution of potato germplasm in the form of breeding lines, new varieties and micro tubers. Because of their small size and weight, micro tubers have tremendous advantages in terms of storage, transportation and mechanization. They can be directly sown into the soil and can be produced in bulk in any season. They have the similar morphological and biochemical characteristics to field produced tubers. Therefore, mass production of potato micro tuber is likely to revolutionize the world potato production (Majid *et al.*, 2014).

Nowadays potato can be rapidly multiplied using nodal cuttings produced *in vitro* and involving following microtubers production. Methods, protocols and conditions to produce *in vitro* plantlets vary across laboratories, as well as methods for obtaining first generation potato seed tubers can be rather different, thus resulting in diverse effects. However, there were many reported media formula which were used for *in vitro* propagation of potato (Badoni and Chauhan, 2010; Molla *et al.*, 2011; Motallebi-Azar *et al.*, 2011; Koleva *et al.*, 2012; Qureshi *et al.*, 2014 and Khadiga *et al.*, 2015).

The program on plant biotechnology in Bangladesh was initiated in late 1970s in the Department of Botany, University of Dhaka with tissue culture of jute. Thereafter within a span of 10-12 years tissue culture research laboratories had been developed in different universities, R&D organizations, private entrepreneurs. A few NGOs are working on plant tissue culture. BRAC & PROSHIKA have already marketed tissue cultured plantlets such as potato, banana and ornamental plants in Bangladesh and neighboring countries.

According to the Tuber Crop Research Center of Bangladesh Agricultural Research Institute (BARI), there are 45-50 tissue culture labs in Bangladesh which are using plant tissue techniques to solve the potato production problems thereby increasing the national average yield of potato in Bangladesh . Some of the public and private organizations conducting potato tissue culture are- Bangladesh Agricultural Research Institute (BARI),Gazipur; Bangladesh Council of Scientific and Research (BCSIR), Dhaka; National Institute of Biotechnology (NIB), Savar; Bangladesh Agricultural University (BAU), Mymensingh; Sher-e-Bangla Agricultural University (SAU), Dhaka; Bangabandhu Shiekh Mujibur Rahman Agricultural University (BSMRAU),Gazipur; Potuakhali Science and Technology University, Potuakhali; Jahangirnagar University (J.U),Savar; Rajshahi University, Rajshahi; Chittagong University, Chittagong; BRAC tissue culture centre, Square Agro- Biotech Division, Giant Agro Ltd., Ejab Agro Industries Ltd., North Bengal Agro Farms Ltd., Lal Teer Seed Ltd., Kishan Botanix Ltd. etc.

Successful plantlets production of different species is achieved on media of different compositions. In this research we will try to identify proper modified culture media which supply a better number of healthy plantlets that will support to reach our agronomic potential. Various mineral formulations are available to culture plant tissues. The major media include MS medium (Murashige and Skoog medium) and Gamborg's B5 medium. Generally, the plant tissue culture media are made up of macro- and micronutrients, vitamins, as sucrose. The nutrient media can be prepared by mixing stock solutions of various chemical ingredients or from commercially available ready mixed powder as recommended by the manufacturers. Among the major salts,very important one is ammonium nitrate (NH_4NO_3) which is now banned from using due to its destructive property. Ammonium nitrate is a rich source of nitrogen and also a strong oxidizing agent. Hence, it is widely used for agricultural as well as blasting purpose. So both the advantages and disadvantages are associated. Using and purchasing this chemical is getting difficult day by day, owing to its unethical use by antisocial entities. It is one of the cheapest crop nourishing fertilizer type and hence it is easily available in the markets and agricultural stores as well. This is the reason why this chemical is easily available for antisocial entities, they can use it the way they want and disturb the social strata. Ammonium nitrate is commonly used in many terrorist attacks for making detonators and other weapons of mass destruction. Apart from being used in suicide bombing, there is always the danger of ammonium nitrate decomposition. If it comes in contact with fire or high temperature, it can explode on its own. Bangladesh, India, Afghanistan and Pakistan have imposed ban on its manufacture, sale and purchase. So not even a small amount is available in Bangladesh. As it has always been prone to misuse, uses of alternate fertilizers and a strict control over the availability of this powerful oxidizing chemical is the only way to control such misuse and get the highest profit. Readymade MS powder can also be used but it is too much expensive. Therefore our aristocratic Professor Dr. Md. Ekramul Hoque, Department of Biotechnology, Sher-e-Bangla Agricultural University decided to idealize a cheap, environment friendly, replacable chemical of Ammonium Nitrate which may be increased concentration of Potassium Nitrate, Ammonium Sulphate or any other chemical. In our research we have used potato because it is the most amenable to tissue culture

manipulation. Cell and tissue culture technology has been the most pronounced in potato compared to any other crop species over the past 50 years. We are trying to see if the regeneration is possible by replacing NH_4NO_3 with another chemical or increasing the concentration of another major salts.

Taking all these mentioned topics into account, the present research has been carried out with the following objectives in view:

- To study the regeneration potentiality of potato in Ammonium nitrate free medium composition,
- To identify the macro nutrient dose for potato regeneration,
- To evaluate *In vitro* regeneration efficiency of potato in different modified macro nutrient formulation,.
- To study the efficiency of a new chemical as a component of stock solution- 01.

CHAPTER II

REVIEW OF LITERATURE

2.1 Idea of *In vitro* regeneration

In vitro regeneration refers to growing and multiplications of cells, tissues and organs on defined liquid or solid media under aseptic and controlled environments. Recent progress in the field of plant tissue culture determined this area to be one of the most dynamic and promising for experimental biology. Objective of this process is to establish an efficient plant regeneration system for rapid propagation and genetic transformation. Tissue culture is considered as a very promising technique for both large-scale clonal propagation of plants and genetic engineering of plant germplasm. This technique has opened a new frontier in agricultural science by addressing food security through biotechnological methods for genetic improvement

2.2 MS medium composition

The investigation for a particular morphogenic response is of great relevance with the proper adjustment of media components. In general, MS (Murashige and Skoog) medium has been used for various *in vitro* growth purposes which was developed for tobacco pith callus. It was suggested that nutrient level in MS media sometimes higher than the required for optimal plant growth (Pierik, 1987). In most of the cases the prescribed MS media are used for various *in vitro* plant growth purposes but the actual elementary investigation is sometimes necessary for a particular developmental study. This research was set to study the regeneration potentiality of potato in ammonium nitrate free medium composition. Different doses and composition of macro salts were used in the present study to study the efficiency of these composition on plant regeneration. The related literatures of this research are inspected under the subsequent heads:

2.3 Review on potato micropropagation

Micropropagation technique permits a huge amount of asexual multiplication of pathogen free tested potato cultivars. Considerable research has been done on the nutritional, hormonal and physical aspect of the culture media and their effects on explants growth. Murashige and Skoog medium is most widely used for potato micropropagation. Semisolid medium is used for initial nodal segment propagation; however liquid medium fosters higher growth rate of potato micro shoots (Rosell *et al.*, 1987). *In vitro* derived microplants can be used as explants source for the production of microtubers *in vitro*, direct transplants in the greenhouse for the production of minitubers, mother plants for further *in vitro* multiplication through single node cuttings and source material for production of synthetic seed.

Mohapatra and Batra (2017) presented a review work on differnt aspects of tissue culture of potato. *In vitro* regeneration process is a commercially viable method for clonal propagation of a wide range of herbaceous and woody plants (Garcia *et al.*, 2010). This technique has been proved to be very effective technique to produce high quality pathogen-free plantlets, in terms of genetic and physiological uniformities (Sathish *et al.*, 2011; Supaibulwattana *et al.*, 2011).

For large scale production of uniform, identical seed material of potato, micro-propagation can be the better alternative over conventional propagation of potato. Potato virus free clones with meristem culture methods were conducted by Nagib *et al.* (2003). The organ that is to serve as tissue source, depends upon the physiological or ontogenic age of the organ, the season in which the explants is obtained, the size of explants and overall quality of the parent plant from which the plant is being obtained (Murashige, 1974).

2.4 Explants for potato tissue culture

Usually, explant is the controlling facor for a effective propagation programme. The efficiency of micropropagation depends on a source of explants and explants itself, treatment of explants while preparing them for *in vitro* culture, composition of culture media, routes of micro propagation followed, and performance of regenerated plantlets (Mohapatra and Batra, 2017). *In vitro* culture of organs (shoot tips, root tips, runner tips, stem segments, flowers, anthers, ovaries, ovules, embryos etc.) tissues, cells and protoplasts is done in propagation technique. In potato, various tissues can be used as explants for shoot generation directly (Anjum and Ali, 2004b). Mohamed *et al.*, (2009) used potato single node as an explant for his experiment. Potato tubers were also used as an explants source (Mutasim *et al.*, 2010).

The use of single-node cuttings excised from tissue cultured plantlets is more common and avoids the influence of tuber tissue from which sprout sections originate (Mohamed and Alsadon, 2010). Nodal cuttings were also used for auxiliary shoot development and suggested to be the best explants source by several researchers (Roca *et al.*, 1978; Hussey and Stacey, 1981) on either liquid or agar solidified medium.

2.5 Disinfection process

For a successful plant regeneration process, sterilization is an important step. It is done before inoculation process. All operations should be carried out in laminar airflow sterile cabinet (Chawla, 2003). Different sterilization agents like $HgCl_2$ (0.1%), NaOCl (5.25% v/v approx.) and 70% ethanol etc. can be used. Yasmin *et al.* (2011) used dissected segments of sprouts as the experimental plant material and were surface sterilized with 10% commercial bleach containing three drops of polyoxyethylene sorbitan monolaurate (Tween-20) for 10 minutes.

Hoque (2010) has practiced sterilization treatment for Solanum tuberosum, which includes the surface sterilization by dipping in 0.5 $HgCl_2$ solution for 3-5 minute and then washed 6-7 times with autoclaved distilled water. Badoni and Chauhan (2010) surface sterilized the explants of potato by treating them with sodium hypochlorite (0.1%) for 8 minutes, followed by 5 minute wash of savlon, and 30 second wash of 70% alcohol, at last 6-7 wash of distilled water followed by every treatment.

2.6 *In vitro* regeneration of potato using modified medium by growth regulators

In a paper, Kikuta and Okazawa (1982) reported the results of studies on the plantlet regeneration in potato tuber tissue cultures *in vitro* research designed to establish culture conditions and controlling factors for shoot-bud formation in excised tuber tissue followed by plantlet regeneration.The amount of medium ingredients adopted for the medium for shoot-bud formation in potato tissue cultures was designated as ZIG medium. Discs (1 x 6 mm, diameter) excised from tubers of potato (*Solanum tuberosum* L. cv. Irish Cobbler) were induced to differentiate *in vitro*, producing shoot-buds or callus cultures in an illuminated growth chamber . The discs were cultured on the nutrient agar medium in 125-ml flasks in a growth chamber at 25°C under 16-hr photoperiod with an intensity of 4,000 Ix (roughly 1,600 fiw/cm2 of irradiance) provided by cool white fluorescent tubes (Sylvania-NEC FL 20 SWj100 V). After 8 weeks of cultivation, shoot-buds produced in potato discs were examined.

The basal nutrient medium (ZIG medium) consisted of inorganic salts according to Okazawa *et al.* (l967), vitamins according to Nitsch and Nitsch (1967), casamino acids, adenosine, mannitol, and agar. Whilst carbohydrate was supported by self-stored starch. Zeatin and indole-3-acetic acid added to the medium induced shoot-buds in potato discs, but the other cytokinins tested did not. Gibberellic acid was effective for shoot-bud induction when discs were excised from the freshly harvested tubers. Plantlets were readily regenerated in the same medium, upon transferring to jiffy-mix and vermiculite bed where they were possible to produce small tubers as plants grew longer.

