COMPARATIVE PERFORMANCE OF AMMONIUM NITRATE (NH_4NO_3) AND OTHER NITROGENOUS SALTS FOR *IN VITRO* REGENERATION OF POTATO (*Solanum tuberosum* L.)

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CERTIFICATE

This is to certify that the thesis entitled **'COMPARATIVE PERFORMANCE OF AMMONIUM NITRATE (NH_4NO_3) AND OTHER NITROGENOUS SALTS FOR *IN VITRO* REGENERATION OF POTATO (*Solanum tuberosum* L.)**' 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,** embodies the results of a piece of bonafide research work carried out by **Miraj Ahmed Wakil,** Registration No. **13-05682** under my supervision and guidance. No part of the thesis has been submitted 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: 07/12/2020
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Dedicated To

My Beloved Parents and Sisters

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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COMPARATIVE PERFORMANCE OF AMMONIUM NITRATE (NH_4NO_3) AND OTHER NITROGENOUS SALTS FOR *IN VITRO* REGENERATION OF POTATO (*Solanum tuberosum* L.)

ABSTRACT

An experiment was undertaken at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh during the period of January, 2018 to January, 2019 to identify a new chemical for the substitute of explosive chemical Ammonium nitrate (NH_4NO_3) in MS media composition for *in vitro* regeneration of potato (*Solanum tuberosum* L.). One potato variety Asterix was used as an experimental materials. The new chemical was denoted as α-chemical for the privacy of the experimental findings. MS powder, MS liquid standard dose, different concentrations (1, 5, and 10 gmL^{-1}) of α chemical which have twice dose of other component in stock solution-I for the preparation of tissue culture media were used as treatment. It was noticed that *in vitro* regeneration of potato has been successfully done with α-chemical in new composition of tissue culture media. The shoot length, node number, number of leaf, root length were highest in MS powder (T_0) at 14, 21 and 28 days after inoculation (DAI). Although, in some parameters MS powder (T_0) gave maximum result but it was statistically non-significant with 5gm of α- chemical/litre in stock solution-I having twice dose of other components in new media composition. The treatment 5gm of α-chemical/litre in stock solution- I having twice dose of other component showed average performance in all the traits under studied. MS powder, Liquid MS standard dose and 1 gmL^{-1} in stock solution- I twice dose of α chemical showed comparatively similar result on most of the parameters under studied. The newly used α-chemical is cheap, non-explosive and environmental friendly which can be an alternative of destructive chemical Ammonium nitrate (NH_4NO_3) for tissue culture media preparation.

CHAPTER I

1 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. 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, 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 tuber 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.. Tissue culture techniques use to obtained good quality seeds through virus eradication using meristem culture, rapid *in vitro* plants multiplication and large scale plantlet production in potato. I*n 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).

Now-a-days 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 4-5 decade 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 & ACI have already marketed tissue cultured plantlets such as potato, banana and ornamental plants in Bangladesh eventually is 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.

Preparation of culture media is the basic foundation for any tissue culture work. At least, 17 different macro and micro plant nutrients are used for the preparation of culture media. Most widely used MS media (1962) composition of nutrients is used for rapid micropropagation and meristem culture technique. The recommended dose of nutrient given by Murashige & Skoog (1962) has been successfully used for last 50-60 year. Ammonium Nitrate (NH_4NO) is an important chemical used as macro nutrient in MS media preparation. The amount of ammonium nitrate per litre is 16.50 gm. It is rich in nitrogen. Near about 35% N present in ammonium nitrate. It is a good source for supply of nitrogen in culture media.But it has a great disadvantage in human civilization. Ammonium nitrate is an explosive chemical. It is used for the production of bomb and in many other destractive activities. Hence, it is not available in our country. Among the major salts,very important one is ammonium nitrate (NH_4NO_3) which is now banned for its destructive property. It is widely used for agricultural as well as blasting purpose. So both the advantages and disadvantages are associated. Owing to its unethical use by antisocial entities, using and purchasing this chemical is getting difficult day by day,. It is one of the cheapest crop nourishing fertilizer type and hence it is easily available in the markets.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 banned on its manufacture, sale and purchase. 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. 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 used for tissue culture media preparation. We try

to find out any alternate chemical which will be replaced NH_4NO_3 on tissue culture media. Cell and tissue culture technology has been the most pronounced in potato compared to any other crop species. Hence, it is used potato plant for *In vitro* regeneration.

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

- ✓ To study the comparative performance of Ammonium Nitrate and other Nitrogenous salts for *In vitro* regeneration of potato.
- ✓ To study the efficiency of α- chemical as a component of stock solution –I
- ✓ To modify the dose of macro nutrient for potato regeneration.
- ✓ To establish an *in vitro* regeneration protocol of potato by applying new chemical.

CHAPTER II

2 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 micro-propagation

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 different 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 factor for an 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/cm^2 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 GA3: 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 GA3 + 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 GA3 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.CH_3(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 T_1, T_2, T_3, T_4, T_5, T_6 and T_7 along with control T_0 (no growth regulators) on mass propagation of two potato cultivars K.CH_3 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 (T_2). This combination took minimum time for regeneration of multiple shoots and roots on internodes of cultivar K.CH_3. 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.

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 mm^2. 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, NH_4NO_3 and KNO_3

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 NH4NO_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 (KNO3 = 3800 mg/l and NH_4NO_3= 3300 mg/l) and NT_3 (KNO_3 = 900 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 (104.25mg) 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.

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.

2.9 Effect of Ammonium Nitrate Free Medium Composition

Hena *et al.* (2011) conducted an experiment to found regeneration capability in Ammonium Nitrate free medium composition of potato.

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.

CHAPTER III

3 MATERIALS AND METHODS

The required components and process applied in this study have been stated in this chapter. 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 were discussed bellow:

3.1 Experiment site and duration:

Comparative performance of Ammonium Nitrate and other Nitrogenous salts for *In vitro* regeneration of potato variety was conducted in Biotechnology laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Sher e-Bangla Nagar, Dhaka-1207 from January, 2018 to January, 2019.

3.2 Materials used in experiment:

Potato is a model plant for tissue culture research. Node & shoot tip of potato was used as an experimental material for *in vitro* regeneration of potato. One potato variety Asterix were used for this experiment. The materials were collected from Tuber Crop Research Center (TCRC), Bangladesh Agricultural Research Institute (BARI), Gazipur. Data were recorded on days to shoot initiation, days to root initiation, number of node per plantlet, length of shoot (cm), length of root (cm), number of shoot, number of leaf, number of root and . Observation was done at 7, 14 and 21 & 28 days after transplant of explant in media.

3.3 Experiment Procedure

As a substitute of NH_4NO_3 we used a new chemical. The name, formula of the chemical is secreted for all upto patenting this technology. For the presentation of the result it is denoted the chemical as α-chemical .

Experiment I : Comparative performance of different formulations of MS media and using α-chemical as a substitute of NH_4NO_3 for *in vitro* regeneration of potato

3.4 Laboratory preparation

Laboratory preparation was started in January 2018 by collecting chemical and instruments. List of chemicals and equipments were presented in **Table 1**.

Table 1.List of the chemicals and instruments used in the experiment

	Chemicals		Instruments
1	α-chemical	1	Autoclave machine
2	Sterilizing chemicals Sodium hypo chloride Potassium hypo chloride Tween-20, $HgCl_2$	2	Hotplate with magnetic stirrer
3	Sucrose	3	Automatic drying oven
4	Agar	4	Freezers
5	NaOH (10 N, 1N)	5	Autoclave
6	HCl	6	Incubators
7	KCl (3M)	7	Laminar Air Flow Chamber
8	Sterilized distiled water	8	Microwave oven
9	Absolute Ethanol	9	Pipettors
10	Ethanol (70%)	10	Plant Growth Chamber
11	Methilated spirit	11	Safety Cabinets
12	Stock Solution I (MS medium ingredients)	12	Shakers
13	Stock Solution II (MS medium ingredients)	13	Shaking Incubator
14	Stock Solution III (MS medium ingredients)	14	Water Purification System
15	Stock Solution IV (MS medium ingredients)	15	pH meter
16	Stock Solution V (MS medium	16	Course and fine electric balances

	ingredients)		
		17	Scalpel, forceps, scissors etc
		18	Culture vials (petridishes, tube)

3.5 Stock solution preparation

The first step in the preparation of the medium is the preparation of stock solutions of the various constituents of the MS medium. As different media constituents were required in different concentrations, separate stock solutions for the macronutrients, micronutrients, Fe-EDTA (Iron stock), vitamins and growth regulators were prepared separately for ready use. MS medium is prepared by the combination of stock solutions with different minerals and hormones required for plant regeneration and growth. Each stock solution is composed of different types and amount of major salt, minor salts, iron and organic, growth regulators etc. respectively.

