Regeneration of potato (*Solanum tuberosum* L.) by utilizing a new composition of stock solution-1 for plant tissue culture medium

BY

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CERTIFICATE

This is to certify that the thesis entitled '**Regeneration of potato (*Solanum tuberosum* L.) by utilizing a new composition of stock solution-1 for plant tissue culture medium**' 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 **Md. Ershad Ali**, Registration No. 19-10022 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:
Dhaka, Bangladesh

Dr. Md. Ekramul Hoque
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Department of Biotechnology
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Dedicated To

- ***My Beloved Parents***
- ***Mr. Md. Shahriar Alam, MP***

  Honorable State Minister, Ministry of Foreign Affairs, The People's Republic of Bangladesh
- ***Professor Dr. Md. Ekramul Hoque***

  Dept. of Biotechnology

  Sher-e-Bangla Agricultural University, Dhaka

ABBREVIATIONS AND ACRONYMS

Acad.	:	Academia
Agril.	:	Agricultural
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 variation
DAI	:	Days After Inoculation
et al.	:	And others
BAP	:	6-Benzyl Amino Purine
KIN	:	Kinetin
IAA	:	Indoleacetic acid
NAA	:	*a*-Napthalen acetic acid
2,4-D	:	2,4- Dichloro phenoxy acetic acid
Int.	:	International
J.	:	Journal
mg/L	:	Milligram per liter
Microbiol.	:	Microbiology
MS	:	Murashige and Skoog
Org.	:	Organ
Physiol.	:	Physiology
Rep.	:	Report
Res.	:	Research
Rev.	:	Review
Sci.	:	Science
Tiss.	:	Tissue
^{0}C	:	Degree Celsius
etc.	:	et cetera

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The Author

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Regeneration of potato (*Solanum tuberosum* L.) by utilizing a new composition of stock solution-1 for plant tissue culture medium

ABSTRACT

An experiment was conducted at North Bengal Agro Farms Ltd., Thakurgaon, Bangladesh, during the period of January to September 2020 for plantlet regeneration of potato using different new composition of stock solution-1. Three chemicals viz. *chemi-i, chemi-ii* and *chemi-iii* were used in stock solution-1 as an alternate of ammonium nitrate. Eight different treatments were designed with those chemicals including Murashige and Skoog (1962) culture media as a check treatment (T_3). Two potato varieties viz. Diamant and Asterix were used for *in vitro* regeneration. The Diamant variety showed the best regeneration potentiality in the T_7, which consists of the *chemi-iii*. The check treatment (T_3) showed 2nd highest performance for all the parameters under studied. The Asterix variety gave the best performance in the check treatment (T_3) and the 2nd highest result was found in the T_4. Different abnormalities were noticed in respect of media and plantlet regeneration. Brown, deep brown, off white and contaminated colour was found in the treatments T_1, T_2, T_5, T_6 and T_8. Plantlet regeneration from those treatments showed aberrancy and it has stunted growth, dwarf size, small leaf formation, black layer at the root tips and finally plantlet died within 4th week of regeneration. The T_7 treatment showed yellow colour and the plantlet regenerated from this treatment were thin, tall with less number of roots. The plantlet regenerated from check treatment (T_3) showed good growth and development throughout the experimental period. Finally, it can be concluded that, the *chemi-iii*, can be used as a component of stock solution-1 which will be a good alternate of ammonium nitrate.

CHAPTER I

INTRODUCTION

The potato is a starchy tuber of the plant *Solanum tuberosum* L. It is a modified stem vegetable and very popular among the Bangladeshi people. The potato was originally believed to have been domesticated by Native Americans independently in multiple locations. Genetic testing of the wide variety of cultivars and wild species traced a single origin for potatoes, in the area of present-day southern Peru and extreme northwestern Bolivia. Potatoes were domesticated approximately 7,000–10,000 years ago there, from a species in the *Solanum brevicaule* (Domingo *et. al*. 2007.). By the end of the 17th century the plant was a major crop in Ireland and by the end of the 18th century it was a major crop in continental Europe, particularly Germany and in the west of England. It continued to spread, in both Western and Eastern hemispheres during the first four decades of the 19th century and the Irish economy itself became dependent upon the potato. It is not known exactly when potato was introduced in this subcontinent. It is assumed that, at the beginning of the 17th century the Portuguese navigators first brought potato to India. Initially, potato was cultivated in areas around Calcutta, from there its cultivation spread to Bangladesh.

The stems of the potato plant extend underground into structures called stolons. The ends of the stolons may enlarge greatly to form a few to more than 20 tubers of variable shape and size, usually ranging in weight up to 300 grams but occasionally to more than 1.5 kg. The skin varies in colour from brownish white to deep purple. The tubers bear spirally arranged buds (eyes) in the axils of aborted leaves, of which scars remain. The buds sprout

to form clones of the parent plant, allowing growers to vegetatively propagate desirable characteristics of the parental type.

Potato is widely cultivated in all the districts of Bangladesh during winter. Well-fertilized, sunny land with sufficient moisture in soil is appropriate for potato plantation. The first fortnight of November is the right time of potato sowing. In certain northwestern areas, farmers even plant potato in October to harvest the crop early. On the basis of the soil quality and potato variety farmers determine the spacing in between the seed tubers and the adjacent rows. Row spacing is usually from 45 to 60 cm. The world potato sector is increasing day by day. Bangladesh is a major potato growing country of the world and it is ranked 7th in the world and 3rd in the Asian countries (FAOSTAT, 2020). In Bangladesh, 4.75 million hectares of land were cultivated and total production has been estimated at 9,470,000 metric tons during 2020 (BBS, 2020).