Badoni and Chauhan (2009) conducted a comparative study on the effect of different hormonal combinations of GA_3: NAA and Kinetin: NAA with MS medium on *in vitro* shoot regeneration of potato cv. Kufri Himalini using meristem tips. In this study the shoot development was studied in terms of different parameter.The best combination of hormones with MS medium was selected and which cultures showed higher growth were further sub-cultured on its parent medium by cutting it in to small pieces in a way that each subsection have at least 1-2 nodes. They concluded that GA_3 + NAA combination is best for shoot regeneration and multiplication of potato cv. Kufri Himalini in comparison to the combination Kinetin + NAA with M. S. Medium.

Molla *et al.,* (2011) reported that *in vitro* regeneration of potato is easily done from different explants on MS medium supplemented with different auxin and cytokinin for diseases free good quality seeds and pathogen free planting materials. They also reported that among the BAP, TDZ (Thidiazuron) and ZR (Zeatinriboside), ZR showed the very good performance in respect of direct regeneration from potato explants.

However, Koleva *et al.,* (2012) conducted a study in which the effect of cytokinins and combination of cytokinins and auxins on *in vitro* microtuber formation and growth of two potato cultivars i.e. Agrija and Andrea were evaluated with the objective of standardizing the media for potato plant growth and microtuber induction. They used MS medium, supplemented with different hormonal combinations where sprouts and nodal explants of potato cultivars were cultured. For sprouts, MS + 4 mg/l KIN and MS + 2 mg/l BAP were Lused as an initial explants, and for development of nodal explants, MS + 4mg/l KIN + 1mg/l IAA and MS + 2 mg/l BAP+1 mg/l NAA were used. For rapid sprouting clean potato tubers were *in vivo* treated with 2 ppm GA_3 which was efficient for the two cultivars. All treated tubers resulted with *de novo* a sprout, which shows effect of 100.00% sprouts formation. A higher number of sprouts were formed from the cultivar Agrija with average of 9.66 sprouts per tuber.

Between the two different explants (nodal segment and sprout) nodal cutting showed the better microtuber formation. The nodal segments as starting explants demonstrated higher efficiency compared to the sprouts. The composition of MS with cytokinin and auxin has shown the best effect, especially MS + 2 mg/l BAP + 1 mg/l NAA where the cultivar Agrija showed greater ability for *in vitro* propagation, with 2.14 tubers per shoot and formed 13.33% microtubers. Agrija cultivar showed maximum potential also for rooting and shoots formation. On MS + 2 mg/l BAP both parameters show 100.00% rooting and formation of start explants.On the tested media the cultivar Agrija has higher potential for *in vitro* micropropagation and microtuberisation. Their study also proved that *in vitro* tuberization capacity of potato depends on the genotype (Koleva *et al.,* 2012).

Chaudhary and Mittal (2014) carried out an investigation to optimize the best combination of growth regulators (GRs) for the multiplication of local potato cultivars K.CH3(Kufri. CHIPSONA 3) and K.Jyoti. (Kufri Jyoti) using stem cutting, internode as explants; the most regenerative variety could be then efficiently micropropagated for commercial purposes and molecular studies. The explants were routinely sub-cultured every 4 weeks on a fresh MS medium , supplemented with 3% sucrose. The cultures were maintained at 23±2 ºC under 300 footcandle for 16 hr lights per day. In this investigation, a high frequency single step and a simple method of direct plant regeneration has been developed for the two potato cultivars. Internodes were excised from field grown plants and cultured on MS media supplemented with different concentrations and combinations of auxins (NAA and IAA), cytokinins (BAP and Zeatin) and GA_3. This study was aimed to evaluate the effect of different growth regulators

(GRs) in seven different combinations named as T1, T2, T3, T4, T5, T6 and T7 along with control T0 (no growth regulators) on mass propagation of two potato cultivars K.CH3 and K.Jyoti. The best regeneration of internodes was obtained when MS medium was supplemented with 1.0 mgL-1 GA_3, 0.01 mgL-1 IAA and 2.0 mgL-1 Zeatin (T2). This combination took minimum time for regeneration of multiple shoots and roots on internodes of cultivar K.CH3. Further, with either decrease, increase or change the combination of growth regulators from the optimum level, a significant decline in percent plant regeneration as well as number of shoots per explant was recorded.

On the other hand, Khadiga *et al.,* (2015) conducted a research to induce *in vitro* microtuber from two potato cultivars grown *in vitro*, to assess the effect of 6-benzylaminopurine (BAP), thiadizuron (TDZ) and sucrose on *in vitro* micro tuber induction of potato (Solanum tuberosum L.) plant under two *in vitro* culture conditions (darkness and light). They used plantlets from Almera and Diamant which were cut to 1.0-2 cm long segments, each with about two nodes (2 axillary buds), incubated on Murashige and Skoog MS medium in order to form micro tubers under two *in vitro* culture conditions (darkness and light). MS media with 6 and 8% sucrose without hormone or supplemented with thiadizuron (TDZ) and benzylaminopurine (BAP) each alone at two concentration (5.0 and 8.0 mg/l) and the two sucrose concentration (6% and 8%). Highest micro tubers number (6.0±0.5 micro tuber/jar) obtained by Almera on MS medium verified with sucrose 8% only under dark, whereas higher micro tuber number obtained by Diamant cultivar is (3.0±0.0 micro tuber/jar) on MS medium verified with sucrose 8% only at dark too. Highest micro tuber number is related to high sucrose concentration than the level of growth hormones in the medium. It was also observed that twenty four hour dark was best for tuber initiation. The result indicated that micro tuber induction of potato was highly dependent on sucrose concentration, dark, growth regulators and genotype interaction.

Ebad *et al.,* (2015) carried out an experiment with the aim of presenting easy protocol for *in vitro* induction of potato plantlets stocks free of pathogens which will be used for selection under abiotic stress. In their study sprouts of four potato genotypes named Lady Rosetta, Jaerla, Cara and Hermis were used. Three concentrations of disinfectant bleach (Clorox) 15, 20, 25% with two exposure time 15 and 20 min were used for disinfecting the isolated potato sprouts. It was found that, as simplest disinfection protocol, concentration 20% Clorox was the suitable one at 20 min of exposure time giving high percentages of survived individuals with low percentage of dead and contaminated individuals.

The sterilized sprouts were cut to isolate apical meristems which were then cultured on shoot induction medium containing solidified MS medium with vitamins and free of exogenous plant growth regulators and incubated in a growth chamber at optimized culture conditions in room culture. The initiated shootlets from the aseptic meristem cultures were cut to nodal cuttings which were culture on the previous MS medium for mass propagation of potato plantlets *in vitro*.

The results cleared that MS medium with vitamins and solidified by agar without exogenous plant growth regulators can be used for mass propagation of free-pathogen true to type of potato genotype *in vitro* under the optimized culture conditions and conserving money which is consumed for the purchase of the exogenous plant growth regulators (Ebad *et al.,* 2015).

Saljooghianpour (2017) conducted a study in which the effects of BAP on the micro tuberization were evaluated using five commercial cultivars of potato i.e. 'Loman', 'Aracy', 'Ranger-russet', 'Agria' and 'Marphona'. Apical and axillary bud explants from greenhouse grown young shoots, were cultured on liquid MS medium containing 2 mg/l GA_3. The medium pH was 5.8±0.1before autoclaving at 121°C and 1-5 kg/cm for 25 min and were maintained in the growth chamber with 24±2°C temperature and a light period of 16 hours (irradiance of 100 µmol $m^{-2}s^{-1}$) and 8 hours darkness period. The explants were subcultured every four weeks on the same medium for plantlet regeneration. Then, for plantlets micro tuberization, plantlets were cultured on liquid MS medium with different concentrations of BAP (0, 5, 10, 15 and 20 mg/l) and 80 g/l sucrose.

Morphological parameters of micro tubers varied significantly among cultivars and BAP concentrations. Results indicated that cultivars produced significantly higher number of micro tubers in the presence of BAP as micro tuberization is positively correlated to the BAP levels.

For the tested cultivars, micro tuberization was the most efficient in liquid MS medium containing 5 mg/l BAP and it was lowest in 0 mg/l BAP. Micro tuberization of the studied potato cultivars is limited without the presence of BAP. Results indicated that 'Agria' and 'Marphona' produced a significantly higher number of micro tubers for almost all BAP concentrations. Phenotypic correlations indicated that some associated genetic factors correlate with each other and contribute in the occurring of these characteristics.

2.7 Medium consistency effect on potato *in vitro* regeneration

Sandra and Maira (2013) carried out a research on effect of media consistency on micropropagation of two potato (*Solanum tuberosum*) cultivars i.e. Granola and Arbolona negra. 30 stem sections with 1 axillary bud, obtained from each cultivar were cultured on the same MS1 semi-solid medium, 1 stem section per tube. In the case of liquid media, 30 explants of each cultivar were cultured on 15 ml of MS medium in 250 ml erlenmeyer flasks, 5 explants per erlenmeyer. Cultures were incubated at 125 rpm on a shaker New Brunswick Scientific®, at 18 ± 1°C under 16 h photoperiod.

Plantlets growing on semisolid medium for eight weeks were taller (12.17±0.58 Granola and 15,13±0.83 in case of Arbolona negra) than plantlets obtained on liquid medium, for both cultivars, but they look fragile and with a foliar area of 12.50 mm2. Plantlets growing on liquid medium had less leaves number than plants growing on solid medium, for both cultivars;

however, leaf area of plantlets developed on liquid medium was 6 to 8 times higher than leaf area of plantlets cultured on semi-solid media. Arbolona negra plantlets showed larger stems than Granola plantlets when cultured on liquid media (Sandra and Maira, 2013).

Qureshi *et al.* (2014) conducted an experiment on effect on media consistency in which the efficacy of liquid MS medium for potato multiplication was evaluated with the objective to find a cost effective multiplication media for potato. The data was recorded for growth parameters i.e. no of days to shoot/root initiation, no of leaves, no of leaves and nodes, intermodal distance, root and shoot length at transplantable stage. Phenotypic differences in growth were observed between the plantlets of both types of media.

Plantlets cultured on liquid media showed better growth of shoot and roots as compared to solid media. The use of growth regulators in liquid cultures also proved to be more effective and it is due to the direct contact of plant with the medium. Liquid media plantlets emerged earlier and having greater number of leaves and nodes per plantlet. Shoot and root length was significantly greater in plantlets of liquid media with mean values 11.34cm and 1.72cm respectively, while in solid media, it was 6.04cm and 1.59cm respectively. The tuber yield and weight was also higher for plantlets developed on liquid media (2.91 and 2.04g) as compared to solid media plantlets (1.76 and 1.12 g). They used (MS) medium containing 1.0 mgl-1Ca-pentothenate, 0.25 mgl-1 Gibberellic acid (GA_3), 100 mgl-1 Myoinsitol and 30 gl-1 sucrose at pH 5.7 was used in this for culturing nodal cuttings of potato cultivar 'Desiree' (Qureshi *et al.* 2014).