All the chemicals used for stock solution is highly purified and labeled as plant tissue culture tested grade. The chemicals are dissolved in double distilled water or highly purified de-ionized water. Each chemical are added according to the list of ingredient presented in Appendix-1. Concentration of α-chemical was measured on the basic of requirement of NH_4NO_3 present in MS standard dose.

Plate 1: Preparing stock solution

3.5.1 Stock solution-I of Major salts

3.5.1.1 Stock solution –I for MS liquid (MSL) medium

Stock solution of macronutrients was prepared with 10 times higher of the final strength for one liter solution. Ten times the weight of the salts required for one liter of medium weighted accurately. Dissolve all the macro-nutrient one by one except $CaCl_2$. The stock solution of $CaCl_2$ was prepared separately in order to avoid precipitation. All the salts were dissolved thoroughly in 750 ml of distilled water and final volume was made up to one liter by further addition of DW. The stock solution was poured into a clean sterilized glass container and stored in a refrigerator at 4°C for ready use.

3.5.1.2 Modification of MS medium by α- chemical

Stock solution –I of MS medium was modified by using new α- chemical. Different concentrations of α- chemical were added as a substitute of NH_4NO_3. Six different concentrations of α- chemical were used for this purpose. The modification of stock –I solution was given below:

3.5.1.2.1 Modification –1 of stock –I solution

The modification-1 of stock solution-I is same as previous of MSL. The only difference was that we used α- chemical 1 gmL^{-1} as a substitute of NH_4NO_3

3.5.1.2.2 Modification –2 of stock –I solution

The modification-2 of stock solution-I was same as standard MSL. Here we used 5gmL^{-1} α - chemical for replacement of NH_4NO_3.

3.5.1.2.3 Modification –3 of stock –I solution

The modification-3 of stock solution-I is same as MSL. The only difference was that we used α - chemical 10 gmL^{-1} as a substitute of NH_4NO_3.

3.5.1.2.4 Modification –4 of stock –I solution

The modification-4 of stock solution-I is twice dose of MSL. The only difference was that we used α - chemical 1 gmL^{-1} as a substitute of NH_4NO_3.

3.5.1.2.5 Modification –5 of stock –I solution

The modification-5 of stock solution-I is twice dose of as MSL. The only difference was that we used α - chemical 5 gmL^{-1} as a substitute of NH_4NO_3.

3.5.1.2.6 Modification –6 of stock –I solution

The modification-5 of stock solution-I is twice dose of as MSL. The only difference was that we used α - chemical 10 gmL^{-1} as a substitute of NH_4NO_3

3.5.2 Micronutrients stock solution (stock II)

A stock solution of all the micronutrients with 100x concentration is generally prepared. Since copper and cobalt are required in very small quantities, it was prepared first to make a separate stock solution of those two salts (100) and then an appropriate volume was pipetted and put into the main micronutrient stock solution. This stock solution was also stored in refrigerator at 4°C.

3.5.3 Iron (Fe-EDTA) stock solution (stock III)

Iron-EDTA was added freshly and it was made 100 times the final strength of the medium in one liter DW. Here, two constituents, $FeSO_4.7H_2O$ and Na_2EDTA, were dissolved in 750 ml of DW in a conical flask by heating in a water bath until the salts dissolved completely and final volume was made up to one liter by further addition of DW. This stock should be stored in an amber color bottle or a bottle covered with an aluminum foil and stored in refrigerator at 4°C.

3.5.4 Vitamins stock solution (stock IV)

The following vitamins were used in the present study for the preparation of MS medium. Myo-inositol (Inositol),Nicotinic acid (Vitamin B_3), Pyridoxin HCl (Vitamin B_6), Thiamine HCl (Vitamin B_1) and Glycine. Each of the vitamins were taken at 100 times of their final strength in measuring cylinder and dissolved in 400 ml of distilled water. The final volume was made up to 1000 ml by further addition of distilled water. This stock solution was also labeled and stored in a refrigerator at 4°C.

3.6 The treatment combinations of experiment

3.6.1 Experiment

Comparative performance of different formulations of MS media and using α-chemical as a substitute of NH_4NO_3 for *in vitro* regeneration of potato

Ammonium nitrate is used as macro nutrient in stock-A solution. So variation of chemical will be in stock- I solution. Rest of nutrients components for the stock solution in MS media preparation will be same as MS, 1962.

We conducted one experiment to fulfill our objectives. The treatment combinations of **experiment** given below:-

T_0= MS powder

T_1= 16.50 gm/L of ammonium nitrate (MS standard dose in stock solution-I)

T_2= 1 gm of α- chemical/litre in stock solution- I

T_3= 5 gm of α- chemical/litre in stock solution- I

T_4=10 gm of α- chemical/litre in stock solution- I

T_5=1 gm of α- chemical/litre in stock solution- I having twice dose of other components

T_6=5 gm of α- chemical/litre in stock solution- I having twice dose of other components

T_7=10gm of α- chemical/litre in stock solution- I having twice dose of other components

3.7 Different Stock solutions for MS media preparation

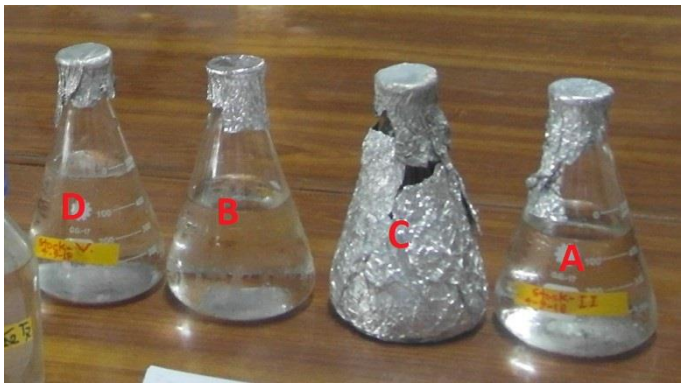


Plate 2: Standard dose of Stock solution-I (16.50 gmL^{-1} of NH_4NO_3) and other stock solution II, III, IV and V for MS media preparation

A= Stock –I (MS standard dose having 16.50 gm/L of NH_4NO_3)

B= Stock-II

C= Stock-III

D= Stock-IV

Plate 3:All modification of stock solution-I along with MS standard dose and Readymade MS powder.

A=MS standard dose in liquid condition.

B=Modification-1 of stock solution -I

C= Modification-2 of stock solution -I

D= Modification-3 of stock solution -I

E= Modification-4 of stock solution -I

F= Modification-5 of stock solution -I

G= Modification-6 of stock solution -I

H= Readymade MS powder

3.8 Other stock solutions preparation

3.8.1 Preparation of IN NaOH

40 g NaOH pellets were weighed and added to the 800 ml of sterilized distilled water and stirred well until dissolved. Sterilized distilled water was added to make volume 1000ml and mixed the closed bottle.

3.8.2 Preparation of IN HCl

To prepare 1L of 1 N solution of HCl, 36.5 g of the substances was dissolved in 1 L of water. It was used for adjusting pH of the cultural medium to decrease pH meter reading.

3.8.3 Preparation of 70% Ethanol

In a 100 ml measuring cylinder 70 ml 99.9% ethanol was poured. Double distilled water was poured up to the level of 100 ml. Store the solution in a sterilized glass bottle. This solution was made fresh each time before use.

3.8.4 Preparation of 10% NaOCl

To prepare 100 ml NaOCl, 10 gm of NaOCl powder was poured in a beaker and added distilled water upto make final volume. Then the solution was kept in Hot Plate magnetic Stirer to dissolve it completely. Then it was cooled in refrigerator at 4^0C.

3.8.5 Preparation of 5% NaOCl

To prepare 5% NaOCl, 5 gm NaOCl was dissolved in 100ml distilled water**.**

3.9 MS Media preparation from readymade MS powder

- ❖ To prepared one liter of MS medium, the following steps were followed:
- ❖ 700 ml double distilled water was taken into 1000 ml beaker
- ❖ 5gm of MS powder and 30 gm of sucrose was added and gently stirred to dissolved ingredients completely with the help of a hot plate magnetic stirrer.
- ❖ The whole mixture was then made up to 1 liter with further addition of double distilled water.
- ❖ pH of the medium was adjusted to 5.80±0.1 by pH meter with the addition of 1 N NaOH or 0.1 N HCl whichever was necessary.
- ❖ Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar

3.10 MS Media preparation from Stock Solution

To prepare one liter of MS medium from stock solution, the following steps were followed:

- ✓ 700 ml double distilled water was taken into 1000 ml beaker
- ✓ 100 mL of Stock solution- I (MSL-I) 10 mL of stock solution- II,10 mL of stock solution- III,10 mL of stock solution- IV + V and 30 gm of sucrose was added and gently stirred to dissolved these ingredients completely with the help of a hot plate magnetic stirrer.
- ✓ The whole mixture was then made up to 1 liter with further addition of double distilled water.
- ✓ pH of the medium was adjusted to 5.80±0.1 by pH meter with the addition of 1 N NaOH or 0.1 N HCl whichever was necessary.
- ✓ Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.
- ✓ According to treatment the modification of stock solution-A were taken.