Nutritionally, potatoes are best known for their carbohydrate content (approximately 26 grams) in a medium potato. Starch is the predominant form of carbohydrate found in potatoes. This portion of starch, referred to as "resistant starch," is considered to have similar physiological effects and health benefits of fiber (e.g., provide bulk, offer protection against colon cancer, improve glucose tolerance and insulin sensitivity, lower plasma cholesterol and triglyceride concentrations and possibly even reduce fat storage) (Cummings *et al*. 1996; Hylla *et al* 1998; Raban *et al.* 1994). The amount of resistant starch found in potatoes is highly dependent upon preparation methods. Cooking and then cooling potatoes significantly increases resistant starch. For example, cooked potato starch contains about 7% resistant starch, which increases to about 13% upon cooling (Englyst *et al*. 1992). Potatoes contain a number of important vitamins and minerals. A 100 gm potato provides 27 mg vitamin C (45% of the Daily Value (DV)),

620 mg of potassium (18% of DV), 0.2 mg vitamin B6 (10% of DV) and trace amounts of thiamin, riboflavin, folate, niacin, magnesium, phosphorous, iron, and zinc.

In Bangladesh, potato is primarily used as a vegetable, although in many countries of the world it constitutes the staple food and contributes more than 90% of the carbohydrate food source. Millions of tons of potatoes are processed annually in Europe into starch, alcohol, potato meal, flour, dextrose and other products. Some are processed into potato chips, dehydrated mashed potatoes, French fries and canned potatoes. Large quantities of potatoes in the Netherlands, Ireland, Germany and other countries of Europe are grown specifically for manufacture of alcohol, starch and for livestock feeding. Europeans consume much larger quantities of potato than the North Americans. Asian countries consume more rice than potato for carbohydrate foods. The principal use of potatoes is to make potato curry along with fish, meat, eggs etc. Potato-based food items are the boiled potato, fried potato, mashed potato, baked potato, potato chop, potato vegetable mix, potato singara, potato chips, french fry etc. In Bangladesh, bakeries and fast food shops have started preparing a wide variety of potato-based food delicacies.

All conventional potato seed production systems are characterized by low multiplication rate and progressive accumulation of degenerative viral diseases during clonal propagations. About 30 viruses and virus like agents infect potato. These being systemic pathogens and are perpetuated through seed tubers and pose a major threat to potato seed production. Potato viruses X, S, Y and PLRV (Potato Leaf Roll Virus) are ubiquitous in the Asia-Pacific region. Other viruses reported from major potato growing countries of the region are- PVA, PVM, PMTV, TRV, PAMV, TNV and PSTV. Consequently, non-availability of quality planting material in adequate

quantities and at affordable prices is the major bottleneck in potato cultivation in many countries. The problem is further aggravated by high seed rate (2 to 3 t/ha) due to which the cost of seed potatoes alone accounts for about 40% to 50% of the total production costs in our country. Shortage of good quality seed has been recognized as the single most important factor limiting potato production in the developing countries. Fortunately, potato has been an early beneficiary of advances in conventional and modern biotechnologies resulting in their use for solving practical problems relating to potato cultivation and improvement. Meristem culture was possibly the first biotechnological approach used to eliminate viruses from systemically infected potato clones. Over the years, this technique has been successfully combined with micropropagation to produce disease-free potato seed. Production of quality planting material is essential not only for improving domestic potato productivity but also to ensure minimum commercial quality as required under international standard.

Potato is a semi-perishable crop susceptible to many diseases, insects and pests. Shortage of good quality seed has been recognized as the single most important factor limiting potato productivity in the developing countries. The availability of tissue culture technology for rapid multiplication of disease-free planting material has facilitated potato seed production to a great extentd (Dodds, 1988). Meristem culture is being successfully employed to obtain virus-free potato clones (Mori *et al*. 1969). Rapid multiplication of these disease-free clones using micropropagation coupled with conventional multiplication methods has now become an integral part of seed production in many countries (Donnelly *et al*. 2003). Seed production, seed certification and quality standards of potato multiplication need to integrated with tissue culturally grown potato plantlet.

The regeneration of plant tissue, cell, organ or whole plant in an artificial nutrient medium under aseptic condition is known as plant tissue culture. The resultant clones are true-to-type of the parental genotype. The new plantlets can be grown in a short period of time. The new plantlets and plants are more likely to be free of viruses and diseases. The process is not dependant on the seasons and can be done throughout the year. Plant tissue culture technology is being widely used for large scale plant multiplication. In addition, plant tissue culture is considered to be the most efficient technology for crop improvement by the production of somaclonal and gametoclonal variants. The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities.

Diseases free and quality plantlets production of potato through tissue culture technology has been well adopted and commercially practices in our country last three decades. At present, more than 50 different Govt. and private tissue culture labs has been established in different corner of Bangladesh. Commercially they were producing millions of potato plantlet for potato seed production. Tissue culture technology is the initial basic step for quality potato seed production.