2.8 Effect of medium modification by Macrosalts, $AgNO_3$ and Nanosilver

Rahman *et al.* (2011) conducted a investigation to determine the effect of key nitrate source (KNO_3 and NH_4NO_3) in MS basal media on micropropagation efficiency of five potato cultivars (Atlanta, Shepody, All Blue, Diamant and Shilbilaty). Three different treatments and a control treatment were given. All components except KNO_3 and NH_4NO_3 were same as the MS media. The effect of the treatments were analysed on three parameters which were - shoot length, shoot fresh weight and multiplication rate of the mentioned potato cultivars. The treatments used in their investigation were- NT_0 (KNO_3 = 0mg/l and NH_4NO_3 = 0mg/l), NT_1 (KNO_3 = 475mg/l and NH_4NO_3 = 413mg/l) , NT_2 (KNO_3 = 3800 mg/l and NH_4NO_3 = 3300 mg/l) and NT_3 (KNO_3 = 1900 mg/l and NH_4NO_3 = 1650mg/l). The amount of KNO_3 and NH_4NO_3 in MS media markedly affected the in vitro growth responses of potato cultivars especially with or without nitrate treatments. It was noticed that no significant differences were raised in shoot length with varied nitrate treatments but zero nitrate media differed significantly from the treated ones.

However, Shepody reached highest shoot length of 8.93 cm in NT_1 (low nitrate) media followed by same cultivar at NT_2 (high nitrate) media. It was observed that shoot fresh weight increased as nitrate content was increased in all varieties except Diamant. Highest shoot fresh weight (104.25

mg) was obtained in Shilbilaty followed by Shepody (97.5 mg) at NT_2 media. The shoot multiplication rate was highest (6-8 fold) in Shepody at NT_2 media followed by same cultivar (5-7 fold) at NT_1 media. The multiplication rate higher in high nitrate media is in agreement with the results obtained by Evans (1993) with different potato genotypes.The multiplication rate was also noted poor in zero nitrate media but the exception to this was Shepody where 3-5 fold multiplication was achieved. It was also observed that internode length increased with the decreased of nitrate content and produced much reduced leaves. Among the cultivars tested the best growth occurred in Shepody at all records.It was noted that low nitrate media (NT_1) produced better shoot length in Shepody and Diamant whereas higher nitrate media (NT_2) resulted maximum shoot fresh weight. The zero nitrate media gave poor performances at all parameters and cultivars and the control responded moderately. This experiment with NT_1, NT_2 and NT_3 media were comparable with respect to growth traits and demonstrated that the micropropogation efficiency did not much improve when the nitrate increased (from NT_1 to NT_3) in the medium. The results suggested that it would be more cost effective to use low level of nitrate in the media and the experiment may give a potential idea to find out the low nitrate salt potato micro-propagation methods effective for some commercial cultivars.

On the other hand, Motallebi –Azar *et al.* (2011) carried out a research in order to develop a protocol for rapid shoot proliferation of potato, the node explants that were cut into pieces of 0.3 – 0.5 cm, containing one axillary bud in each explant and were cultured on MS media containing three concentrations of NH_4NO_3 (800, 1900 and 2400 mg/l) and three concentrations of hydrolyzed casein (0.0, 100 and 200 mg/l), 3% sucrose, 0.8% agar and supplemented with two concentrations of BAP (0.0 and 2 mg/l). They reported the effects of different concentrations of NH_4NO_3, hydrolyzed casein and BAP on *in vitro* shoot proliferation in potato cv. Agria, for improving the micropropagation procedure. The most effective concentrations as regards the number of lateral shoots were media supplemented with 2400 mg/l NH_4NO_3, without hydrolyzed casein, or with 800 mg/l NH_4NO_3 and 200 mg/l hydrolyzed casein, both media containing 2 mg/l BAP. Maximum percentage of root formation and minimum percentage of callus formation was observed on media without BAP. The maximum number of roots per shoot was recorded at 800 mg/l and 1900 mg/l NH_4NO_3 in media without BAP. Minimum callus production percentage was observed in culture media containing 1900 mg/l or 2400 mg/l NH_4NO_3, in the absence of BAP.

Previously mentioned experiment of Sandra and Maira (2013) the effect of $AgNO_3$ on micropropagation of two potato (*Solanum tuberosum*) cultivars i.e. Granola and Arbolona negra was also evaluated. Different concentrations of silver nitrate for both cultivars were tested.As the $AgNO_3$ concentration increased, leaf number diminished, stem length diminished and leaf area increased for both cultivars. Plantlets growing on MS medium supplemented with 2 mg/l $AgNO_3$ showed an adequate stem length and a high leaf area. After eight weeks of culture, these plants did not show symptoms of ethylene growth inhibition: epinasty or hyperhydricity.

Nanotechnology is concerned as a key technology which will have wide usage like economic, social and ecological implication. Antibacterial activity is one of the important abilities of nanomaterial and bacterial contamination is a serious problem in plant tissue culture procedures. Safavi and Mortezaeinezhad (2012) conducted a research to evaluate the potential of nano silver to remove bacterial contaminants that exist in plant tissue culture media. Experiment involved Murashige and Skoog (MS) media with five rates (5, 25, 50, 75 and 100 mg/l) of nano silver. Potato explants were cultured on this modified MS medium and evaluated after four weeks. The results showed that nano silver had a good potential for removing the bacterial contaminants in plant tissue culture procedures.

With 5 mg/l nano silver added in tissue culture media, the growth was very well in each four weeks. When 25, 50, 75 and 100 mg/l nano silver was added in tissue culture media, the potato had not very good growth. Their results showed that Nano Silver can reduce and remove micro-organisms in MS media and the best results can be achieved by using 5 mg/l nano silver in potato tissue culture media.

CHAPTER III

MATERIALS AND METHODS

The required components and process applied in this study have been stated in this step. In this chapter we will discuss about the materials used for research, the experiment scheme, the parameters on which the data will be collected, system used for data collection and data analysis method.

3.1 Experiment site and duration:

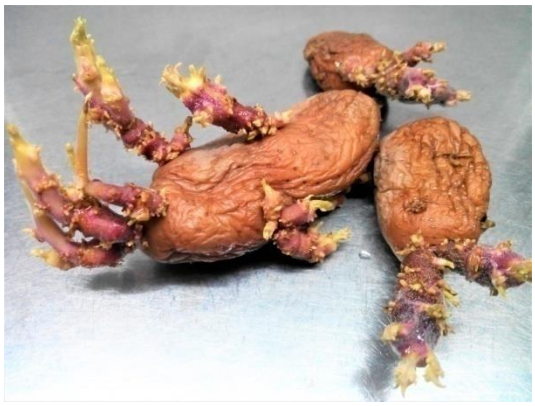
My experiment on performance of ammonium nitrate (NH_4NO_3) free medium in *in vitro* regeneration of a potato variety was done in nicely co-ordinated Biotechnology laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Sher e-Bangla Nagar, Dhaka-1207 from January, 2016 to June, 2017. Two sub-experiments were done under this study to meet the purpose of the research-

Sub-experiment 01: *In vitro* regeneration of potato in modified stock solution-01 using sprout as explant.

Sub-experiment 02: *In vitro* regeneration of potato in modified stock solution-01 using nodal segment as explant.

3.2 Materials used in experiment:

Tissue culture technique is the most evident and easy in potato plant, that's why potato sprout and nodal segment were used as explant. Asterix variety was used for study purpose. The sprouted potato was collected from the Department of Biotechnology (Plate 1). Data on days to shoot initiation, shoot length (cm), no. of shoot/explant, shoot diameter, no. of node/plantlet, no. of leaves/explant, no of root/plantlet and root length (cm)/plantlet were listed. The inspection was done on 7, 14, 21 and 28 days after inoculation of explant on culture media.



A

B

Plate 1. (A) Sprouted potato tubers and (B) separated potato sprouts for micropropagation

3.3 Chemicals, glasswares and instruments:

Arrangements for the required chemicals, glasswares and instruments were done in January, 2016 and then the research was started. An elaborared list of the used chemicals, glasswares and instruments is given below-

Necessary chemicals:

- ✓ Readymade MS powder
- ✓ MS medium components
- ✓ Disinfecting chemicals (70% Ethanol, $HgCl_2$, tween-20)
- ✓ Sucrose
- ✓ Agar
- ✓ NaOH (10N, 1N)
- ✓ HCl
- ✓ Sterilized and deionized distilled water (DDH_2O)
- ✓ Absolute Ethanol
- ✓ Methilated spirit
- ✓ Stock solution - 01
- ✓ Stock solution - 02
- ✓ Stock solution - 03
- ✓ Stock solution - 04
- ✓ Stock solution - 05

Used glasswares, instruments and equipments:

- ✓ Measuring cylinders
- ✓ Petridishes
- ✓ Beaker (250 ml, 500 ml and 1 L)
- ✓ Culture vials
- ✓ Autoclave
- ✓ Microwave oven
- ✓ Laminar Air Flow Cabinet
- ✓ Freezer
- ✓ Hotplate with magnetic stirrer
- ✓ Electric balances
- ✓ Pipettors
- ✓ Plant growth chamber
- ✓ pH meter
- ✓ Water distillation plant
- ✓ Automatic drying oven
- ✓ Forceps, needles, spatula, brush, scissors,cotton, surgical blade.

3.4 Disinfecting glasswares:

The glasswares and the small instruments like forceps, scissors etc. are washed by Trix. Then they are rinsed thoroughly with clean tap water to remove the soap properly. Finally they are dipped in distilled water to remove any ionization. Then these glasswares are dried in drying oven. After drying them properly all these are palced in the autoclave machine for sterilization process at 121^0 C temperature for 20 minutes at 15 psi.

3.5 MS media preparation:

Media preparation is one of the primary and most essential steps in tissue culture. Media is prepared based on the type of tissue being cultured. Murashige and Skoog medium (MS medium) is a most commonly used plant growth medium in the laboratories for regeneration of plant cell culture. All plant cells requires water, nutrients and plant growth regulators. To meet up this requirement some stock solutions are needed to be made which consist of different concentrations of inorganic nutrients (major elements, minor elements), organic nutrients (sucrose, vitamins, supplements) and plant growth regulators. Five stock solutions were prepared with these nutrients and growth regulators. The patented and the usual concentrations of these chemicals are stated in Appendix -1.

3.6.1 Stock solution-01 of Major salts:

This stock solution was prepared in 10 times more concentrated than the concentration to be used in the medium. So the required salts were weighed ten times higher of the actual weight needed and liquefied in distilled water in a 1L beaker with the help of the magnetic stirrer. Because of precipitation problem $CaCl_2$ was liquefied separately and then added with the main solution. Finally the volume was made upto 1L and transferred in the main glass vessel and then placed in refrigerator at 4^0 C. It was prepared in five different formulations which were denoted as treatment-01, treatment-02, treatment-03, treatment-04 and treatment-05 .

3.6.1.1 Treatment composition for stock solution-01:

The composition of these five treatments are enlisted in tabular form-

Treatment no.	Chemical name	Chemical formula	Amount (gm)
Treatment-01	Potassium nitrate	KNO_3	19.00
	Ammonium nitrate	NH_4NO_3	16.50
	Magnesium sulphate	$MgSO_4.7H_2O$	3.70
	Calcium chloride	$CaCl_2.2H_2O$	4.40
	Potassium dihydrogen phosphate	KH_2PO_4	1.70
Treatment-02	Potassium nitrate	KNO_3	19.00
	Magnesium sulphate	$MgSO_4.7H_2O$	3.70
	Calcium chloride	$CaCl_2.2H_2O$	4.40
	Potassium dihydrogen phosphate	KH_2PO_4	1.70
Treatment-03	Potassium nitrate	KNO_3	38.0
	Magnesium sulphate	$MgSO_4.7H_2O$	7.40
	Calcium chloride	$CaCl_2.2H_2O$	8.80
	Potassium dihydrogen phosphate	KH_2PO_4	3.40
Treatment-04	Stock solution-01 developed from the department of Biotechnology , SAU, Dhaka	-	-
Treatment-05	Readymade MS powder (Duchefa Biochemie, Netherland)	-	-

3.6.2 Stock solution-02 of Minor salts

These salts are required in very small amount in the medium. Thats why they are called minor salts. This stock solution was 100 times more concentrated than the concentration to be used in the medium. So the required salts were weighed 100 times higher of the actual weight needed and liquefied in distilled water in a 1L beaker with the help of a magnetic stirrer. Then this solution was labeled, the date was indicated and preserved in refrigerator at 4^0C for use in upcoming research.