3.11 Agar

The media was gelled with 8 g/L agar and the whole mixture was gently heated on microwave oven at 250°C Temperature for 8-10 minutes.

3.12 Sterilization

3.12.1 Sterilization of culture media

One liter of MS medium were divided into conical flasks and capped with aluminium foil. Then the conical flasks were autoclaved at 15 psi pressure at 121^0C for 20 minutes. The medium was then transfer into the culture room and cooled at 24^0C temperature. The media was aliquot fixed volume into culture vial. After dispensing the vial were covered with thin polythene cap and marked with different codes with the help of a permanent glass marker to indicate specific treatment.

Plate 4: Preparation of culture media

3.12.2 Sterilization of glassware and instruments

All types of glassware instrument was washed properly by liquid detergent, cleaned with running tap water and finally washed with distilled water and dried in automatic drying oven. Glassware, culture vessels, beakers, petridishes, pipettes, slides, plastic caps, other instruments such as forceps, needles, scissor, spatula, surgical blades,

brush, cotton, instrument stand were sterilized in an autoclave at a temperature of 121°C for 20 minutes at 15 psi pressure.

3.12.3 Sterilization of culture room and transfer area

At the starting time, the culture room was spray with formaldehyde and then the room was kept closed for one day. Then the room was cleaned through gently washing the floors walls and rakes with a detergent. This is followed by careful wiping them with 70% ethanol. This process of sterilization of culture room was repeated at regular intervals. The transfer area was also cleaned with detergent and also sterilized twice in a month by 70% ethanol. Laminar air flow cabinet was usually sterilized by switching on the cabinet. The ultra-violate ray kills the microbes inside the laminar airflow. It switches on 30 minutes before working in empty condition and for 20 minutes with all the instruments. The working surface was wiping with 70% ethanol, 30 minutes before starting the transfer work.

3.13 Sterilization of Laminar Air Flow Cabinet

The laminar air flow cabinet was started half an hour before working. The air flow cabinet surface was cleaned with cotton soaked with70% ethanol. All glassware was kept on the cabinet to reduce contamination except culture media. The lid of cabinet was closed well and UV was switched on for 30 minutes while turning off the air flow. After required time was over, UV was switched off, opened the door and switched on the air flow. Within 5 minutes, work was started. The forearms and hands were sterilized by rubbing 70% ethanol before started working. During the culture all equipment were frequently flamed after dipping with 95% ethanol.

3.14 Preparation of explants

The sprouts of potato were used as explants. The sprouts were separated from the potato and washed thoroughly with double distilled water into laminar airflow cabinet for surface sterilization, potato sprouts were first sterilized with 70% (v/v) ethanol for one minute. The sprouts were then rinsed twice with sterile distilled water. Afterwards the sprouts were surface sterilized by immersing in 0.1% $HgCl_2$ solution containing three drops of tween-20 solution and then finally rinsed and

washed four times with sterilized distilled water. The surface sterilized disinfected sprouts were then cut into small segments and kept under sterilized distilled water into sterilized petri dishes to make the sprout alive . Then the explants were ready for inoculation.

3.15 Inoculation of culture

The explants were prepared carefully under aseptic condition inside the laminar airflow cabinet. Explants were directly inoculated to each vial containing 25 ml of MS medium. The vials were plugged crocked and total operation was done in the laminar airflow cabinet in sterile condition.

Plate 5: Inoculation of culture

3.16 Sub-culture of the plantlet

The regenerated plantlets were sub-cultured after 4 week of inoculation. The shoot was cut into small pieces and placed on prepared sterilized MS medium. The sub-cultured vials were then inoculated at 25 ± 1^{0}C with 16 h photoperiod. Repeated

subculture was attended at regular interval of 28 days. The observations and data collection were noted regularly.

3.17 Culture of the regenerated shoot for root induction

After 5 weeks of proper development, shoot grew about 4-5 cm in length were excised from the culture vial and transferred to root induction media aseptically in the laminar air flow cabinet. Data was recorded after 4 and 6 weeks of subculture.

3.18 Experimental design

In laboratory condition, the one factors experiment was laid out in Completely Randomized Design (CRD) with three replications.

3.19 Data collection

Data on the following parameters were recorded under *in vitro* condition.

3.19.1 Days to Shoot Initiation

Days to shoot initiation was recorded by using a plastic scale in laminar airflow cabinet at until 7 DAS from each subculture.

3.19.2 Length of Shoot

The length of shoot was recorded by using a plastic scale in laminar airflow cabinet at 14, 21 & 30 days after sub-culture (DAS).

3.19.3 Number of leaves per plant

Total number of leaves per plant was recorded by visual observation at 14, 21 & 30 days after sub-culture (DAS) from each subculture.

3.19.4 Days to Root Initiation

Days to root initiation were recorded by using a plastic scale in laminar airflow cabinet at until 10 DAS from each subculture.

3.19.5 Length of Root

The length of root was recorded by using a plastic scale in laminar airflow cabinet at 14, 21 & 30 days after sub-culture (DAS).

3.19.6 Number of Root

Total number of root was recorded by visual observation at 14, 21 & 30 days after sub-culture (DAS) from each subculture. The mean value of the data provided the number of root.

3.19.7 Number of shoot

Total number of shoot was recorded by visual observation at 14, 21 & 30 days after sub-culture (DAS) from each subculture. The mean value of the data provided the number of shoot.

3.19.8 Number of node

Total number of internode was recorded by visual observation at 14, 21 & 30 days after sub-culture (DAS) from each subculture. The mean value of the data provided the number of internode.

3.20 Statistical analysis

The data obtained for different characteristics were statistically analyzed to find out the significance difference among the treatments. The mean values of all the recorded characteristics were evaluated and analysis of variance was performed by the 'F' (variance ratio) test using MSTAT-C software. The significance of the difference among the treatments means was estimated by Duncan's Multiple Range Test (DMRT) at 5% level of probability. LSD was also calculated to compare the differences between two treatment means.

CHAPTER IV

4 RESULTS AND DISCUSSION

The present experiment was conducted to comparative performance of Ammonium Nitrate and other Nitrogenous salts for study the *In vitro* regeneration of potato (*Solanum tuberosum* L.). One experiment was conducted under the laboratory condition. The analysis of variance (ANOVA) of the data has been presented in Appendix II-X. The results have been presented and discussed and possible interpretations were given experiment wise under the following parameters:-

4.1 Days to shoot initiation

Statistical variations were observed among different treatments on days to shoot initiation (Table 1). The maximum days to shoot initiation (6.67) was recorded in T_4 (NH_4NO_3 free stock solution), followed by T_1 (6.33) 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 (2.33days) was recorded in T_6 (stock solution-01 with α-chemical) and followed by T_0 (readymade MS powder) also followed by T_5 (3.67). Both T_3 and T_7 are 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.

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

Treatments	Days to shoot initiation
T_0	3.33 e
T_1	6.33 a
T_2	5.33 b
T_3	4.33 cd
T_4	6.67 a
T_5	3.67 de
T_6	2.33 f
T_7	4.67 bc
LSD $_{(0.05)}$	0.99
CV(%)	9.60

T_0= MS powder
T_1= 16.50 gmL^{-1} of Ammonium nitrate
T_2= 1 gm of α- chemical/litre
T_3= 5 gm of α- chemical/litre
T_4= 10 gm of α- chemical/litre

T_5= 1gm of α- chemical/litre in stock solution- I having twice dose of other components
T_6=5gm of α- chemical/litre in stock solution-I having twice dose of other components
T_7=10gm of α-chemical/litre in stock solution-I having twice dose of other components

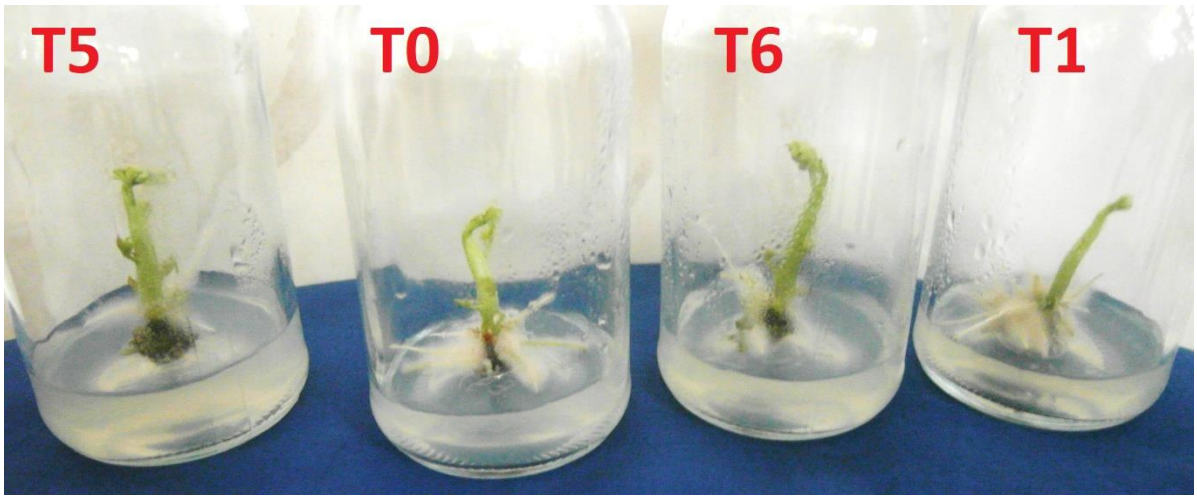


Plate 6: Shoot initiation from sprout of potato in different treatment.