Murashige & Skoog (1962) media composition has been used for long time for regeneration of plantlet. Preparation of culture media needs different macro and micro plant nutrients. Ammonium Nitrate (NH_4NO_3) is an important chemical used as macro nutrient in MS media preparation. 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 as an oxidizing agent in explosive. It is also used for the production of bomb and in many other destructive activities. Although ammonium nitrate (NH_4NO_3) is used as a fertilizer and is an important

ingredient of tissue culture media but due to explosive nature, it is now totally band in our country. The supplier or importer are not selling a single gram of ammonium nitrate (NH_4NO_3) in our country. Hence, the tissue culture works all over the country gets seriously hampered. Different research Institutes and private tissue culture companies adopted alternate approach for their ongoing tissue culture program. Ready made MS powder is an alternative of this problem but those ready made MS media are very expensive. In contrast, manually prepared stock solution is very cheap and user friendly. Generally, the students, teachers, researchers, lab technicians are familiar with this method. It has been practiced for last 30-40 years in our country. This long time adopted technology is tremendously hampered due to non availability of ammonium nitrate (NH_4NO_3).

Ammonium nitrate (NH_4NO_3) of stock solution-1 supply NH_4^+ ion to the explants for its growth and development. The aim of the present study is to develop a new chemical composition of stock solution-01 where ammonium nitrate (NH_4NO_3) is lacking. Hence, three different inorganic nitrogenous salt with the ability to dissociate as NH_4^+ ion in the presence of water were used. For the privacy and the secrecy of the research the salts were named as *Chemi-i*, *Chemi-ii* and *Chemi-iii* and formatted as italic form. The stock solution will be prepared with those chemicals. Different combination and concentration of those chemical will be the treatment of the present research to find out any alternate source of NH_4NO_3 for the preparation of tissue

Considering the above prospects and problems, the present experiment has been undertaken with the following specific objectives:

- To find out any alternate chemical of NH_4NO_3 for tissue culture media preparation.
- To identify the suitable concentration of new chemical for culture media.
- To study the effect of new chemical on *in vitro* regeneration of potato.

- To study the *in vitro* performance of two potato varieties using new chemical.

CHAPTER II

REVIEW OF LITERATURE

The potato (*Solanum tuberosum* L.) is an important vegetable and cash crop in our country. The conventional planting material for potato (potato seed) is the vegetative tuber. Tissue culture is considered as a very promising technique for both large-scale clonal propagation of plants for quality seed potato production. This technique has opened a new frontier in agricultural science by addressing food security through biotechnological methods for commercial application. More than 60 years ago potato tissue culture research was started. Unlimited reviews were available in this regard. But development a new culture media composition for rapid *in vitro* regeneration in potato is limited. However, the relevant literatures are given below:

2.1 Concept of potato tissue culture

Tissue culture or cell culture is the process where cells are grown and maintained in a controlled environment such as a laboratory, outside of their natural and original source. *In vitro* produced disease free potato clones combined with conventional multiplication methods has become an integral part of seed production in many countries (Naik and Sarkar, 2000). The key finding of the development and achievements of various lines of potato biotechnology research through the common use of *in vitro* culture techniques were summarized. Starting with the early research on the induction and differentiation of callus tissues, review sequentially and chronologically presents the advance of various *in vitro* culture techniques and their practical applications in clonal propagation, germplasm storage, production of healthy virus-free plants and breeding were reported by Vinterhalter *et al*., (2011).

2.2 Disinfection process

Sterilization is an important step for a successful plant regeneration process. It is done before inoculation process. All operations should be carried out in laminar airflow sterile cabinet. Yasmin *et al*. (2003) 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.

Different sterilization agents like $HgCl_2$ (0.1%), NaOCl (5.25% v/v approx.) and 70% ethanol etc. can be used. 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 (2009) 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.

2.3 Review on potato micropropagation

Micropropagation technique permits a huge amount of asexual multiplication of pathogen free tested potato cultivars. Considerable research has been done on the nutritional, hormonal and physical aspect of the culture media and their effects on explants growth. Murashige and Skoog (1962) medium is most widely used for potato micropropagation. *In vitro* derived microplants can be used as explants source for the production of microtubers in *in vitro* condition. 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.*, 1995). 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).

2.4 Explants for potato tissue culture

Explant is the key factor for successful micropropagation program. The potentiality of *in vitro* regeneration depends on a source of explants and its property. Treatment of explants while preparing them for *in vitro* culture and composition of culture media, also another important factor 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. They used potato single node as an explant for his experiment. Potato tubers were also used as an explants source (Anjum and Ali, 2004). 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. Nodal cuttings were also used for auxiliary shoot development and suggested to be the best explants source by several researchers (Hussey and Stacey, 1981) on either liquid or agar solidified medium.). The efficiency of *in vitro* shoot regeneration and microtuber production of potato (*Solanum tuberosum* L.) from nodal explants was studied using agar and other new and cheaper gelling agents- tapioca and sago in Murashige & Skoog (MS) salt medium (Garcia and Martinez, 1995). For shoot regeneration, agar was maintained at 8 mgL-1, tapioca and sago were varied between 9-18 and 10-14 % (w/v), respectively supplemented with 3% sucrose, 0.03 mgL-1NAA, 0.25 mgL-1 GA3 and 2.5 mgL-1 Ca-panthothnate. The microtuberisation study was done using agar, tapioca and sago at 0.8, 14 and 10% concentration (w/v), respectively, in the presence of benzylaminopurine and

paclobutrazol. Type of gelling agent significantly affected *in vitro* plant regeneration. After 32 days, shoot height and number of nodes respectively were 8.86 cm and 10.5 in agar, and 8.9-12.1 in 11-15% tapioca, respectively.