3.6.3 Stock solution-03 of Ferus

Ferus is usually present in the form of $FeSO_4.7H_2O$ and Na_2EDTA. In some cases, ferus is prepared together with micronutrient. Ferus oxidises in the presence of sunlight. Furthermore, high concentrations will cause precipitation to occur. Therefore, preparing fresh ferus at 100 times higher concentration than the required medium concentration and storing the stock solution in dark environment or cloaking the container with silver foil can prolong the shelf life of ferus stock solution. The solution was prepared with the help of a magnetic stirrer. The container was labeled and the date of preparation was indicated and stored in a chiller at 4^0C.

3.6.4 Stock solution-04 of Myo-Inositol

This solution was prepared with a single component Myo- Inositol which was weighed 100 times higher than the required amount in the medium. It is one kind of hexitol and an important component of the tissue culture media. It is often used as a vitamin, but very recent findings reveals that, it is actually a sugar alcohol. The required weight was dissolved in distilled water and final volume was made up to 1L, then the vial was labeled with date and preserved in refrigerator for later use.

3.6.5 Stock solution-05 of vitamins

The last stock solution was prepared with vitamins such as- Nicotinic acid, Pyridoxine HCL, Thiamine HCL and Glycine. These were measured at 100 times higher of the required weight in media. Then all these were added in distilled water and liquefied with the help of Hot plate magnetic stirrer. Then 1L volume was made with distilled water and labeled and the preparation date was noted and kept in freezer for subsequent use.
All stock solutions together with different treatments of stock solution-01 have been shown in plate no-2 to 5.

Plate 2. Treatment-01 (stock solution-01) and other stock solutions 2, 3, 4 and 5 for MS media preparation

Plate 3.Treatment-02 (stock solution-01) and other stock solutions 2, 3, 4 and 5 for MS media preparation

Plate 4. Treatment-03 (stock solution-01) and other stock solutions 2, 3, 4 and 5 for MS media preparation

Plate 5. Treatment-04 (stock solution-01) and other stock solutions 2, 3, 4 and 5 for MS media preparation

3.7 Solutions for pH adjustment

For adjusting the pH of the culture media NaOH and HCL were used. Stock solution for NaOH and HCl are given below-

3.7.1 Stock solution of 1N NaOH

To prepare 1N NaOH 40gm of this chemical was dissolved in distilled water in a 1L vial and the final volume was made up to 1L.

3.7.2 Stock solution of 1N HCL

36.5 gm equivalent weight of HCL was added in 1L sterilized distilled water with precaution. It was used for lowering pH.

3.8 Flowchart of MS media preparation from the prepared stock solution

The diagram of the sequences of 1 litre MS media preparation is given below-

1st step: 800 ml sterilized distilled water was taken in a 1L glass beaker,
2nd step: 100 ml Stock solution-01(specific modified stock-01 in case of specific treatment) was added and the rest stock solutions were added at a quantity of 10 ml each. Then the solution is mixed with the help of a magnetic stirrer.
4th step: 30 gm of sucrose was added in the mixture.
3rd step: The solution volume was brought up to 1L with autoclaved distilled water.
5th step: pH of the MS media was fixed at 5.8 with pH meter by adding NaOH or HCl.
6th step: At last 8 gm agar was put together with the prepared medium and heated for some minutes in oven for liquification.

3.9 Sequences of media preparation from MS powder

The sequences of Murashige and Skoog culture media (1962) preparation process for 1L were-

- 800 ml sterilized distilled water was poured into a 1L glass beaker,
- 30 gm sucrose and 5 gm MS powder were weighed separately with the help of the electric balance and transferred in the beaker for making the solution,
- The beaker is placed on the magnetic stirrer for a good mixing,

- Then the solution volume was made 1L by adding more distilled water,
- The medium pH was fixed at 5.8 with the help of HCL or NaOH before autoclaving.
- At last as gelling agent agar was selected. 8 gm agar was weighed and mixed with the prepared solution. The solution was heated in oven for melting of agar.

3.10 Stock solution of disinfecting chemical

Usually ethanol is used as an alcohol as sterilization agent which is required to sterilize worker's hand during manipulation work. Washing hands with antibacterial liquid hand wash followed by spraying on hands with 70% ethanol was quite effective. The Laminar airflow cabinet was also sprayed with 70% ethanol for decontamination process. The required instruments were also sterilized by flame sterilization process where the instruments were soaked in 70% ethanol followed by flaming on methylated spirit in the laminar airflow hood which was done repeatedly with the work in progress.

3.11 Moist heat sterilization of culture media

The culture media was distributed equally in the culture vials. Then the vials were tightly capped and well-arranged in the busket of autoclave machine. Then the machine was set to sterilize the vials at 121^0C for 20 minutes at 15 psi. After decontaminating, the vials were transferred in the culture room for cooling and placement of the explants.

3.12 Disinfection of culture room and laminar airflow cabinet

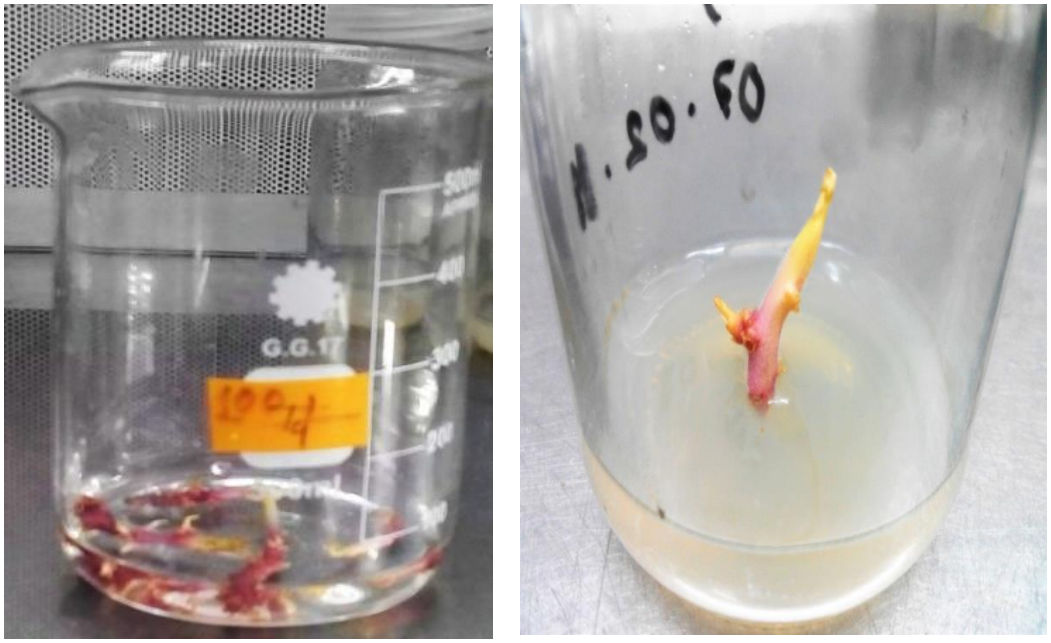
The tissue culture room was kept closed for an entire day after formaldehyde spraying in the whole room. The floor and rake were wiped with antibacterial detergent followed by 70% ethanol.

The whole laminar airflow cabinet was wiped with 70% ethanol before starting inoculation of explant. The UV ray was kept switched on for 30 minutes without anything in it, then 20 minutes with the necessary instruments and glasswares right before starting work.

3.13 Sterilization of the explants

The sprouts of Asterix variety of potato were separated from the potato tuber and cleaned with tap water and then rinsed with sterilized distilled water for several times. Then the explants were transferred in another beaker for further work. At first they were sterilized with 70% ethanol for 1 or 2 minutes. After discarding the ethanol,, they were rinsed with

autoclaved water for 2 or 3 times. Then the sprouts were surface sterilized with 0.1% $HgCl_2$ with a few drops of tween-20 in it for 2-3 minutes and those were washed again with distilled water to get rid of the chemicals from the explants. At last the sprouts were ready for transfer in the media.



A **B**

Plate 6. (A) Disinfection process of potato sprouts and (B) inoculation in MS media

3.14 Transferring of explants to the media

After sterilizing the explants under controlled environment, the sprouts were transferred into the culture vials containing media. Then the cap was flame sterilized and the vial was tightly closed in the laminar airflow cabinet.

3.15 Subculturing of the regenerated shoot

After 3 to 4 weeks of plant regeneration, the cultured shoots were cut into small pieces containing conspicuous nodes.The cutting was done in laminar airflow cabinet with autoclaved scalpel blade, scissor, forcep to maintain sterilized condition. Then these new explants i.e. nodal segments were then transferred in newly made culture media for further regeneration.

Plate 7. Preparing nodal segments for the subculture process

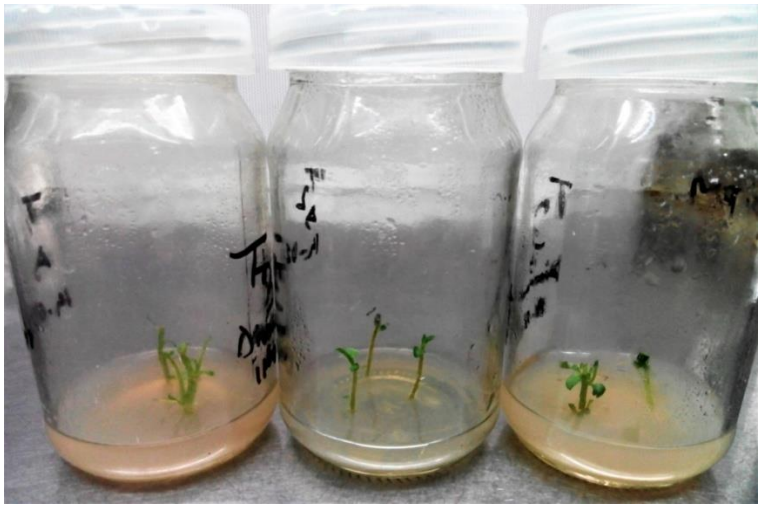


Plate 8. Inoculation of the nodal segments in MS media

Plate 9. Incubation process of the sub-experiment-02 ongoing in the sterilized culture room

3.16 Incubation

The culture vials were placed on the racks in aseptic environment (Plate 9). The temperature was maintained at 25±2 °C with an air conditioner and light intensity varied from 2000–3000 Lux. White fluorescent lights were used for growth of the culture. The photoperiod was generally maintained at 16 hours and 70% relative humidity (RH) for better growth and development.

3.17 Acclimatization

Regenerated plantlets were transplanted to pots containing soil and cowdung in 1:1 ratio and soil mixture were treated with a solution of 1% IBA. Occasional spray of water was done to prevent sudden desiccations and maintain high humidity around the plantlets. Initially the plantlets were hardened in growth chamber. Then after 2 weeks, exposed to lower humidity and higher light intensity. Finally, after 20 days plantlets were transferred to natural environment.

3.18 Scheme of the experiment:

In lab situation, the two factors experiment was arranged in Completely Randomized Design (CRD) with five replications.