4.2 Effect of treatment on shoot length (cm)

The effect of treatment in shoot length is presented in Table 02. At 14 days after inoculation, the maximum shoot length (6.80 cm) was found in 5gm of α-chemical/litre in stock solution- I, The treatment (T_6) which was statistically similar with (T_0) and different from all other treatments and followed by T_5 and T_7. The minimum shoot length (4.10 cm) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically different from all other treatments and followed by T_1.

At 21 and 28 days after inoculation, the maximum shoot length (9.50 cm) and (11.40 cm) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6. The minimum shoot length (5.39 cm) and (7.29 cm) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically different from all other treatments and followed by T_1. The robust plantlet was observed in 5gm of α- chemical/litre in stock solution- I (T_6) and T_0 (MS powder) treatment (Plate 7). Hence, reference related to α-chemical in culture media preparation is quite unavailable. Here, we mentioned few references of α-chemical used in field condition for potato production. Oliveira *et al.* (2000) reported that maximum stem elongation was

reached quickly with double density and had the tendency to keep constant at the highest (3%) and lowest (1%) nitrogen levels 70 days after planting. The rate of leaf appearance increased drastically due to more branching caused by high nitrogen level, and increased above ground dry matter per plant. Rizk *et al.* (2014) reveal that Urea as foliar spraying resulted the vigor potato plant, i.e. the tallest plants and that carried largest number, fresh and dry weight of leaves and stems. Moreover, the better plant growth was recorded with that plants received the higher urea level, i.e. 3%. The application of urea within 2 – 3 % as foliar spraying, had an increase in tuber yield.

Table 2. Effect of treatments on shoot length (cm) of potato plantlets at different days after inoculation (DAI) in Asterix variety of potato

Treatments	Shoot length (cm) at different days after inoculation (DAI)		
	14 DAI	**21 DAI**	**28 DAI**
T_0	6.50 a	9.50 a	11.40 a
T_1	4.47 cd	5.80 f	8.80 e
T_2	5.00 bc	6.06 e	8.06 ef
T_3	5.30 b	8.06 c	10.06 cd
T_4	4.10 d	5.39 g	7.29 f
T_5	5.50 b	8.95 b	11.00 b
T_6	6.80 a	9.00 a	11.10 a
T_7	5.17 b	7.50 d	9.06 de
LSD $_{(0.05)}$	0.55	0.19	1.09
CV(%)	5.93	1.43	6.40

T_0= MS powder T_1= 16.50 gmL^{-1} of Ammonium nitrate
T_2= 1 gm of α- chemical/litre T_3= 5 gm of α- chemical/litre
T_4= 10 gm of α- chemical/litre
T_5= 1gm of α- chemical/litre in stock solution- I having twice dose of other components
T_6=5gm of α- chemical/litre in stock solution-I having twice dose of other components
T_7=10gm of α-chemical/litre in stock solution-I having twice dose of other components