2.5 *In vitro* callus induction of potato

Yasmin *et al.,* 2003 conducted an experiment to observe the effect of NAA and BAP on callus formation and regeneration from leaf and inter nodal segment as explants. Five levels of each of NAA (0, 1.25, 2.5, 5 and 10 mgL-1 and BAP (0, 0.5, 1, 2 mgL-1) were applied to MS media for callus induction and plantlet regeneration. Twenty explants were cultured in each combination. Leaf showed better performance in callus induction and plantlet regeneration. Highest percentage of callus (95%) and minimum time (8.13days) were recorded with 2.5 mgL-1 NAA + 2 mgL-1 BAP. The percentage of regeneration (80%) was highest with 2.5 mgL-1 NAA + 2 mgL-1 BAP treatment among the combinations.

2.6 Subculture for callus induction and plantlet regeneration

Shahab-ud-din *et al*., 2011 was conducted an experiment to investigate the effects of different concentrations of plant growth regulators and their combinations on callus induction of potato (*Solanum tuberosum* L.). The explants of potato tuber were cultured on Modified MS medium supplemented with different concentrations of 2, 4-D, NAA, BA for callus induction. Among the treatments 2, 4-D at different concentrations produced different degree of callus but comparative massive amount of callus was formed on MS medium supplemented with 2, 4-D at 3.0 mg L-1. Khalafalla *et al*., 2010 reported the procedure of plant regeneration from callus culture of potato (*Solanum tuberosum* L.). Calli were induced from 1.0 -2.00 cm tuber segment of potato cultivar Almera on MS medium supplemented with different levels (1.0-5 mgL-1) of 2,4-D. The hundred percent explants

produced nodular calli within 7- 12 days on MS medium when supplemented with 2.0-5.0 mgL-1 of 2, 4-D. Callus derived shoots were rooted most effectively in full-strength MS medium containing 1.0 mgL-1 IBA. The success of plant tissue culture for *in vitro* culture of potato was encouraged by acclimatization of the plantlets in the greenhouse conditions. Regenerated plants were morphologically uniform with normal leaf shape and growth pattern (Avila *et al*., 1996).

In vitro microtuber formation potentiality of potato was investigated to establish a rapid disease free seed production system in potato. MS medium supplemented with 4 mgL-1 of kinetin showed best performance in respect of multiple shoot regeneration and microtuber formation (Hoque, 2010). Simple MS medium was notable to produce any micro tuber under *in vitro* condition. Dark condition better responded to tuberization than light condition. Three potato cultivars *viz.*, Cardinal, Altamash and Diamant were selected for *in vitro* responses. High regeneration and morphogenic potential of different explants i.e., shoot tips, leaf discs, nodes and internodes have been tested for direct regeneration. The shoot regeneration *in vitro* of potato cultivars Chieftain, Desiree, Kennebec, Lenape, Niska, Russet Burbank and Shepody from petioles with intact leaflets was assessed by using six treatment combinations a basal medium with or without silver thiosulphate or thidiazuron at two concentrations (2.0 or 0.5 mgL-1) of IAA (Yee *et al*., 2001

2.7 Effect of Growth Regulators

Although there are several reports for the use of hormone free MS medium during potato proliferation (Yasmin *et al.,* 2003). However, the growth of explants is slow in such hormones free, cost effective media. Otherwise, the growth rate of explant can be improved by supplementing medium with growth regulators (Hoque, 2010). An experiment was conducted by Molla *et*

al., 2011 to find out a suitable growth regulators and its optimum concentration for direct regeneration. Seven different concentrations of BAP, six different concentrations of Thidiazuron (TDZ) and Eight different concentrations of Zeatin riboside (ZR) were tested separately for in vitro direct regeneration of potato along with GA3 (0.2 mgL-1)and IAA (0.01 mgL-1). Among the different concentrations of BAP, TDZ and ZR, MS medium supplemented with 3 mgL-1 of BAP, 0.3 mgL-1 TDZ and 5 mgL-1 ZR showed very good shoot initiation.

Khadiga *et al.,* 2009 reported that initially potato *in vitro* culture was started from nodal cuttings and maintained on a hormone free media at 23±2 ºC for 2-weeks. It is clearly evident from the data that, direct shoot regeneration was remarkably influenced by type and concentrations of the auxins, cytokinins and GA3 used and no organogenesis was recorded in the basal MS media. Maximum number of shoots per explants after 30 days were recorded in cultivar Diamant followed by Cardinal i.e. 18 and 16, respectively in comparison to control (T0) treatment.

2.8 Effect of Growth Regulators on Shoot induction

It is a well known fact that the regeneration potential of micropropagated plants is genotype dependent and the chemical composition of the culture medium is perhaps the most studied aspect of potato tissue culture. It plays an important role in success of potato regeneration.

An experiment was conducted by Sarkar and Mustafa, 2002 where maximum shoot induction in two indigenous potato varieties was observed on MS semi-solid medium supplemented with 1.0 mgL-1 BAP and 0.1 mgL-1 GA3. Between two varieties, namely Lal Pakri and Jam Alu, the farmer showed the best performance in terms of number of shoots/explants, nodes/shoots and shoot length. It is very obvious from the results shown that the treatment (T_2) i.e. MS+GA3 (1.0) mgL-1 +IAA (0.01 mgL-1)+Zeatin

(2.0 mgL-1) induced the highest number of shoots and roots in studied cultivars. Pereira and Fortes, 2003 reported MS liquid medium supplemented with 0.25 mgL-1 gibberellic acid and 5.0 mgL-1 pantothenic acid as the most suitable regime for potato micro propagation. Águila *et al*., 2001 cultured potato explants on MS media supplemented with 1 mg gibberellic acid per litre in solid MS media.