3.19 Data compilation:

Under *in vitro* situation, the research data of the following parameter were listed by using scale in case of length and by visual observation in case of other parameters. The inspection was done on 7, 14, 21 and 28 days after inoculation of explant on culture media. The mean value of the data was calculated in case of each parameter. The parameters were-

- Data on days to shoot initiation
- Shoot length (cm)
- No. of shoot per explant
- Shoot diameter
- No. of node per plantlet
- No. of leaves per explant
- No of root and
- Root length (cm) per plantlet

3.20 Statistical analysis:

The acquired data on various parameters were analyzed statistically by ANOVA which was performed by 'F' test where MSTAT-C software to detect the difference among the treatments. The mean values of the listed parameters were assessed. The significance of the difference among the treatment means were estimated by LSD test at 5% level of probability. LSD (Least Significant Difference) was also calculated to compare the differences between two treatment means.

CHAPTER IV

RESULTS AND DISCUSSION

The present research was carried out to evaluate the regeneration capability of potato (*Solanum tuberosum* L.) in ammonium nitrate free culture medium. Subsequently two sub-experiments were conducted under the *in vitro* condition. The analysis of variance (ANOVA) of the data has been submitted in Appendix II-XII. The results have been submitted and discussed and feasible explanation have been given experiment wise under the following parameters.

4.1 Sub-experiment 01: *In vitro* regeneration of potato in modified stock solution-01 using sprout as explant.

4.1.1 Days to shoot initiation

Statistical variations were observed among different treatments on days to shoot initiation (Table 1). The maximum days to shoot initiation (8.67) was recorded in T_2 (NH_4NO_3 free stock solution), followed by T_1 (7.30) which was different from all other treatments. It indicates that NH_4NO_3 is an important ingredient which is needed for proper shoot initiation in potato regeneration. In contrast, the minimum data (5.00 days) was recorded in both T_4 (stock solution-01 with β chemical) and T_5 (readymade MS powder) followed by T_3 (5.67). T_3 performed moderately which might be due to the lower concentration of nitrogen as it was formulated without NH_4NO_3. But due to the increased concentration of KNO_3, this treatment performed better than the T_2.

The highest shoot number (1.67) was recorded in T_4 which was statistically non significant with other treatments and the lowest (1.00) was recorded in T_1(standard MS dose,1962) and T_2 which was also non significant with other treatments (Table 1).

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Table 1: Days to shoot initiation and number of shoot at one week after inoculation in Asterix variety of potato

Treatment	Days to shoot initiation	No of shoot/explants
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	7.30 b	1.00 a
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	8.67 a	1.00 a
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	5.67 c	1.33 a
T_4 (Stock solution -01, developed in the Department of Biotechnology, SAU, Dhaka)	5.00 c	1.67 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	5.00 c	1.33 a
LSD $_{(0.05)}$	0.91	0.80
CV(%)	7.63	7.80

4.1.2 Treatment effect on shoot length (cm)

The treatment effect on shoot length is presented in Table 2. The maximum shoot length (8.00 cm) was found in T_4 (stock solution-01 with newly developed chemical) which was statistically different from all other treatments at 21 days after inoculation. Highest length (9.17 cm) was also noticed at 28 DAI from the same treatment. It may be due to higher nitrogen percentage of the new β chemical. In this respect, the presented work is in contrast with Rahman *et al.* (2011) who reported that low nitrate media produced better shoot length in Shepody and Diamant whereas higher nitrate media resulted maximum shoot fresh weight. Though zero nitrate media gave poor performances at all parameters and cultivars and the control responded moderately. They demonstrated that the micropropogation efficiency did not much improve when the nitrate increased in the medium. In our study T_3 (NH_4NO_3 free stock solution with double dose of other ingredients) performed moderately in case of shoot length (6.67 cm and 8.57 cm) both at 21 DAI and 28 DAI respectively which may be due to the double dose of KNO_3.

A comparison between different treatment is given in plate 10. The minimum shoot length (4.67cm) was found in T_2 (NH_4NO_3 free stock solution) which was statistically different from all other treatments at 21 days after sub-culture. Same treatment showed minimum shoot length (6.60 cm) at 28 DAI too. It indicates the importance of NH_4NO_3.

The experiment is based on the effect of modified treatments on potato sprouts. The treatments are modified with different concentration of the major salts like KNO_3 , NH_4NO_3 and β chemical. In a report on the effect of key nitrate source (KNO_3 and NH_4NO_3)

in MS basal media on micropropagation efficiency of five potato cultivars, it was mentioned that no significant differences were raised in shoot length with varied nitrate treatments but zero nitrate media differed significantly from the treated ones. In the case of β chemical, an experiment was conducted by my senior Md. Abul Bashar under direct supervision of our honourable Chairman Professor Dr. Md. Ekramul Hoque. The report indicated that the treatment having 5 gmL^{-1} and 1 gmL^{-1} of β chemical showed better result than the other treatments having more β chemical (Bashar, 2016).

Table 2: Shoot length of potato sprouts at different days of regeneration

Treatment	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	4.00 ab	6.83 ab	7.90 a
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	2.33 b	4.67 c	6.60 b
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	4.00 ab	6.67 b	8.57 a
T_4 (Stock solution -01, developed in the Department of Biotechnology, SAU, Dhaka)	5.00 a	8.00 a	9.17 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	5.33 a	7.33 ab	9.00 a
LSD $_{(0.05)}$	1.79	1.22	1.27
CV(%)	12.95	9.83	8.21

T_1

T_2

T_3

T_4

T_5

Plate 10. Shoot length (cm) measurement of the Asterix variety at 21 DAI

4.1.3 Treatment effect on number of shoot/ explant

The maximum shoot number (2.33) found in T_4 (stock solution-01 with newly developed chemical) was statistically similar with all other treatments at 14 days after inoculation (DAI). The minimum shoot number (1.00) was recorded in T_2 (NH_4NO_3 free stock solution) (Table 3). The modification did not show much difference at 14 DAI in case of shoot number. But at 21 DAI and 28 DAI the modifications affected the shoot number to a certain extent. Highest shoot number (4.07 and 6.05) was recorded in Treatment-04 which was statistically different from other treatments at 21 DAI and 28 DAI respectively which may have been caused by the effect of new β chemical. Whereas the minimum data (3.00) was recorded in T_2. The absence of NH_4NO_3 may be the reason of its poor performance. T_3 (NH_4NO_3 free stock solution with double dose of other ingredients) performed moderately both at 21 DAI (3.33) and 28 DAI (5.46) as this treatment was formulated without NH_4NO_3. But having double dose of KNO_3 may be cause of the leading of T_3 form T_1 (5.05) at 28 DAI.

Table 3: Number of shoot/explant of potato sprouts at different days of regeneration

Treatment	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	1.33 a	3.33 bc	5.05 c
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	1.00 a	3.00 c	4.29 d
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	2.00 a	3.33 bc	5.46 b
T_4 (Stock solution -01, developed in the Department of Biotechnology, SAU, Dhaka)	2.33 a	4.07 a	6.05 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	2.00 a	3.67 ab	5.73 ab
LSD $_{(0.05)}$	1.52	0.56	0.38
CV(%)	6.51	7.15	3.13

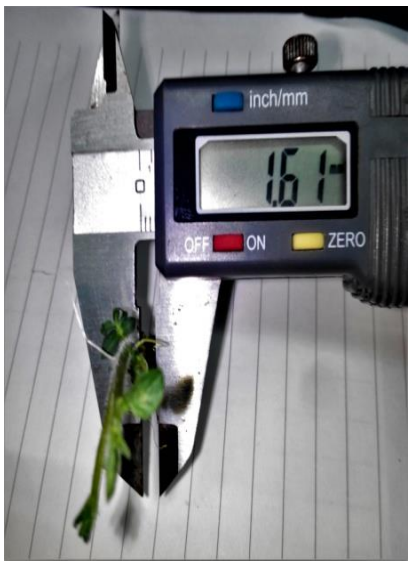
4.1.4 Treatment effect on shoot diameter (mm)

At 21 days after inoculation (DAI), the maximum shoot diameter (1.81 mm) was recorded in T_4 (stock solution-01 with newly developed chemical) which was statistically non significant with T_3 (NH_4NO_3 free stock solution with double dose of other ingredients) and T_5 (readymade MS powder) but statistically different from other treatments (Plate 11). On the other hand, the minimum shoot diameter (0.80 mm and 0.81 mm) was recorded in T_2 (NH_4NO_3 free stock solution) at 21 DAI and 28 DAI respectively (Table 4) which was statistically different from all other treatments and might be caused by the absence of NH_4NO_3. The maximum shoot diameter (2.00) was found again in T_4 which was statistically similar with T_5 (readymade MS powder) but different from all other treatments at 28 days after inoculation (DAI).

Both at 21 DAI and 28 DAI, Treatment-04 and Treatment-05 showed statistically similar results. As Treatment-05 is the readymade MS powder, it was significantly different and showed better result than Treatment-01(Formulated recommended MS dose,1962). Treatment-03 also performed at a satisfactory level but not up to the highest probably due to the absence of key nitrogen source - NH_4NO_3.

Table 4: Shoot diameter (mm) of potato sprouts at different days of regeneration

Treatment	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	1.14 b	1.17 b
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	0.80 c	0.81 c
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	1.34 b	1.62 a
T_4 (Stock solution -01, developed in the Department of Biotechnology, SAU, Dhaka)	1.81 a	2.00 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	1.76 a	1.97 a
LSD $_{(0.05)}$	0.29	0.29
CV(%)	10.35	10.56



T_1 **T_2** **T_3**

T_4 **T_5**

Plate 11. Measuring the shoot diameter (mm) of the Asterix variety at 28 DAI (Days After Inoculation) in different treatments

4.1.5 Treatment effect on number of node/plantlet

At 14 days after inoculation (DAI), the maximum node number (3.33) was found in T_4 (stock solution-01 with newly developed chemical) which was statistically different from all other treatments which may be because of the newly used β chemical. On the other hand, the minimum node number (1.33) was found in T_2 (NH_4NO_3 free stock solution) which was statistically different from all other treatments. Even though Treatment-02 was formulated without the key nitrogen source NH_4NO_3 but KNO_3 was same as its standard MS dose, 1962; may be it was not enough for a satisfactory regeneration in potato. The maximum node number (7.67) was found in T_4 which was statistically different from T_2 (4.00) at 21 days after inoculation (DAI) which emphasizes the importance of a proper nitrogen source which provides a well established regeneration in potato. Same highest node number (8.00) was found in T_4 and T_5 at 28 DAI (Table 5).

Table 5: Number of node/plantlet of potato sprouts at different days of regeneration

Treatment	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	2.33 ab	6.00 a	6.67 ab
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	1.33 b	4.00 b	5.00 b
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	2.33 ab	6.67 a	7.33 a
T_4 (Stock solution -01, developed in the Department of Biotechnology, SAU, Dhaka)	3.33 a	7.67 a	8.00 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	2.67 a	7.00 a	8.00 a
LSD $_{(0.05)}$	0.97	1.79	1.75
CV(%)	21.52	5.14	13.30

4.1.6 Treatment effect on number of leaves /plantlet

Among the five treatments, the maximum leaf number (4.33) was found in T_4 (stock solution-01 with newly developed chemical) which was statistically different from all other treatments at 14 DAI and was followed by T_5 (3.67). While minimum leaf number (1.33) was found in T_2 (NH_4NO_3 free stock solution) which was statistically different from all other treatments at 14 DAI. This poor regeneration may be caused by the absence of NH_4NO_3. At 21 days after inoculation (DAI), the maximum leaf number (9.00) was found in T_4 which was statistically different from all other treatments. On the other hand, minimum leaf number (5.33) was found in T_2 which was statistically different from all other treatments. Treatment-03 (NH_4NO_3 free stock solution with double dose of other ingredients) performed better (8.00) than Treatment-02, may be due to the double dose of KNO_3.