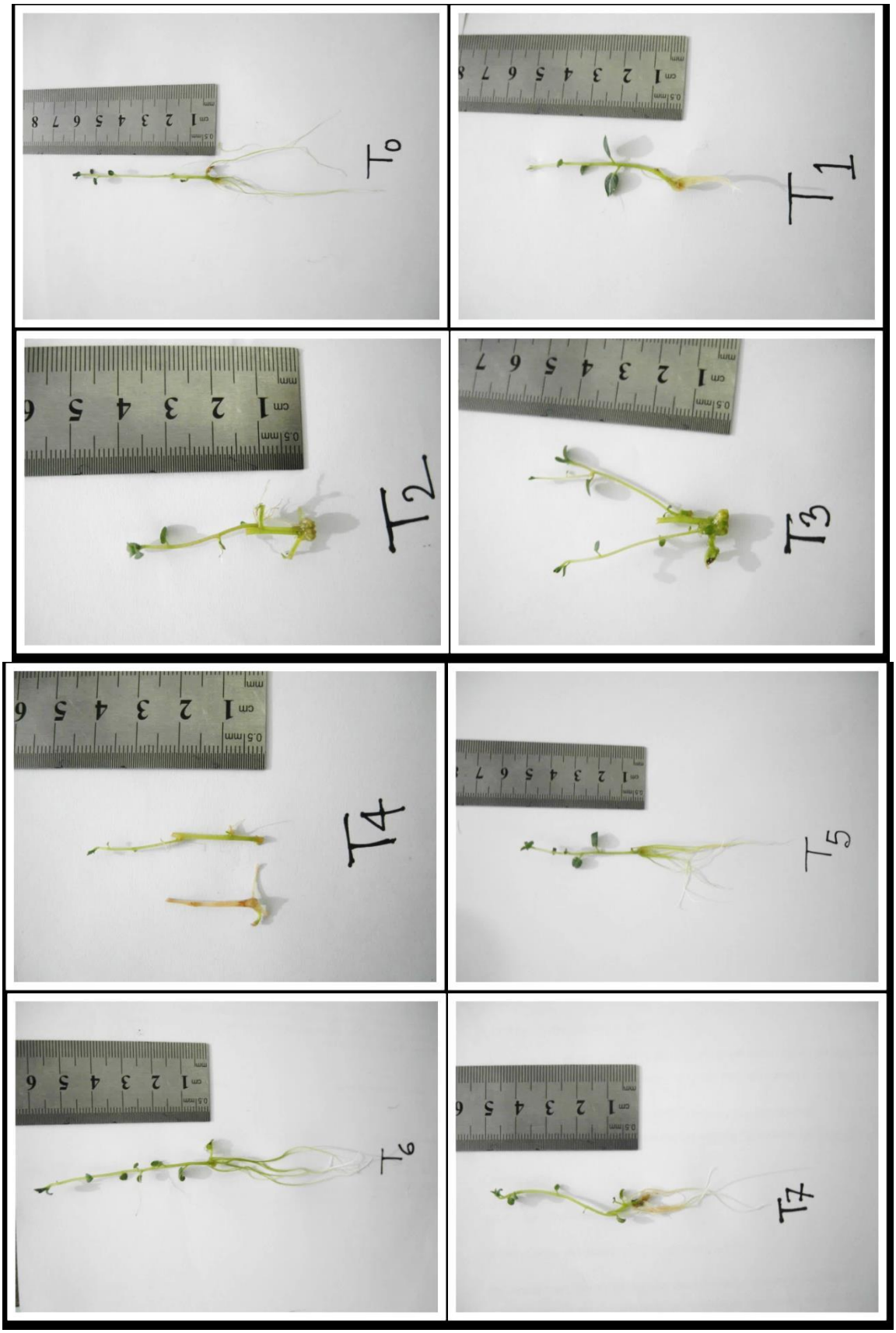


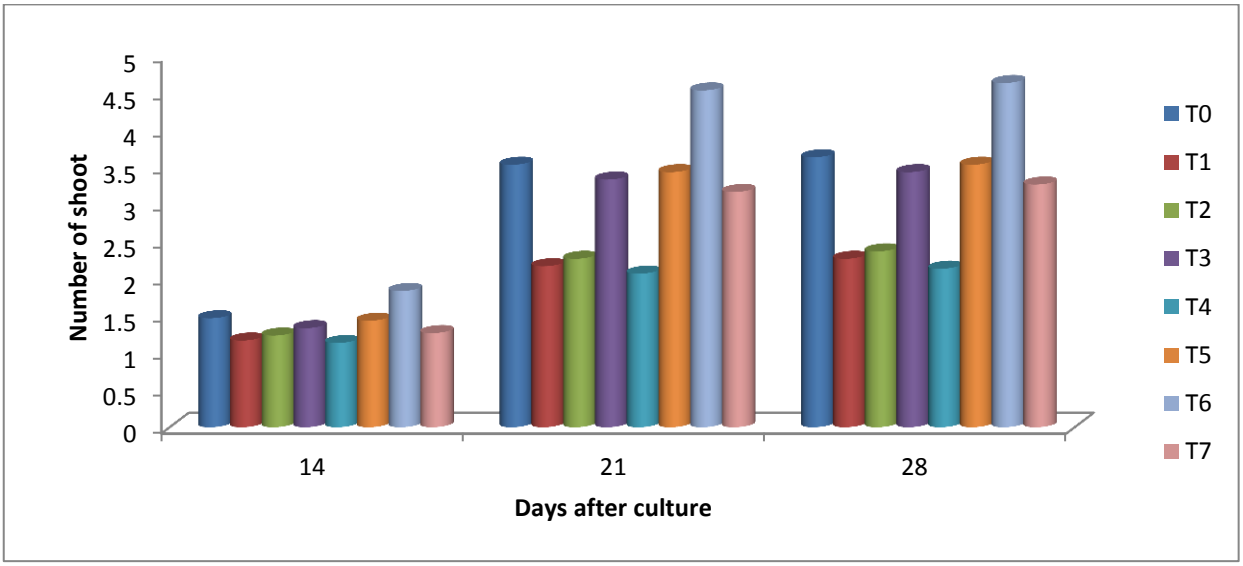
Plate7: Shoot length of potato plantlet in different treatment

4.3 Effect of treatments on the number of shoots

The effect of treatment on the number of shoot is presented in graph 01. At 14 days after inoculation, the maximum number of shoot (1.83) was found in 5gm of α-chemical/litre in stock solution- I, The treatment (T_6) which was statistically different from all other treatments and followed by T_0, T_5 and T_3. The minimum number of shoot (1.13) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically similar with T_1 and different from all other treatments.

At 21 and 28 days after inoculation, the maximum number of shoot (4.53) and (4.63) was found in 5gm of α- chemical/litre in stock solution- I. The treatment (T_6) which was statistically different from all other treatments and followed by T_0 also followed by T_5 and T_3 . The least number of shoot (2.07) and (2.13) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments and followed by T_1.

Graph 1.Shoot number of potato plantlet on various treatment at different days after initiation



T_0= MS powder
T_1= 16.50 gmL^{-1} of Ammonium nitrate
T_2= 1 gm of α- chemical/litre
T_3= 5 gm of α- chemical/litre
T_4= 10 gm of α- chemical/litre
T_5= 1gm of α- chemical/litre in stock solution- I having twice dose of other components
T_6=5gm of α- chemical/litre in stock solution-I having twice dose of other components
T_7=10gm of α-chemical/litre in stock solution-I having twice dose of other components

Plate 8: Number of shoots of potato plantlet.

4.4 Effect of treatments on the number of leaf

The effect of treatment on the number of leaf is presented in Table 03. At 14 days after inoculation, the maximum number of leaf (10.17) was found in T_0 (MS powder) which was statistically different from all other treatments and followed by T_6, T_5 and T_3.On the other hand, the minimum number of leaf (5.00) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically similar with T_1 and different from all other treatments.

At 21 and 28 days after inoculation, the maximum number of leaf (11.17) and (12.16) was found in T_0 (MS powder) which was statistically different from all other treatments and followed by T_6 also followed by T_5 and T_3 .In contrast, the least number of leaf (5.58) and (6.08) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically different from all other treatments and followed by T_1.

Table 3. Leaf number of potato plantlet in different treatment at different days after initiation

Treatments	number of leaf at different days after initiation (DAI)		
	14 DAI	**21 DAI**	**28 DAI**
T_0	10.17 a	11.17 a	12.16 a
T_1	5.25 e	6.25 d	7.25 e
T_2	6.42 d	8.00 c	9.08 d
T_3	7.33 cd	9.00 b	10.08 c
T_4	5.00 e	5.58 d	6.08 f
T_5	8.08 bc	9.00 b	10.50 bc
T_6	9.00 b	9.75 b	11.08 b
T_7	6.83 d	7.33 c	8.83 d
LSD $_{(0.05)}$	0.98	0.94	0.81
CV(%)	7.76	6.60	4.98

T_0= MS powder T_1= 16.50 gmL^{-1} of Ammonium nitrate
T_2= 1 gm of α- chemical/litre T_3= 5 gm of α- chemical/litre
T_4= 10 gm of α- chemical/litre
T_5= 1gm of α- chemical/litre in stock solution- I having twice dose of other components
T_6=5gm of α- chemical/litre in stock solution-I having twice dose of other components
T_7=10gm of α-chemical/litre in stock solution-I having twice dose of other components

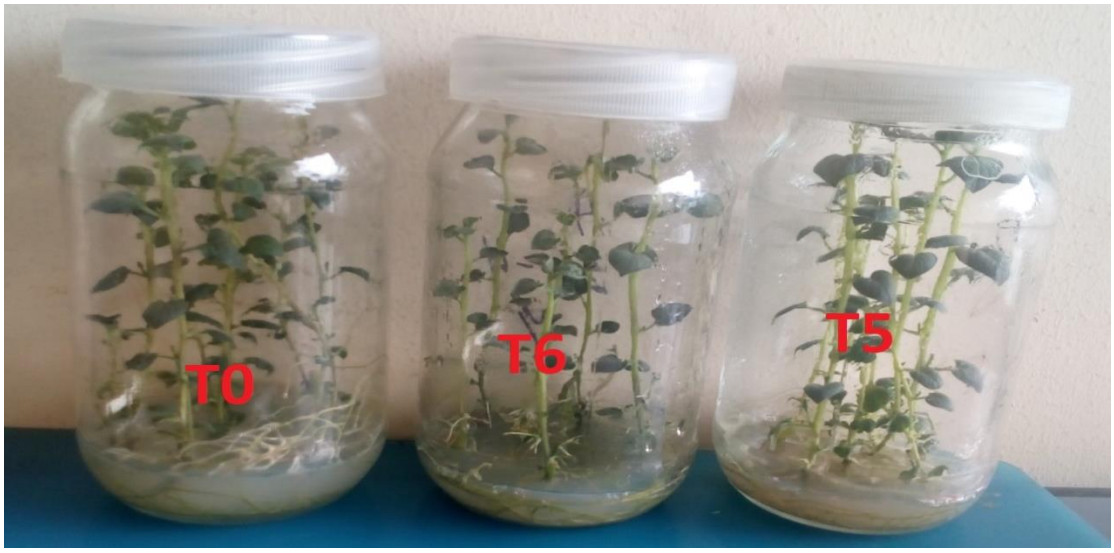


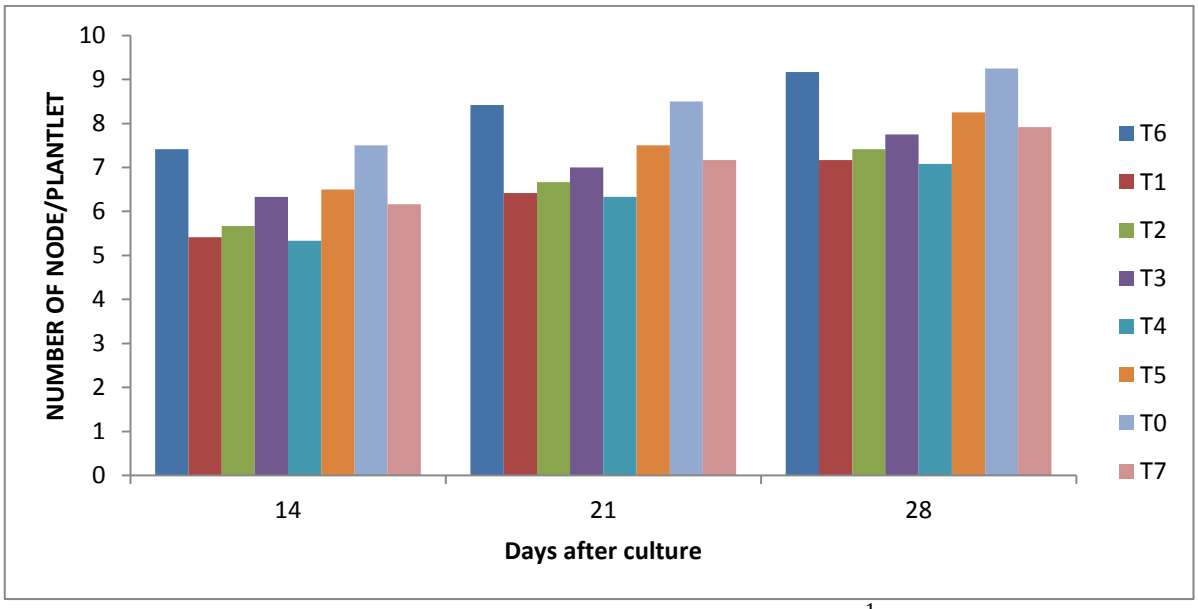
Plate 9: Number of leaves potato plantlet in different treatment

4.5 Effect of treatment on number of node

The effect of treatment on the number of node is presented in graph 02. At 14 days after inoculation, the maximum number of node (7.50) was found in the treatment T_0 (MS powder) which was statistically similar with T_6 treatment and different from all other treatments and the value was followed by T_5 and T_3.On the other hand, the minimum number of node (5.33) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically similar with T_1 and different from all other treatments.