Asma *et al*., 2001 studied an experiment with the effect of different concentrations (1.0, 2.0, 3.0, 4.0 mgL-1 of GA3 and BAP) on the *in vitro* multiplication of nodal fragments and stem segments of potato cv. Desiree. The maximum shoot length was obtained when 4.0 mgL-1 of GA3 was applied. The maximum number of shoots (4) was obtained when 2.0 mgL-1 BAP was applied.

A study was conducted by Pandey *et al.,* 2009 *in vitro* shoot regeneration by using nodal explants of potato. The regeneration medium was supplemented with 0.03 mgL-1 of NAA and 0.25 mgL-1 GA3. Highest number of shoot (3.11) was obtained with the high concentration of GA3. Internodes and leaf explants of potato in combinations with different plant growth regulators specially different concentrations of Zeatin riboside (ZR) were tested when shoot induction was most successful on callus derived from internodes tissue cultured on induction medium supplemented with 2.5 mgL-1 ZR, 0.2 mgL-1 NAA, 0.02 mgL-1 of GA3 for two weeks and then transferred to shooting medium with 2.5 mgL-1. From the above reviews, it is appeared that different media, culture condition, different concentrations and combinations of growth regulators, modifications of MS media composition have great remarkable influence on *in vitro* regeneration of potato.

2.9 Effect of Growth Regulators on Number of Roots per Explant

(Yousef *et a*l., 2011) and (Pereira *et al.,* 2003) reported that the data presented in the number of roots per explant were obtained on all tested

media compositions except in T0 which was lack of plant growth hormones. The highest number of roots i.e., 28 per explant was recorded on media composition in which MS media was supplemented with GA3 (1.0 mgL-1)+IAA (0.01 mgL-1)+ Zeatin (2.0 mgL-1). Variety K. CH3 was followed by K. Jyoti (26 average number of roots). A decline was recorded in average number of roots with either increase or decrease from these hormonal concentrations in both the varieties. The number of roots was the lowest in T0 as compared to other treatments. Genotypes were found detrimental for in vitro growth responses. The genotypic differences in the ability to regenerate roots were also reported by various workers. (Sidikou *et al*., 2003) in their study founded potato genotypes micropropagated *in vitro* in medium with sucrose at 2-12% and BA at 0-5 mgL-1. All cultivars showed 100% regeneration. Regeneration frequency increased with increasing concentration of BA and sucrose.

Sarkar and Mustafa, 2002 observed that 0.5 strength of MS containing 0.1 mgL-1 NAA appeared to be the best for root induction from excised shoots in two indigenous variety namely Lal Pakri and jam Alu. Shibili *et al.,* 2001 was conducted an experiment that cuttings of 3 cm from glass house grown plantlets were successfully rooted by treating with 1.0 mgL-1 IBA + 0.5 mgL-1 IAA for 5 seconds. (Salaiz *et al.,* 2005) investigated *in vitro* regeneration ability of the explants of potato cv. Desiree using different combinations and concentrations of different growth regulators. After 9 weeks callus were transferred in MS media with 1.0 mgL-1 BAP with addition of 2, 4-D showing root formation. An experiment was conducted by (Sharma and Sarjeet, 2010) to show the effect of 2.0 mgL-1 IBA, 1.0 mgL-1 IBA + 1.0 mgL-1 NAA or 1.0 mgL-1 IBA + 160 mgL-1 floroginical on rooting of nodal cuttings. It was recorded that supplemented MS media with 2.0 mgL-1 IBA produce higher numbers of roots with 1.0 mgL-1 IBA + 1.0 mgL-1 NAA.

2.10 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. Thirty stem sections with one axillary bud was used as explants and were cultured on the same MS semi-solid medium (1 stem section per test 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 higher were than plantlets obtained on liquid medium for both cultivars. Plantlets growing on liquid medium had less leaves number than plants growing on solid medium.

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 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-1 Ca- pentothenate, 0.25 mgl-1 Gibberellic acid (GA3), 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).

2.11 Effect of medium modification by Macro salts, 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 NH_4NO_3 were same as the MS media. The effect of the treatments were analyzed 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- NT0 (KNO_3 = 0mg/l and NH_4NO_3 = 0mg/l), NT1 (KNO_3 = 475mg/l and NH_4NO_3 = 413mg/l), NT2 (KNO_3 = 3800 mg/l and NH_4NO_3= 3300 mg/l) and NT3 (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 NT1 (low nitrate) media followed by same cultivar at NT2 (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 NT2 media. The shoot multiplication rate was highest (6-8 fold) in Shepody at NT2 media followed by same cultivar (5-7 fold) at NT1 media. 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 (NT1) produced better shoot length in Shepody and Diamant whereas higher nitrate media (NT2) 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 NT1, NT2 and NT3 media were comparable with respect to growth traits and demonstrated that the micropropogation efficiency did not much improve when the nitrate increased (from NT1 to NT3) 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.

2.12 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 and root length showed lowest performance in this treatment. It indicates that, NH_4NO_3 is essential for *in vitro* regeneration of potato.