The maximum leaf number (11.00) was found in both T_4 and T_5 (readymade MS powder) which was statistically different from T_2 (6.00) at 28 days after inoculation (DAI) (Table 6). Treatment-05 was formulated with ready made MS powder and may be that is why it gave statistically similar result with the Treatment-04 which we designed by β chemical for stock solution-01 preparation. At 28 DAI both T_1 (9.00) and T_3 (9.00) were statistically similar. This statistically similar result may be caused by the double dose of KNO_3 and the absence of NH_4NO_3.

Table 6: Number of leaves/plantlet of potato sprouts at different days of regeneration

Treatment	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	1.67 cd	7.00 bc	9.00 a
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	1.33 d	5.33 c	6.00 b
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	2.67 bc	8.00 ab	9.00 a
T_4 (Stock solution -01, developed in the Department of Biotechnology, SAU, Dhaka)	4.33 a	9.00 a	11.00 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	3.67 ab	8.67 ab	11.00 a
LSD $_{(0.05)}$	1.03	1.75	2.06
CV(%)	10.04	12.25	11.91

T_1

T_2

T_3

T_4

T_5

Plate 12. Plantlet regeneration from Asterix variety on five different formulations at 21 DAI

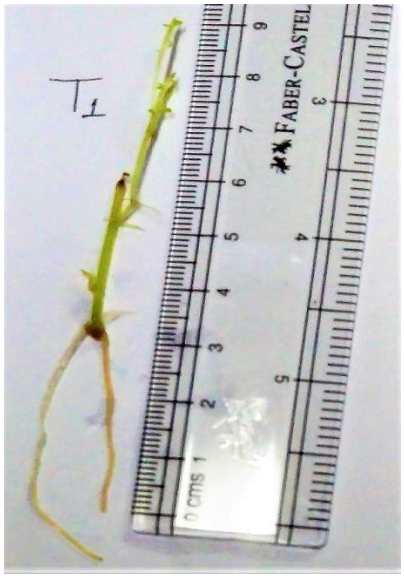
4.1.7 Treatment effect on root length (cm)

The maximum root length (2.67 cm) was found in T_5 (readymade MS powder) which was statistically similar with T_4 (stock solution-01 with newly developed chemical) and different from all other treatments at 14 DAI. On the other hand, minimum root length (1.17 cm) was found in T_2 (NH_4NO_3 free stock solution) which was statistically different from all other treatments (Table 7 and Plate 13). The maximum root length (9.90 cm and 13.50 cm) was found in T_5 (readymade MS powder) which was statistically similar with T_3 (8.60 cm and 11.40 cm) and T_4 (9.63 cm and 13.00 cm) and different from other two treatments at both 21 days and 28 days after inoculation (DAI).

In case of root length Treatment-05 had been found to give the best result among the five treatments. Treatment-04 which we designed by β chemical for stock solution-01 preparation gave the second best result. Perhaps it was caused by the newly used β chemical. So it can be concluded that the β chemical did not give a vigorous root growth like it gave in case of the shoot characters as mentioned above.

Table 7: Length of root (cm) of potato sprouts at different days of regeneration

Treatment	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	1.67 ab	6.37 b	8.67 b
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	1.17 b	5.00 b	7.10 b
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	1.83 ab	8.60 a	11.40 a
T_4 (Stock solution -01, developed in the Department of Biotechnology, SAU, Dhaka)	2.33 a	9.63 a	13.00 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	2.67 a	9.90 a	13.50 a
LSD $_{(0.05)}$	1.01	2.02	2.72
CV(%)	7.73	13.60	13.47



T_1 **T_2** **T_3**

T_4 **T_5**

Plate 13. Measuring the root length (cm) of the Asterix variety at 21 DAI

4.1.8 Treatment effect on root number

At 21 days after inoculation (DAI), the maximum root number (10.00) was found in T_5 (readymade MS powder) which was statistically similar with all other treatments. On the other hand, minimum root number (6.67) was found in T_2 (NH_4NO_3 free stock solution) which was statistically similar with all other treatments. Same minimum result (6.67) was recorded for the same treatment, T_2 at 28 DAS respectively which was statistically different from all other treatments(Table 8). The maximum root number (13.67) was found in T_4 and T_5 which was statistically different from all other treatments at 28 days after inoculation (DAI).

Treatment-03 (NH_4NO_3 free stock solution with double dose of other ingredients) performed moderately at both 21 DAI (8.67) and 28 DAI (10.33) which may be caused by the dose of this treatment.

Table 8: Number of root / explant of potato sprouts at different days of regeneration

Treatment	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	7.00 a	7.00 c
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	6.67 a	6.67 c
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	8.67 a	10.33 b
T_4 (Stock solution -01, developed in the Department of Biotechnology, SAU, Dhaka)	9.00 a	13.67 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	10.00 a	13.67 a
LSD $_{(0.05)}$	3.21	2.50
CV(%)	10.60	12.95

4.2 Sub-experiment 02: *In vitro* regeneration of potato in modified stock solution-01 using nodal segment as explant

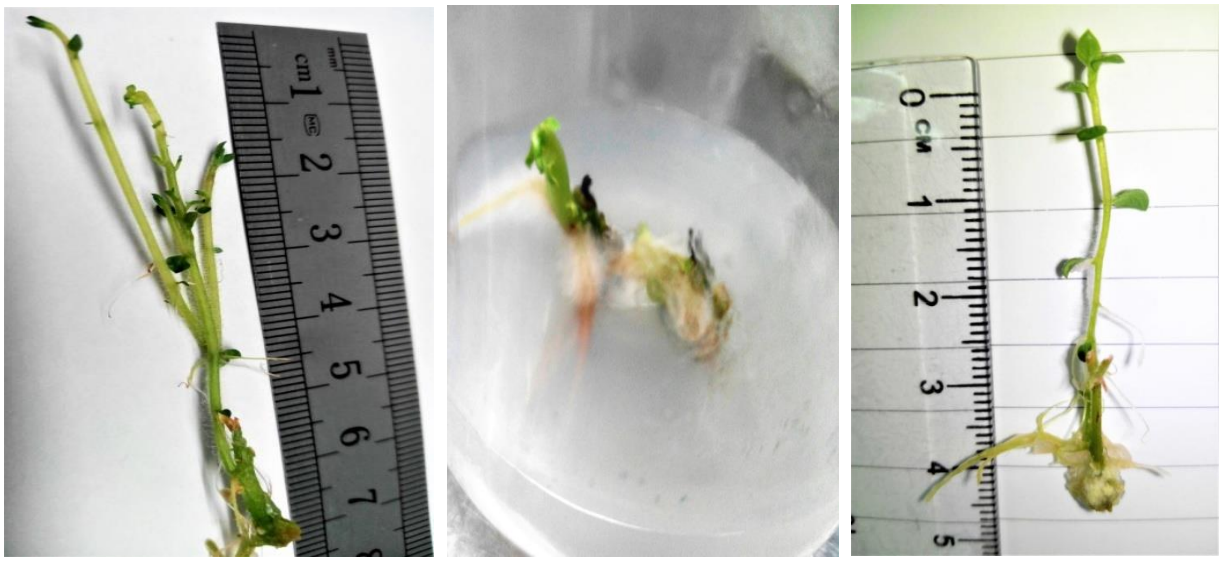
4.2.1 Treatment effect on shoot length (cm) using node as explants

The treatment effect on shoot length is presented in Table 9. The maximum shoot length (3.97cm) was found in T_4 (stock solution-01 with newly developed chemical) which was statistically different from all other treatments at 14 days after inoculation (DAI). Same treatment showed maximum shoot length (4.17 cm and 4.27 cm) at 21 DAI and 28 DAI respectively. A comparison between different treatment was given in plate 14. The minimum shoot length (1.97cm) was found in T_2 (NH_4NO_3 free stock solution) which was statistically different from all other treatments at 14 days after inoculation. Same treatment showed minimum shoot length (2.77 cm) at 28 DAI too. This poor performance may be caused by the absence of NH_4NO_3 as it is the key nitrogen source of the MS medium. Since KNO_3 was present in its standard MS dose, 1962; it may have not been enough for a better regeneration in Asterix variety.

T_3 (NH_4NO_3 free stock solution with double dose of other ingredients) performed moderately (3.30 cm and 3.43 cm) at 14 DAI and 21 DAI which may be because of its formulation without NH_4NO_3.

Table 9: Shoot length (cm) of potato at different days of regeneration using nodal segments as explant

Treatment	7 DAI	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	1.67 c	2.63 c	3.20 b	3.90 a
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	1.17 d	1.97 d	2.33 c	2.77 b
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	2.20 b	3.30 b	3.43 ab	4.00 a
T_4 (Stock solution -01, developed in the Department of Biotechnology,SAU,Dhaka)	2.40 a	3.97 a	4.17 a	4.27 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	2.20 b	3.27 b	3.50 ab	4.03 a
LSD $_{(0.05)}$	0.15	0.37	0.81	0.42
CV(%)	3.85	6.50	12.85	5.91



T_1 **T_2** **T_3**

T_4 **T_5**

Plate 14. Shoot length (cm) of Asterix variety under different treatment at 28 DAI

4.2.2 Treatment effect on number of node/plantlet using node as explants

At 14 days after inoculation (DAI), the maximum node number (2.67) was found in T_4 (stock solution-01 with newly developed chemical) which was statistically similar with all other treatments except T_2 (NH_4NO_3 free stock solution), in which the minimum node number (1.67) was found. The maximum node number (8.33) was found in T_4 which was statistically similar with T_5 (8.00) and different from all other treatments at 21 days after inoculation (DAI). The minimum node number (4.33) was recorded in T_2 which was statistically different from other treatments(Table 10).

At 21 DAI and 28 DAI same moderate node number (7.33) was found in T_3 (NH_4NO_3 free stock solution with double dose of other ingredients) and it was statistically similar with T_1 (7.33), T_4 (8.67) and T_5 (8.00). Though Treatment-03 was formulated without NH_4NO_3 but the double dose of the other ingredients may have caused this statistically similar result.

Table 10: Number of node/plantlet of potato at different days of regeneration from nodal segment as explant

Treatment	7 DAI	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	1.33 a	2.67 a	5.67 bc	7.33 a
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	1.33 a	1.67 a	4.33 c	5.67 b
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	1.33 a	2.33 a	7.33 ab	7.33 a
T_4 (Stock solution -01, developed in the Department of Biotechnology,SAU,Dhaka)	2.00 a	2.67 a	8.33 a	8.67 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	1.67 a	2.33 a	8.00 a	8.00 a
LSD $_{(0.05)}$	1.35	1.03	1.95	1.38
CV(%)	6.88	8.47	5.34	9.87

4.2.3 Treatment effect on number of leaves/plantlet using node as explants

The maximum leaf number (7.00) was found in T_4 (stock solution-01 with newly developed chemical) which was followed by T_5 (6.33) and was statistically different from T_2 (NH_4NO_3 free stock solution) which showed minimum leaf number (3.33) at 14 DAI. At 21 days after inoculation (DAI), the maximum leaf number (13.00) was found in T_4 which was statistically different from all other treatments. On the other hand, minimum leaf number (8.00) was found in T_2 which was statistically different from all other treatments too. The maximum leaf number (13.00) was found in T_4 and T_5 which were statistically different from T_2 (8.67) at 28 days after inoculation (DAI) (Table 11). Absence of NH_4NO_3 may have been the cause of the poor performance of Treatment-02.