At 21 and 28 days after inoculation, the maximum number of node (8.50) and (9.25) was found in T_0 (MS powder) which was statistically similar with T_6 treatment and different from all other treatments and followed by T_5 and T_3 .In contrast, the least number of node (6.33) and (7.08) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically similar with T_1 and different from all other treatments and followed by T_2.

Graph 2. Node number of potato plantlet on various treatment at different days after initiation



T_0= MS powder T_1= 16.50 gmL^{-1} of Ammonium nitrate
T_2= 1 gm of α- chemical/litre T_3= 5 gm of α- chemical/litre
T_4= 10 gm of α- chemical/litre
T_5= 1gm of α- chemical/litre in stock solution- I having twice dose of other components
T_6=5gm of α- chemical/litre in stock solution-I having twice dose of other components
T_7=10gm of α-chemical/litre in stock solution-I having twice dose of other components

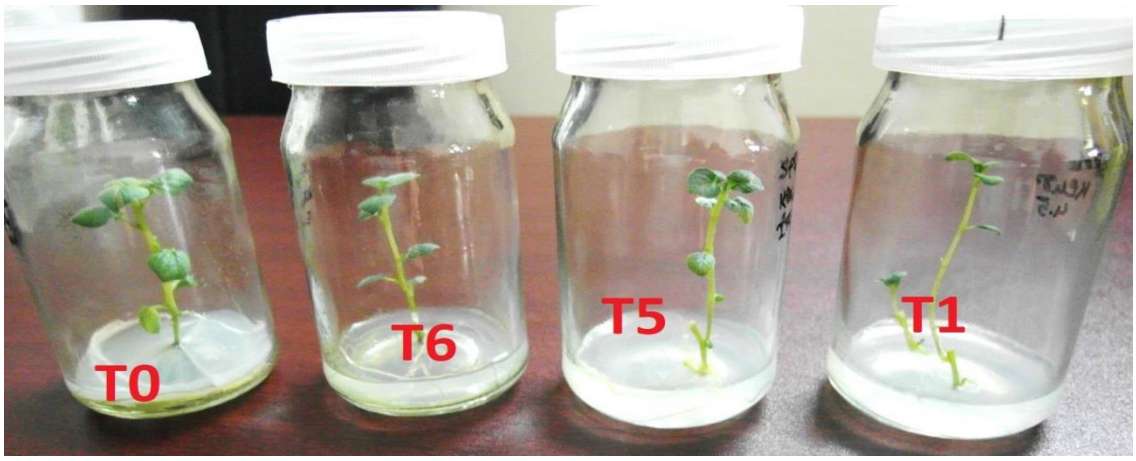


Plate 10: Number of node of potato plantlet in different treatment

4.6 Days to Root initiation

Statistical variations were observed among different treatments on days to root initiation (Table 4). The maximum days to root initiation (10.33) was recorded in T_4 (NH_4NO_3 free stock solution), followed by T_1 (9.67) which was different from all other treatments. It indicates that NH_4NO_3 is an important ingredient which is needed for proper root initiation in potato regeneration. In contrast, the minimum data (6.67days) was recorded in T_6 (stock solution-01 with α- chemical) and followed by T_0 (readymade MS powder) also followed by T_5 (7.67). Both T_3 and T_7 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.

Table 4: Days to root initiation in Asterix variety of potato

Treatments	Days to root initiation
T_0	7.33 fg
T_1	9.67 ab
T_2	9.33 bc
T_3	8.33 de
T_4	10.33 a
T_5	7.67 ef
T_6	6.67 g
T_7	8.67 cd
LSD $_{(0.05)}$	0.99
CV(%)	6.79

T_0= MS powder
T_1= 16.50 gmL^{-1} of Ammonium nitrate
T_2= 1 gm of α- chemical/litre
T_3= 5 gm of α- chemical/litre
T_4= 10 gm of α- chemical/litre

T_5= 1gm of α- chemical/litre in stock solution- I having twice dose of other components
T_6=5gm of α- chemical/litre in stock solution-I having twice dose of other components
T_7=10gm of α-chemical/litre in stock solution-I having twice dose of other components

4.7 Effect of treatment on the root length (cm)

The effect of treatment in root length is presented in Table 05. At 14 days after inoculation, the maximum root length (7.58 cm) was found in 5gm of α-chemical/litre in stock solution- I twice dose (T_6) which was statistically similar with T_0 (MS powder) and different from all other treatments and followed by T_5 and T_7. The minimum root length (1.52 cm) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically different from all other treatments and followed by T_1.

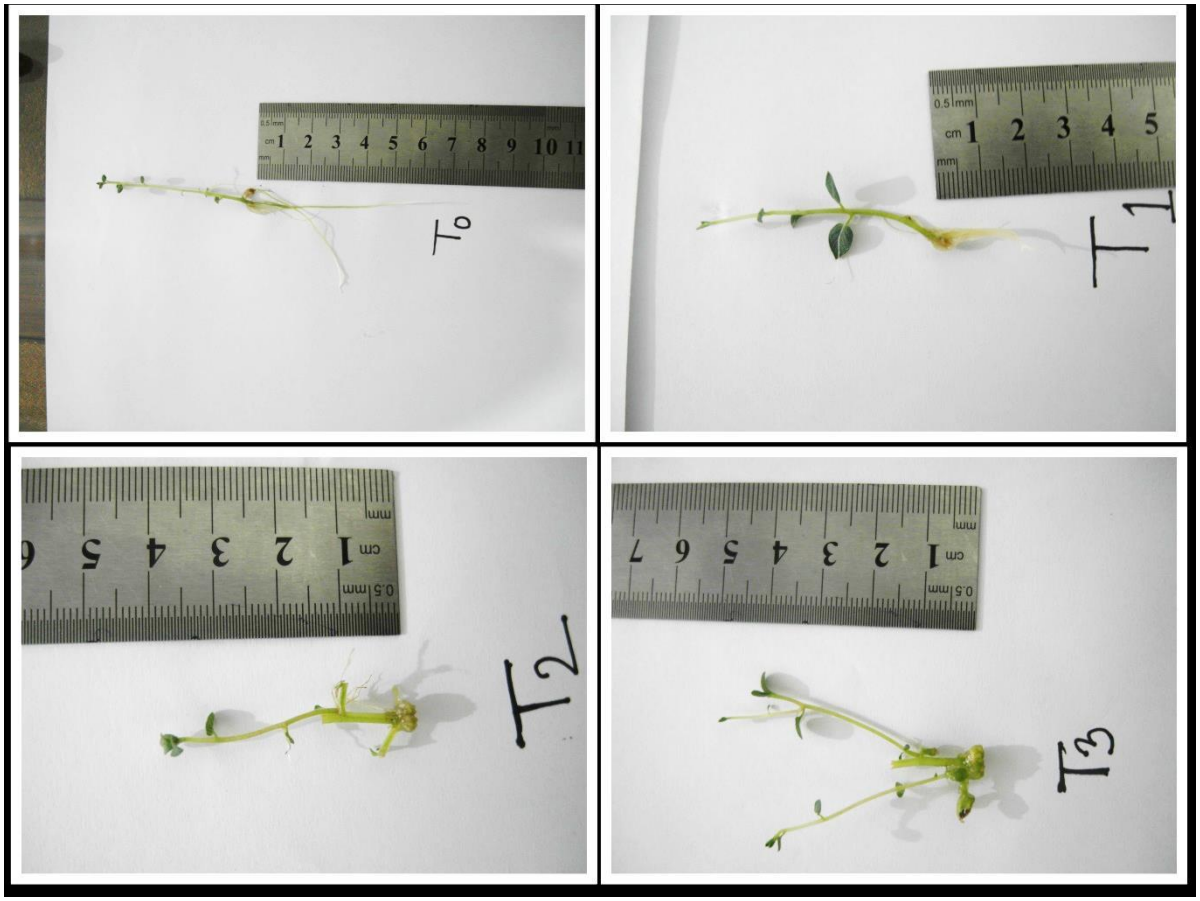
At 21 and 28 days after inoculation, the maximum root length (9.67 cm) and (11.58 cm) was found in 5gm of α- chemical/litre in stock solution- A twice dose (T_6) which was statistically similar with (MS powder) and different from all other treatments and followed by T_5 and T_7 . The minimum root length (1.75 cm) and (2.08 cm) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments and followed by T_1.