CHAPTER III

MATERIALS AND METHODS

The research work was carried out at North Bengal Agro Farms Ltd., Thakurgaon, Bangladesh. The experiment was conducted for *in vitro* regeneration of potato in new media composition which has different composition of stock solution-01. Duration of the experiment was from January, 2020 to September, 2020. The materials and methods that were used for conducting the experiment have been presented in this chapter. It included a short description of the experiment, materials used for the experiment, design of the experiment, data collection procedure and procedure of data analysis.

3.1 Time and location of the experiment

The present research was carried out at Tissue Culture Laboratory of North Bengal Agro Farms Ltd., Thakurgaon, Bangladesh. It is a tissue culture based potato seed production company. Every year they produce huge number of potato plantlet for commercial minituber production. The duration of the experiment was from January, 2020 to September, 2020.

3.2 Experimental materials

Potato is a model plant for tissue culture research. The regenerated shoot tip and nodal segments were used as explants for this experiment. Two potato varieties *viz.* Diamant and Asterix were used as experimental materials.

3.3 Media Composition

Growth and development of plant cell under *in vitro* condition are largely governed by the composition of the culture media. A crucial factor in establishing and maintaining culture is the formulation of the media. Hence,

the media composition, formulation and concentration for regeneration of any plantlet are major factor. The principal components of most of the plant tissue culture media are inorganic and organic nutrients, growth regulators and vitamins etc. The Murashige and Skoog (MS) (1962) is the most common tissue culture medium for rapid micropropagation of plant species. The MS medium has 17 different plant nutrients which has four different categories of stock solutions. Among them stock solution-1 is the major composition and it has the following nutrient combination and concentration.

SL No.	Name of chemical	Supply of nutrient	Concentration (gm/litre)	Category
01	KNO_3	Nitrogen (N) and Potassium (P)	19.00	Stock solution-01
02.	NH_4NO_3	Nitrogen (N)	16.50	
03.	$MgSO_4.7H_2O$	Megnesium (Mg) and Sulpher (S)	3.70	
04	$CaCl_2.2H_2O$	Calcium (Ca)	4.40	
05	KH_2PO_4	Phosphorus (P)	1.70	

Ammonium Nitrate (NH_4NO_3) is an important salt in stock solution-1 for MS media preparation. But it has some major demerits. It is used in making bomb for terrorist attack and other weapons of mass destruction. Hence, it is a totally band chemical in our country since last 5-7 years. Due to unavailability of this chemical plant tissue culture research and commercial application of tissue culture technology are seriously hampered in our country. Thus any alternative of Ammonium Nitrate (NH_4NO_3) is badly needed for smooth running of plant tissue culture activities. Therefore, remarkable efforts were made by Dr. Md. Ekramul Hoque, an expert of plant tissue culture and Professor Department of Biotechnology, Sher-e-Bangla Agricultural University conducting several researches to establish alternate chemical to be used in stock solution-01 for plant tissue culture media. As a part of this effort, three different chemicals having the potentiality to supply

nitrogen in the media were used to design this experiment. Name of the chemicals and their composition is secret for the privacy of the research. Hence, we denoted those three chemicals as *Chemi-i*, *Chemi-ii* and *chemi-iii* and written as italic form. Those three chemicals individually will be a component of stock solution-01 and there will be no Ammonium Nitrate (NH_4NO_3). The variation of stock solution-01 will be the different treatments for this experiment. Variation will be created by different concentrations of those three chemicals and other component of stock solution-01.

3.4 Brief introduction about different treatments

Treatment-01

The nutrient composition of treatment-01 is given in table 1. Here *chemi-i* was used instead of Ammonium Nitrate (NH_4NO_3). The amount of *chemi-i* is 1 gm/litre. The rest of the component of stock solution-1 was same as MS (1962) medium but concentration was different.

Treatment-02

Here, *chemi-i* was used instead of Ammonium Nitrate (NH_4NO_3) but it was different from treatment-01 in respect of concentration of *chemi-i*. The amount of *chemi-i* was 5.00 gm/litre. Rest of the nutrients were like treatment-01.

Treatment-03

Murashige and Skoog (1962) standard dose used as treatment-03. It was used as a check treatment.

Treatment-04

The nutrient composition was same as treatment-03 but it has totally different in respect of the amount of each nutrient ingredient. It was different from MS standard dose.