Regeneration was successful to a certain extent in Treatment-03 (NH_4NO_3 free stock solution with double dose of other ingredients). It performed (11.00) better than Treatment-02 at 21 DAI. But at 28 DAI the node number (10.67) decreased in case of this treatment.

Table 11: Number of leaves/plantlet of potato at different days of regeneration from nodal segment as explants

Treatment	7 DAI	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	3.00 ab	4.33 b	9.67 b	10.00 b
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	2.67 b	3.33 b	8.00 c	8.67 b
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	4.00 ab	6.00 a	11.00 b	10.67 ab
T_4 (Stock solution -01, developed in the department of Biotechnology,SAU,Dhaka)	4.33 a	7.00 a	13.00 a	13.00 a
T_5 (Ready made MS powder, the Duchefa, Netherland)	4.33 a	6.33 a	12.67 a	13.00 a
LSD $_{(0.05)}$	1.50	1.58	1.52	2.56
CV(%)	11.70	5.49	7.42	12.29

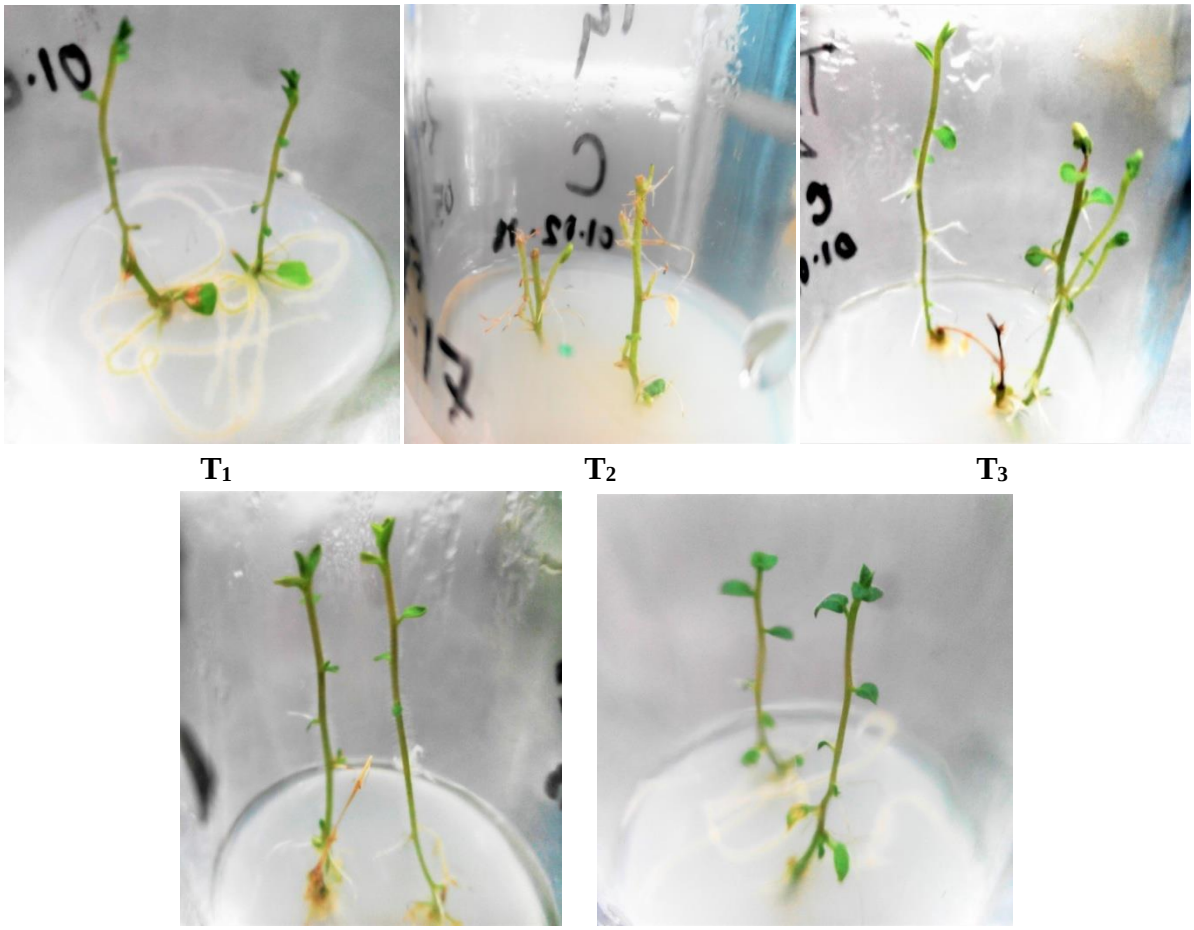


Plate 15. Effect of different treatments on Asterix variety at 14 DAI

4.2.4 Treatment effect of on root length (cm)

At 14 days after inoculation (DAI), the maximum root length (5.60 cm) was found in T_5 (readymade MS powder) which was statistically different from all other treatments. On the other hand, minimum root length (3.20 cm) was found in T_2 (NH_4NO_3 free stock solution) which was statistically different from all other treatments too (Table 12 and Plate 16). The maximum root length (7.67 cm and 15.77 cm) was found in T_5 which was statistically different from all other treatments at both 21 days and 28 days after inoculation (DAI). The minimum root length (5.47cm and 12.63 cm) was recorded in T_2 at 21 DAI and 28 DAI which was statistically different from all other treatments too.

In case of root related parameters Treatment-05 performed better than Treatment-04 which is not like the other parameters. Perhaps the β chemical is the reason of this fluctuation.

In this case T_1 (7.23cm and 14.80cm) which was formulated with standard MS dose, 1962; performed better than T_3 (6.77cm and13.47) at 21 DAI and 28 DAI respectively. New composition of Treatment-03 may have been the cause of this fluctuation in case of root length.

T_1 **T_2** **T_3**

T_4 **T_5**

Plate 16. Assessing the root length (cm) of the Asterix variety under different treatment at 21 DAI

Table 12: Length of root (cm) of potato at different days of regeneration from nodal segment as explant

Treatment	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	5.10 b	7.23 b	14.80 c
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	3.20 d	5.47 d	12.63 e
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	4.73 c	6.77 c	13.47 d
T_4 (Stock solution -01, developed in the department of Biotechnology,SAU,Dhaka)	5.17 b	7.10 bc	15.20 b
T_5 (Ready made MS powder, the Duchefa, Netherland)	5.60 a	7.67 a	15.77 a
LSD $_{(0.05)}$	0.30	0.40	0.21
CV(%)	3.33	3.12	5.76

4.2.5 Treatment effect on number of root

At 21 days after inoculation (DAI), the maximum root number (12.00) was found in T_5 (readymade MS powder) which was statistically different from all other treatments. On the other hand, minimum root number (6.33) was found in T_2 (NH_4NO_3 free stock solution) which was statistically different from all other treatments. The maximum result (13.33) was recorded for the same treatment T_5 (readymade MS powder) at 28 DAI too which was statistically different from all other treatments (Table 13). The minimum root number (8.00) was found in T_2 (NH_4NO_3 free stock solution) which was statistically different from all other treatments at 28 days after inoculation (DAI).

Treatment-04 (stock solution-01 with newly developed chemical) was the second best treatment. It (10.67 and 11.67) performed better than T_1 (9.00 and 10.33) and T_3 (9.67 and 10.67) both at 21 DAI and 28 DAI respectively. It may be due to the new β chemical but it did not performed best which is unlike other shoot related parameters.

Table 13: Number of root of potato at different days of regeneration from nodal segment as explant

Treatment	14 DAI	21 DAI	28 DAI
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	6.00 ab	9.00 b	10.33 b
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	3.33 b	6.33 c	8.00 c
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	6.67 a	9.67 ab	10.67 b
T_4 (Stock solution -01, developed in the department of Biotechnology,SAU,Dhaka)	6.67 a	10.67 ab	11.67 b
T_5 (Ready made MS powder, the Duchefa, Netherland)	7.00 a	12.00 b	13.33 a
LSD $_{(0.05)}$	2.97	2.65	1.61
CV(%)	6.56	14.77	7.93

4.3 Acclimatization and plant establishment in field condition

After sufficient plant development at 28 days after sub culture, the regenerated plantlets were taken to growth cabinet for acclimatization and maintained for further observations under controlled conditions of light, temperature and humidity. Then the plantlets transferred to pot filled with sterilized soil: cowdung (1:1) and after 2 weeks, exposed to lower humidity and higher light intensity. Finally, after 20 days plantlets were transferred to natural environment. The survival rate of the regenerated plants in growth chamber (Table 14) and in field condition (Table 15) has been shown below:

Table 14. Survival rate of *in vitro* regenerated plantlets in growth chamber

Treatment	Number of plants transplanted	Number of plants survived	Survival percentage (%)
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	20	16	80%
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	20	15	75%
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	20	17	85%
T_4 (Stock solution -01, developed in the department of Biotechnology,SAU,Dhaka)	20	18	90%
T_5 (Ready made MS powder, the Duchefa, Netherland)	20	18	90%

Table 15. Survival rate of *in vitro* regenerated plantlets in field condition

Treatment	Number of plants transplanted	Number of plants survived	Survival percentage (%)
T_1 (Stock solution -01 formulated as recommended dose of MS 1962)	15	9	60%
T_2 (Stock solution-01 without NH_4NO_3 and other ingredients same as MS 1962 dose)	15	3	20%
T_3 (Stock solution -01 without NH_4NO_3 but other ingredients have double dose of MS 1962 formulation)	15	9	60%
T_4 (Stock solution -01, developed in the department of Biotechnology,SAU,Dhaka)	15	12	80%
T_5 (Ready made MS powder, the Duchefa, Netherland)	15	12	80%

CHAPTER V
SUMMARY AND CONCLUSION

5.1 SUMMARY

The present experiment was onducted to study the performance of Ammonium Nitrate free media for *in vitro* regeneration of potato in the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Sher e-Bangla Nagar, Dhaka-1207 from January, 2016 to June, 2017. Two sub-experiments were done under this study and the key findings are-

5.1.1 Sub-experiment 01: *In vitro* regeneration of potato in modified stock solution-01 using sprout as explants.

The Treatment-04 (stock solution-01 with newly developed chemical) and Treatment-05 (readymade MS powder) showed better result in respect of different parameters under investigation. On the contrary, in case of the Treatment-02 (NH_4NO_3 free stock solution), the sprouts of Asterix variety did not regenerate well, may be because of the low concentration of nitrogen. The absence of NH_4NO_3 may have been the triggering factor of this poor performance. Treatment-03 (NH_4NO_3 free stock solution with double dose of other ingredients) performed at satisfactory level but not upto maximum level because it was a NH_4NO_3 free treatment. Treatment-01(standard MS dose,1962) performed at medium level and differed significantly than Treatment-05. In spite of having the same MS medium composition the ready made MS powder performed much better than the formulated Treatment-01. It can be concluded that, different modifications of stock solution-01 has significant effect on plantlet regeneration and its development.

The maximum shoot length (8.00cm) was found in Treatment-04 which was statistically different from all other treatments at 21 days after inoculation. Highest length (9.17 cm) was also noticed at 28 DAI from the same treatment. The minimum shoot length (4.67cm) was found in Treatment-02 which was statistically different from all other treatments at 21 days after inoculation. Same treatment showed minimum shoot length (6.60 cm) at 28 DAI too. Moderate shoot length (6.67 cm and 8.57 cm) were recorded in Treatment-03 both at 21 DAI and 28 DAI respectively.