Table 5.Root length (cm) of potato plantlets at different days after inoculation in the Asterix variety

Treatments	Root length (cm) at different days after inoculation (DAI)		
	14 DAI	21 DAI	28 DAI
T_0	7.50 a	9.65 a	11.40 a
T_1	2.50 f	4.58 e	6.75 e
T_2	4.95 e	7.25 d	9.42 d
T_3	6.10 c	8.08 c	10.00 c
T_4	1.52 g	1.75 f	2.08 f
T_5	6.82 b	8.83 b	10.75 b
T_6	7.58 a	9.67 a	11.58 a
T_7	5.50 d	7.58 cd	9.58 d
LSD $_{(0.05)}$	0.37	0.52	0.35
CV(%)	4.06	4.27	2.29

T_0= MS powder T_1= 16.50 gmL^{-1} of Ammonium nitrate
T_2= 1 gm of α- chemical/litre T_3= 5 gm of α- chemical/litre

T_4= 10 gm of α- chemical/litre
T_5= 1gm of α- chemical/litre in stock solution- I having twice dose of other components
T_6=5gm of α- chemical/litre in stock solution-I having twice dose of other components
T_7=10gm of α-chemical/litre in stock solution-I having twice dose of other components



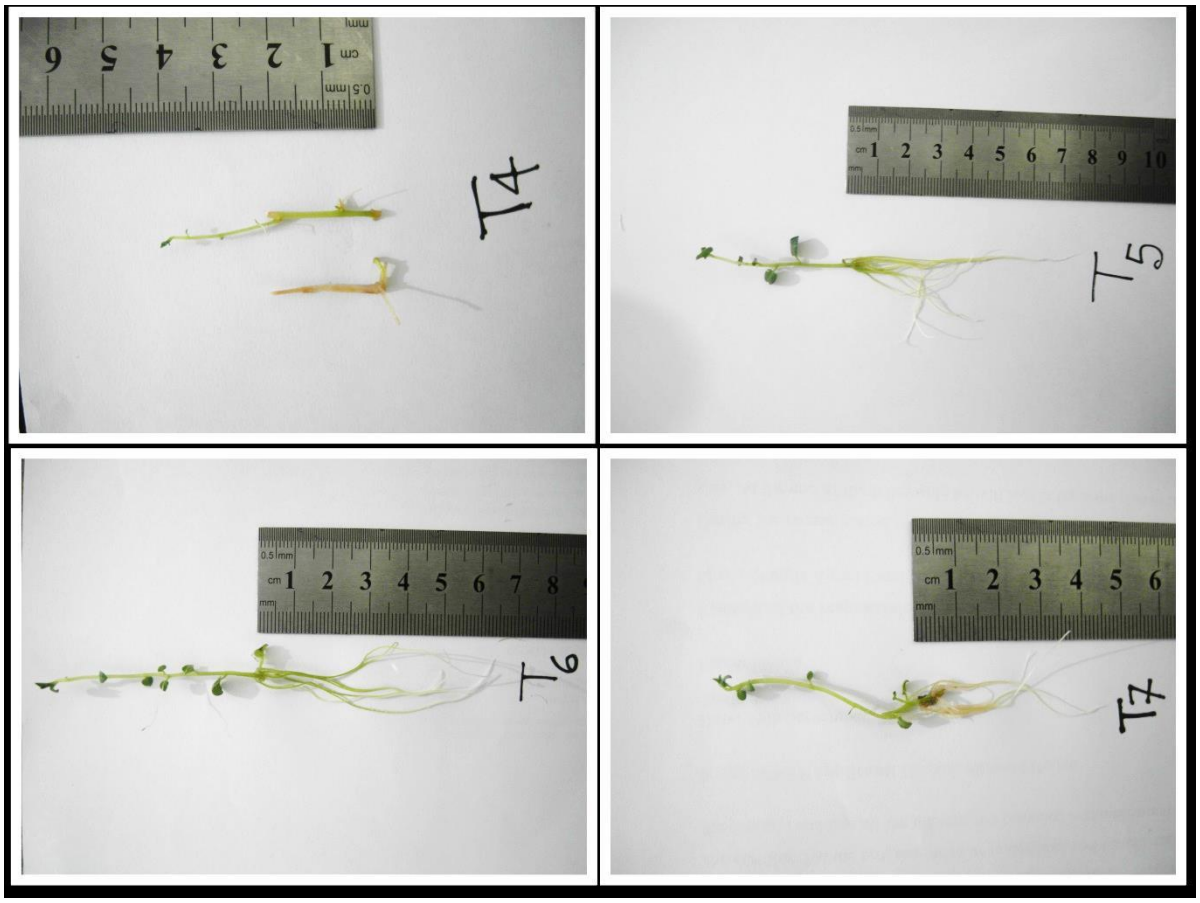


Plate11: Root length of potato plantlet in different treatment

4.8 Effect of treatments on the root number

The effect of treatment in root length is presented in Table 06. At 14 days after inoculation, the maximum number of root (9.33) was found in T_0 which was statistically different from all other treatments and followed by T_6, T_5 and T_3. On the other hand, the minimum number of root (4.33) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments.

At 21 and 28 days after inoculation, the maximum number of root (10.50) and (11.33) was found in T_0 which was statistically different from all other treatments and followed by T_6 also followed by T_5 and T_3 . The least number of root (2.07) and (2.13) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments and followed by T_1.

Table 6. Root number of potato plantlet on various treatment at different days after initiation

Treatments	Number of root at different days after initiation (DAI)		
	14 DAI	21 DAI	28 DAI
T_0	8.50 b	9.59 b	10.50 b
T_1	5.41 e	6.42 e	7.42 e
T_2	6.42 d	7.33 d	8.42 d
T_3	7.50 c	8.67c	9.50 c
T_4	4.33 f	5.42 f	6.75 f
T_5	8.42 b	9.33 b	10.42 b
T_6	9.33 a	10.50 a	11.33 a
T_7	7.33 c	8.50 c	9.25 c
LSD $_{(0.05)}$	0.37	0.31	0.54
CV(%)	3.03	2.15	3.37

T_0= MS powder T_1= 16.50 gmL^{-1} of Ammonium nitrate
T_2= 1 gm of α- chemical/litre T_3= 5 gm of α- chemical/litre
T_4= 10 gm of α- chemical/litre
T_5= 1gm of α- chemical/litre in stock solution- I having twice dose of other components
T_6=5gm of α- chemical/litre in stock solution-I having twice dose of other components
T_7=10gm of α-chemical/litre in stock solution-I having twice dose of other components

Plate 12: Number of root of potato plantlet in different treatment

4.9 Acclimatization and establishment of plantlets on soil

After a satisfactory number of shoot and root development at 7 weeks of culture the individual plantlets were moved from vial carefully without any root damage. The roots were washed with running tap water for removing surplus media. The plantlets were then transplanted into small plastic pot prepared with a standard ratio of cowdung and soil in a shade condition. The plantlets were sprayed occasionally with water for maintaining humidity. At first 15 plants of each treatment were hardened in glass house. Among them, 14, 10, 11, 13, 9, 13, 14 and 12 plants survived respectively in T_0, T_1, T_2, T_3, T_4, T_5, T_6 and T_7 treatment. They were hardened in netting condition and 12, 8, 9, 10, 5, 10, 12 and 9 plants survived respectively in T_0, T_1, T_2, T_3, T_4, T_5, T_6 and T_7 treatment. So, in glass house survival rate was (93.33%, 66.67%, 73.33%, 86.67%, 60.00%, 86.66%, 93.33% and 85.78%) at T_0, T_1, T_2, T_3, T_4, T_5, T_6 and T_7 treatment respectively and in netting condition, survival rate was (92.30%, 88.87%, 90.00%, 83.33%, 71.42%, 83.33%, 92.30% and 81.81%) at T_0, T_1, T_2, T_3, T_4, T_5, T_6 and T_7 treatment respectively. Finally in open atmospheric condition the plants were transplanted in the main field. Getu *et al.* (2012) found that after one month of acclimatization in glasshouse, 90% and 100% of plantlets originally derived from shoots of petiole and leaf of Beletech, respectively, survived. Similarly, 80% and 90% of plantlets originally derived from shoots of petiole and leaf of Awassa-83, respectively, survived. So, analyzing the survival rate it can be concluded that acclimatization potentiality of potato was satisfactory.