Table 1. List of treatment and their chemical composition

Sl no.	Treatment	Chemical composition	Concentration (gm/Litre)	Source of nitrogen
01	Treatment-01	KNO_3	19.00	*Chemi-i*
		Chemi-i	1.00	
		$MgSO_4.7H_2O$	3.70	
		$CaCl_2.2H_2O$	4.40	
		KH_2PO_4	1.70	
02	Treatment-02	KNO_3	38.00	*Chemi-i*
		Chemi-i	5.00	
		$MgSO_4.7H_2O$	7.40	
		$CaCl_2.2H_2O$	8.80	
		KH_2PO_4	3.40	
03	Treatment-03 (MS,1962 standard dose) Check treatment	KNO_3	19.00	NH_4NO_3
		NH_4NO_3	16.50	
		$MgSO_4.7H_2O$	3.70	
		$CaCl_2.2H_2O$	4.40	
		KH_2PO_4	1.70	
04	Treatment-04	KNO_3	Different amount of MS standard dose	NH_4NO_3
		NH_4NO_3	Different amount of MS standard dose	
		$MgSO_4.7H_2O$	Different amount of MS standard dose	
		$CaCl_2.2H_2O$	Different amount of MS standard dose	
		KH_2PO_4	Different amount of MS standard dose	
05	Treatment-05	KNO_3	19.00	*Chemi-ii*
		Chemi-ii	1.00	
		$MgSO_4.7H_2O$	3.70	
		$CaCl_2.2H_2O$	4.40	
		KH_2PO_4	1.70	
06	Treatment-06	KNO_3	38.00	*Chemi-ii*
		Chemi-ii	5.00	
		$MgSO_4.7H_2O$	7.40	
		$CaCl_2.2H_2O$	8.80	
		KH_2PO_4	3.70	
07	Treatment-07	KNO_3	19.00	*Chemi-iii*
		Chemi-iii	1.00	
		$MgSO_4.7H_2O$	3.70	
		$CaCl_2.2H_2O$	4.40	
		KH_2PO_4	1.70	
08	Treatment-08	KNO_3	38.00	*Chemi-iii*
		Chemi-iii	5.00	
		$MgSO_4.7H_2O$	7.40	
		$CaCl_2.2H_2O$	8.80	
		KH_2PO_4	3.70	

Treatment-05

The second alternate chemical (*chemi-ii)* of Ammonium Nitrate (NH_4NO_3) was used in this treatment. The amount was 1gm/litre. Rest of the component of stock solution was same as MS standard dose but has different amount.

Treatment-06

The same *chemi-ii* was used here but the amount was 5.0gm/litre. Rest of the ingredient was same as MS (1962) standard dose.

Trearment-07

The third alternate chemical of Ammonium Nitrate (NH_4NO_3) was *chemi-iii* in this treatment. The amount of this *chemi-iii* was 1.0gm/litre. Rest of the component was same as MS (1962) standard dose but the amount is different.

Treatment-08

The *chemi-iii* was also used in this treatment but it was 5.0gm/litre. Rest of the component of stock solution was same as MS standard dose.

3.5 Experiments

Two sub-experiments were conducted to fulfill the mentioned objectives.

Sub-experiment-01: Plantlet regeneration in Diamant potato variety using new stock solution-01.

Sub-experiment-02: Plantlet regeneration in Asterix potato variety using new stock solution -01.

3.6 Preparation of different stock solution

Stock solution is the initial step for the preparation of culture medium. Separate stock solutions for the macronutrients, micronutrients, Fe-EDTA (Iron stock), vitamins and growth regulators were prepared separately for further use. All the chemicals used for stock solution is highly purified and labeled as plant tissue culture tested grade. The chemicals were dissolved in

double distilled water or highly purified de-ionized water. Each chemical was added according to the list of ingredient presented in Appendix-vii.

3.6.1 Stock solution–1 for culture medium (Macro nutrients)

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 was weighted accurately. All the macronutrient were dissolved 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.

Variation in stock solution-01 is the major concern of this experiment. Those variations were used as different treatment. Separate marking was made on the basic of treatment and denoted as Treatment-01, 02, 03, 04, 05, 06, 07 and 08.

3.6.2 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.

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

Iron-EDTA was added freshly and it was made 100 times higher 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.

3.6.4 Vitamins stock solution (stock-IV)

The following vitamins were used in the present study for the preparation of MS culture medium. Myo-inositol (Inositol), Nicotinic acid (Vitamin B3), Pyridoxin HCl (Vitamin B6), Thiamine HCl (Vitamin B1) 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.

3.6.5 Other stock solutions preparation

Preparation of IN NaOH

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

Preparation of IN HCl

The commercial liquid concentrated HCl was used for the preparation of 1L of 1 N solution of HCl. Dilution was made by using the formula of $S_1V_1 = S_2V_2$. S_1 = Strength of commercial HCl, V_1 = Volume of commercial HCl for the preparation of target amount. S_2 =Target strength (IN) of HCl, V_2 = Volume (ml) of HCl (on the basis of researcher requirement).

Preparation of 70% Ethanol

In a 100ml measuring cylinder 70ml 99.9% ethanol was poured. 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.

Preparation of 10% NaOCl

To prepare 100 ml NaOCl, 10gm 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 stirrer to dissolve it completely. Then it was cooled in refrigerator at 4^0C.

3.6.6 Tissue culture medium preparation from new stock solution

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

1. 700 ml sterilized distilled water was taken into 1000 ml beaker.
2. 100 mL of stock solution-1 (Treatment wise different chemical used), 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.
3. The whole mixture was then made up to 1 liter with further addition of distilled water.
4. The pH of the medium was adjusted to 5.80 by pH meter with the addition of 1 N NaOH or 0.1 N HCl.
5. Finally, 8 gm agar was added to the mixture and heated for 12 minutes in an electric oven for melting of agar.
6. Stock solution-1 was marked according to treatment and treatment wise different media were prepared and level accurately.

Agar

The media was gelled with 8 g/L agar and the whole mixture was gently heated on microwave oven at 280°C temperature for 10 minutes.

3.6.7 Sterilization

3.6.7.1 Sterilization of culture media

One liter of new tissue culture medium were divided into two conical flasks and capped with aluminium foil. Then the conical flasks were autoclaved at 15 psi pressure at 121 ^{0}C for 20 minutes. The medium was then transfer into

the culture room and cooled at 23^0C temperature. The media was aliquot 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

3.6.7.2 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 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 45 minutes at 15 psi pressure.