The maximum node number (3.33) was found in Treatment-04 which was statistically different from all other treatments at 14 days after inoculation (DAI). On the other hand, the minimum node number (1.33) was found in Treatment-02 which was statistically different from all other treatments. The maximum node number (7.67) was found in Treatment-04 which was statistically different from all other treatments at 21 days after inoculation (DAI). The maximum

leaf number (4.33) was found in Treatment-04 which was statistically different from all other treatments at 14 DAI. The minimum leaf number (1.33) was found in Treatment-02 which was statistically different from all other treatments at 14 DAI. At 21 days after inoculation (DAI), the maximum leaf number (9.00) was found in Treatment-04 which was statistically different from all other treatments.

At 14 days after inoculation (DAI), the maximum root length (2.67 cm) was found in Treatment-05 which was statistically similar with Treatment-04 and different from all other treatments. On the other hand, minimum root length (1.17 cm) was found in Treatment-02 which was statistically different from all other treatments. The maximum root length (9.90 cm and 13.50 cm) was found in Treatment-05 which was statistically similar with Treatment-03 (8.60 cm and 11.40 cm) and Treatment-04 (9.63 cm and 13.00 cm) and different from all other treatments at both 21 days and 28 days after inoculation (DAI).

At 21 days after inoculation (DAI), the maximum root number (10.00) was found in Treatment-05 which was statistically similar with other treatments. On the other hand, minimum root number (6.67) was found in Treatment-02 which was statistically similar with all other treatments. Same minimum result(6.67) was recorded for the same treatment Treatment-02 at 28 DAI too which was statistically different from all other treatments.

5.1.2 Sub-experiment 02: *In vitro* regeneration of potato in modified stock Solution-01 using nodal segment as explant

The objective of present study was to evaluate the performance of Ammonium Nitrate free media in case of *in vitro* regeneration potentiality of Asterix variety. The key findings of this experiment proved that Treatment-04 containing 5 gm/l of β chemical showed the best performance on *in vitro* regeneration of potato in Asterix variety. The parameters showed significant difference among other treatments. It proves that β chemical has positive influence on growth of potato plantlets.

The maximum shoot length (3.97cm) was found in Treatment-04 which was statistically different from all other treatments at 14 days after inoculation. Same treatment showed maximum shoot length (4.17 cm and 4.27 cm) at 21 DAI and 28 DAI respectively . The minimum shoot length (1.97cm) was found in Treatment-02 which was statistically different from all other treatments at 14 days after inoculation. Same treatment showed minimum shoot length (2.77 cm) at 28 DAI respectively. The maximum node number (8.33) was found in Treatment-04 which was statistically similar with Treatment-05 (8.00) and different from all other treatments at 21 days after inoculation (DAI).

The maximum leaf number (7.00) was found in Treatment-04 which was followed by Treatment-05 (6.33) and was statistically different from Treatment-02 which showed minimum leaf number (3.33) at 14 DAI. The maximum leaf number (13.00) was found in Treatment-04 and Treatment-05 which was statistically different from Treatment-02 (8.67) at 28 days after inoculation (DAI).

At 14 days after inoculation (DAI), the maximum root length (5.60 cm) was found in Treatment-05 which was statistically different from all other treatments. On the other hand, minimum root length (3.20 cm) was found in Treatment-02 which was statistically different from all other treatments too. The maximum root length (7.67 cm and 15.77 cm) was found in Treatment-05 which was statistically different from all other treatments at both 21 days and 28 days after inoculation (DAI).

At 21 days after inoculation (DAI), the maximum root number (12.00) was found in Treatment-05 which was statistically different from all other treatments. On the other hand, minimum root number (6.33) was found in Treatment-02 which was statistically different from all other treatments. The maximum result (13.33) was recorded for the same treatment Treatment-05 at 28 DAI too which was statistically different from all other treatments. The minimum root number (8.00) was found in Treatment-02 which was statistically different from all other treatments at 28 days after inoculation (DAI).

5.2 CONCLUSION

In vitro regeneration potentiality was studied in different modified stock solution-01. It revealed that MS 1962 dose of stock solution-01 has positive effect on *in vitro* regeneration of potato. All the parameters under studied performed at medium level.

Without NH_4NO_3 along with standard dose of other ingredients for the preparation of stock solution-01 has tremendous negative effect on *in vitro* regeneration of potato. All the morphological parameters *viz* shoot length, number of leaf, root length etc. studied showed lowest performance in this treatment. It indicates that NH_4NO_3 is essential for *in vitro* regeneration of potato.

In the 3rd treatment where stock solution-01 was made without NH_4NO_3 but other ingredients had double dose of MS 1962 formulation, *in vitro* regeneration of potato occurred at satisfactory level but not up to maximum level. It showed that minimum level of potato regeneration can be done without NH_4NO_3 by using this new dose of stock solution-01.

In contrast, when NH_4NO_3 was replaced by β chemical in stock solution-01 which was developed from the Department of Biotechnology, SAU, Dhaka, the treatment showed the best performance for all the parameters studied. It proved that we can replace NH_4NO_3 with β chemical for preparation of stock solution-01.

Ready made MS powder (The Duchefa) had positive effect on *in vitro* regeneration in potato. Some of the parameters showed highest performance in regeneration. This treatment showed statistically similar result with the treatment which we designed by
β chemical for stock solution-01 preparation.

Finally, it can be concluded that *in vitro* regeneration of potato from sprout and nodal segment can be done successfully by replacing NH_4NO_3 with β chemical for stock solution-01 (solution developed from the Department of Biotechnology, SAU, Dhaka). Stock solution-01 (without NH_4NO_3 and double dose of other ingredients has capacity to regenerate plantlet of potato but the morphological appearance of those plantlet were lower than the MS powder or β chemical containing stock solution-01.

CHAPTER VI
RECOMMENDATIONS

1. As only one variety has been used in this experiment other potato varieties should be brought under this investigation. Other commercial crops also should be tried out by this modified media.

2. Other possible modifications also should be tried in case of potato as potato is easy to regenerate.

3. In addition to sprout and nodal segment other explants like meristem, shoot tip can be practiced for plantlet regeneration. More specific parameters for the effect of variety and treatments of subculture can be studied.

4. Further experiments should be tried with different growth regulators for *in vitro* regeneration.

CHAPTER VII
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CHAPTER VIII
APPENDICES

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

Components	**Concentrations**	
Major Salts(10×)	**mg/L**	**g/L**
KNO_3	1900	19.00
NH_4NO_3	1650	16.50
$MgSO_4.7H_2O$	370	3.70
$CaCl_2.2H_2O$	440	4.40
KH_2PO_4	170	1.70
Minor Salts(100×)	-	**mg/L**
KI	0.83	83
H_3BO_3	6.2	620
$MnSO_4.H_2O$	22.3	2230
$ZnSO_4.7H_2O$	8.6	860
$Na_2MoO_4.2H_2O$	0.25	25
$CuSO_4.5H_2O$	0.025	2.5
$CoCl_2.6H_2O$	0.025	2.5
Iron EDTA solution(100×)	-	**g/L**
$FeSO_4.\ 7H_2O$	27.8	2.78
$Na_2EDTA.\ 2H_2O$	37.3	3.73
Vitamins(100×)	-	**mg/L**
Myo-Inositol	100	10000
Nicotinic acid	0.5	50
Pyridoxine HCl	0.5	50
Thiamine HCl	0.1	10
Glycine	2.0	200

Total concentration of Micro and Macro salts including Iron sources and Organics (vitamins): 4405.19 mg/L.

Appendix II: Mean square value of the data of Asterix potato explants at 7 DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of	
		Days to shoot initiation	No of shoot/explant
Replication	2	2.067	0.267
Treatment	4	7.833*	0.233 ^{NS}
Error	8	0.233	0.183

*significant at 5% level of probability

NS- Non significant

Appendix III: Mean square value of the data on Asterix potato explants on shoot length(cm) at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of		
		14 DAI	21 DAI	28 DAI
Replication	2	1.067	0.35	0.173
Treatment	4	4.1*	4.683*	3.261*
Error	8	0.9	0.433	0.458

*significant at 5% level of probability

Appendix IV: Mean square value of the data on Asterix potato explants on number of shoot/explant at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of		
		14 DAI	21 DAI	28 DAI
Replication	2	0.067	0.266	0.1
Treatment	4	0.9 ^{NS}	0.489*	1.394*
Error	8	0.65	0.062	0.028

*-Significant at 5% level

NS- Non significant

Appendix V: Mean square value of the data on Asterix potato explants on shoot Diameter (mm) at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of	
		21 DAI	28 DAI
Replication	2	0.016	0.004
Treatment	4	0.429*	0.801*
Error	8	0.023	0.024

*significant at 5% level of probability

Appendix VI: Mean square value of the data on Asterix potato explants on number of node/plantlet at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of		
		14 DAI	21 DAI	28 DAI
Replication	2	0.6	0.067	0.2
Treatment	4	1.567*	5.9*	4.667*
Error	8	0.267	0.9	0.867

*significant at 5% level of probability

Appendix VII: Mean square value of the data on Asterix potato explants on number of leaves/plantlet at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of		
		14 DAI	21 DAI	28 DAI
Replication	2	0.467	0.2	0.2
Treatment	4	4.9*	6.567*	12.6*
Error	8	0.3	0.867	1.2

*significant at 5% level of probability

Appendix VIII: Mean square value of the data on Asterix potato explants on length of root (cm) at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of		
		14 DAI	21 DAI	28 DAI
Replication	2	0.017	0.026	3.421
Treatment	4	1.025*	13.692*	23.032*
Error	8	0.288	1.155	2.091

*significant at 5% level of probability

Appendix IX: Mean square value of the data on Asterix potato explants on umber. of root/explant at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of	
		21 DAI	28 DAI
Replication	2	0.067	3.267
Treatment	4	5.9 ^{NS}	35.067*
Error	8	2.9	1.767

*significant at 5% level of probability

NS- Non significant

Appendix X: Mean square value of the data on Asterix potato explants on shoot length (cm) at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of			
		7 DAI	14 DAI	21 DAI	28 DAI
Replication	2	0.165	0.369	1.049	0.949
Treatment	4	0.764*	1.721*	1.312*	1.042*
Error	8	0.006	0.039	0.183	0.05

*significant at 5% level of probability

Appendix XI: Mean square value of the data on Asterix potato explants on number of node/plantlet at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of			
		7 DAI	14 DAI	21 DAI	28 DAI
Replication	2	0.267	0.467	0.067	0.2
Treatment	4	0.267 ^{NS}	0.5 ^{NS}	8.567*	3.733*
Error	8	0.517	0.3	1.067	0.533

*significant at 5% level of probability

NS- Non significant

Appendix XII: Mean square value of the data on Asterix potato explants on number of leaves/plantlet at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of			
		7 DAI	14 DAI	21 DAI	28 DAI
Replication	2	0.467	0.2	1.067	0.267
Treatment	4	1.833*	6.9 *	13.1 *	10.9*
Error	8	0.633	0.7	0.65	1.85

*significant at 5% level of probability

Appendix XIII: Mean square value of the data on Asterix potato explants on Length of root (cm) at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of		
		14 DAI	21 DAI	28 DAI
Replication	2	0.446	1.081	0.513
Treatment	4	2.566*	2.098*	4.992*
Error	8	0.025	0.046	0.012

*significant at 5% level of probability

Appendix XIV: Mean square value of the data on Asterix potato explants on Number of root at several DAI (Days after inoculation) as influenced by different treatment

Sources of Variation	Degrees of freedom	Mean Square value of		
		14 DAI	21 DAI	28 DAI
Replication	2	0.067	6.067	11.4
Treatment	4	6.733*	13.433*	11.433*
Error	8	2.483	1.983	0.733

*significant at 5% level of probability