Table 7. Survival rate of *in vitro* regenerated plantlets of potato

Treatment	Hardening in Glass house			Hardening in Netting Condition		
	No. of plantlets transfered in plastic pot	No. of survived plantlets	Survival rate (%)	No. of plantlets transferred	No of seedlings established	Survival rate (%)
T_0	15	14	93.33	13	12	92.30
T_1	15	10	66.67	9	8	88.87
T_2	15	11	73.33	10	9	90.00
T_3	15	13	86.67	12	10	83.33
T_4	15	9	60.00	7	5	71.42
T_5	15	13	86.67	12	10	83.33
T_6	15	14	93.33	13	12	92.30
T_7	15	12	85.78	11	9	81.81

T_0= MS powder T_1= 16.50 gmL^{-1} of Ammonium nitrate

T_2= 1 gm of α- chemical/litre T_3= 5 gm of α- chemical/litre

T_4= 10 gm of α- chemical/litre

T_5= 1gm of α- chemical/litre in stock solution- I having twice dose of other components

T_6=5gm of α- chemical/litre in stock solution-I having twice dose of other components

T_7=10gm of α-chemical/litre in stock solution-I having twice dose of other components

CHAPTER V

5 SUMMARY AND CONCLUSION

5.1 SUMMARY

The experiment was conducted at the Biotechnology Laboratory and Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh during the period of January, 2018 to January, 2019 to identify a new chemical for the comparative performance of Ammonium Nitrate and other Nitrogenous salts for *In vitro* regeneration of potato (*Solanum tuberosum* L.). The key findings were given below.

The treatment 5gm of α- chemical/litre in stock solution- I twice dose (T_6) which was statistically similar with T_0 (MS powder) showed better result in respect of different parameters under investigation. On the contrary, 10 gm of α-chemical/litre in stock solution- A showed leat effect on plantlets. It can be concluded that, different modifications of stock solution-A by α-chemical has significant efft on plantlet regeneration and its development. The amount of α-chemical in stock solution-A has tremendous role on *in vitro* regeneration of potato.

At 14 days after inoculation, the maximum shoot length (6.80 cm) was found in 5gm of α- chemical/litre in stock solution- I, The treatment (T_6) which was statistically similar with (T_0) and different from all other treatments and followed by T_5 and T_7. The minimum shoot length (4.10 cm) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments and followed by T_1.

At 21 and 28 days after inoculation, the maximum shoot length (9.50 cm) and (11.40 cm) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6. The minimum shoot length (5.39 cm) and (7.29 cm) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments and followed by T_1.

At 14 days after inoculation, the maximum number of shoot (1.83) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and different from all other treatments. The minimum number of shoot (1.13) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically similar with T_1 and different from all other treatments.

At 21 and 28 days after inoculation, the maximum number of shoot (4.53) and (4.63) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and different from all other treatments. The least number of shoot (2.07) and (2.13) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments and followed by T_1.

At 14 days after inoculation, the maximum number of leaf (10.17) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and different from all other treatments. On the other hand, the minimum number of leaf (5.00) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically similar with T_1 and different from all other treatments.

At 21 and 28 days after inoculation, the maximum number of leaf (11.17) and (12.16) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and different from all other treatments. In contrast, the least number of leaf (5.58) and (6.08) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments and followed by T_1.

At 14 days after inoculation, the maximum number of node (7.50) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and similar with T_0 and different from all other treatments. On the other hand, the minimum number of node (5.33) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically similar with T_1 and different from all other treatments.

At 21 and 28 days after inoculation, the maximum number of node (8.50) and (9.25) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and different from all other treatments. In contrast, the least number of

node (6.33) and (7.08) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically similar with T_1 and different from all other treatments and followed by T_2.

At 14 days after inoculation, the maximum number of root (9.33) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and different from all other treatments. On the other hand, the minimum number of root (4.33) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments.

At 21 and 28 days after inoculation, the maximum number of root (10.50) and (11.33) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and different from all other treatments. The least number of root (2.07) and (2.13) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically different from all other treatments and followed by T_1.

At 14 days after inoculation, the maximum root length (7.58 cm) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and different from all other treatments. The minimum root length (1.52 cm) was found in 10 gmL^{-1} of α-chemical (T_4) which was statistically different from all other treatments and followed by T_1.

At 21 and 28 days after inoculation, the maximum root length (9.67 cm) and (11.58 cm) was found in MS powder (T_0) treatment which was statistically similar with the treatment T_6 and different from all other treatments. The minimum root length (1.75 cm) and (2.08 cm) was found in 10 gmL^{-1} of α- chemical (T_4) which was statistically different from all other treatments and followed by T_1.

For shoot and root initiation T_6 and T_0 treatment was best. In contrast, T_4 treatment was comparatively lowest.

5.2 CONCLUSION

In vitro regeneration capacity, shoot and root morphology of potato plantlet were investigated in this experiment. The key findings revealed that, MS powder showed the best performance on *in vitro* regeneration of potato. Although, in some cases there was no significant difference among the MS powder and the treatment T_6. α-chemical can be used as a substitute of NH_4NO_3 for the preparation of tissue culture media. Formulation of α- chemical concentration in stock solution-I is our new development. Hence, it is patentable discovery of our experiment. The regenerated plantlets reach optimum growth condition at 21 days after inoculation (DAI). Our present finding proved that, α- chemical can be used as a substitute of NH_4NO_3 for the preparation of tissue culture media.

5.2.1 Merits or advantages of newly identified α- chemical for tissue culture

1. It is very cheap as compare to MS powder.
2. It is aviable in all over the country.
3. It is familiar to all levels of user.
4. It is required a very small amout compared to ammonium nitrate for stock solution preparation.
5. The regeneration performance using this chemical is as similar to as with the ready made MS powder.
6. It has no harmful effect on environment.
7. It is user friendly to all.

RECOMMENDATIONS

The present investigation revealed that 5gm of α- chemical/litre in stock solution- I twice dose (T_6) chemical showed near about similar performance in compare with MS powder under *in vitro* condition of potato. But further research may be carried out on the following mentioned points:

1. Regeneration potentiality of new chemical should be validate with some other variety of potato. More investigation is needed to re-check the present finding of new chemical.

2. The validation of present findings can be done in some other crop for *in vitro* regeneration

3. More specific parameters for the effect of variety and treatments of subculture can be studied.

4. Furthur experiments must be tried with different growth regulators for *in vitro* regeneration of potato.

CHAPTER VII

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APPENDICES

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

Components	Concentrations (mg/L)	Concentrations
Micro Elements	**mg/L**	**µM**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H2O$	8.60	29.91
Macro Elements	**mg/L**	**mM**
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
NH_4NO_3	1650.00	20.61
Vitamins	**mg/L**	**µM**
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	0.10	0.30

Total concentration of Micro and Macro elements including vitamins: 4405.19 mg/L

Manufacturing Company: Duchefa Biochem

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
		Days to shoot initiation
Replication	2	2.067
Treatment	7	6.643*
Error	16	0.333

*significant at 5% level of probability

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

Sources of Variation	Degrees of freedom	Mean Square value
		Days to root initiation
Replication	2	2.067
Treatment	7	4.667*
Error	16	0.333

*significant at 5% level of probability

Appendix IV: 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		
		14 DAI	21 DAI	28 DAI
Replication	2	1.067	0.35	0.173
Treatment	7	2.549*	11.806*	11.061*
Error	16	0.101	0.012	0.397

*significant at 5% level of probability

Appendix V: 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	7	0.152*	2.157*	2.186*
Error	16	0.014	0.003	0.003

*-Significant at 5% level

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

Sources of Variation	Degrees of freedom	Mean Square value		
		14 DAI	21 DAI	28 DAI
Replication	2	0.067	0.266	0.1
Treatment	7	14.312*	20.778*	29.171*
Error	16	0.045	0.091	0.041

*significant at 5% level of probability

Appendix VII: 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		
		14 DAI	21 DAI	28 DAI
Replication	2	0.6	0.067	0.2
Treatment	7	2.083*	2.113*	2.113*
Error	16	0.032	0.083	0.083

*significant at 5% level of probability

Appendix VIII: 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		
		14 DAI	21 DAI	28 DAI
Replication	2	0.467	0.2	0.2
Treatment	7	9.514*	10.241*	12.089*
Error	16	0.318	0.297	0.219

*significant at 5% level of probability

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

Sources of	Degrees of	Mean Square value		
Variation	freedom	14 DAI	21 DAI	28 DAI
Replication	2	0.2	1.067	0.267
Treatment	7	8.497*	8.773*	7.547*
Error	16	0.047	0.031	0.096

*significant at 5% level of probability