3.6.7.3 Sterilization of culture room and transfer area

At the beginning, the culture room was spray with formaldehyde and then the room was kept closed for two 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 (uv) 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.

3.6.7.4 Sterilization of Laminar Air Flow Cabinet

The laminar air flow cabinet was started half an hour before working. The air flow bench was cleaned with cotton soaked with 70% ethanol. All glassware were 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 and opened the door and switched on the air flow. 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.

3.6.8 Preparation of explants and inoculation of explant onto culture vial

The shoot tip of potato were used as explants. The shoot tips were washed thoroughly with double distilled water into laminar airflow cabinet for surface sterilization. The shoot tips were then rinsed twice with sterile distilled water. Afterwards the explant 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 shoot tips were trimmed and lower one or two leaf were removed to made the explant as 0.5-1.0 cm in size (Plate 1). The explants were transfer or inoculated in a culture vial (Plate 2). Explants were 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 1: Diamant variety shoot tip used as explant for plantlet regeneration in potato.

Plate 2: Inoculated Diamant shoot tip into culture vial in different treatments for *in vitro* regeneration of potato

Plate 3: Asterix variety nodal segment used as explant for plantlet regeneration in potato.

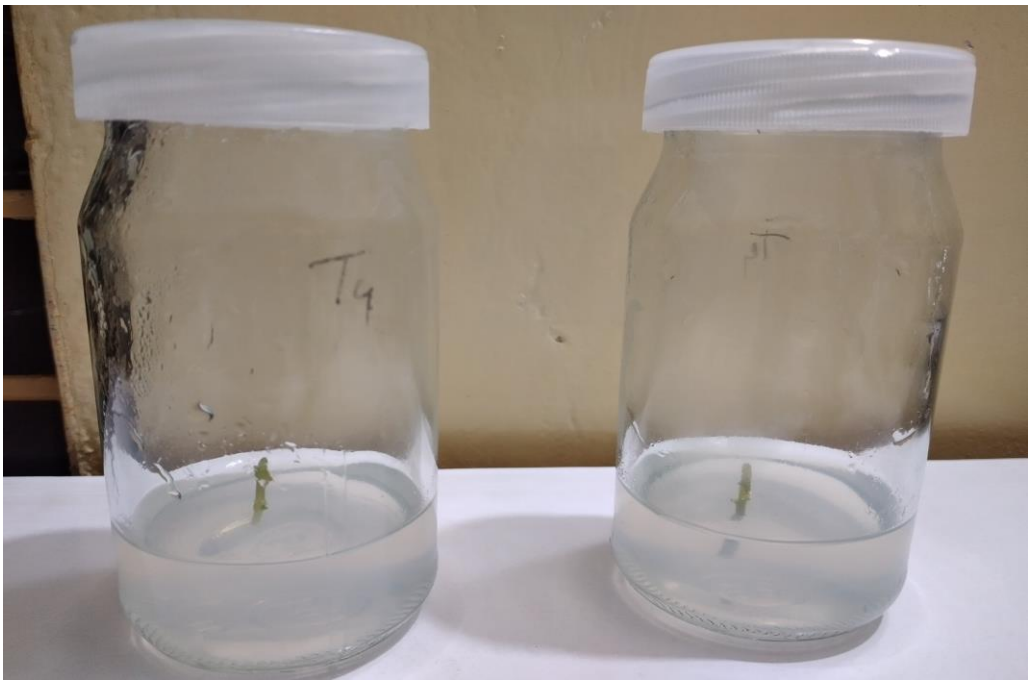

Plate 4: Inoculated Asterix nodal segment into culture vial in different treatments for *in vitro* regeneration of potato

3.6.9 Placement of culture vial in growth room

The inoculated culture vial were kept in growth room for shoot and root induction. Temperature, light intensity and humidity was properly maintained in culture room. Temperature were fixed on 21 ± 1^0C. Light intensity was 4000-5000 lux. Humidity was 60-70%. All the parameter were checked by thermometer, lux meter and hygrometer, respectively. The culture vials were arranged properly in the culture rack. Observation was made in every 3 days after interval. Contamination and other abnormal vials were removed from the culture room as and when necessary.

3.6.10 Experimental design

In laboratory condition, the one factors experiment was laid out in Completely Randomized Design (CRD) with three replications. Ten culture vial were used as one replication for each treatment.

3.6.11 Data collection

Data on the following parameters were recorded for further analysis and interpretation.

3.6.12 Length of shoot

The length of shoot was recorded by using a plastic scale in laminar airflow cabinet at 2^{nd}, 3^{rd} and 4^{th} week from each inoculation.

3.6.13 Number of shoot

Total number of shoot was recorded by visual observation at 2^{nd}, 3^{rd} and 4^{th} week from each inoculation date of explant in cultural vial. The mean value of the data provided the number of shoot.

3.6.14 Number of node

Total number of internode was recorded by visual observation at 2^{nd}, 3^{rd} and 4^{th} week from each inoculation. The mean value of the data provided the number of internode.

3.6.15 Number of leaves per plantlet

Total number of leaves per plantlet was recorded by visual observation at 2^{nd}, 3^{rd} and 4^{th} week from each inoculation of explant.

3.6.16 Number of root

Total number of root was recorded by visual observation at 2^{nd}, 3^{rd} and 4^{th} week from each inoculation. The mean value of the data provided the number of root.

3.6.17 Length of root

The length of root was recorded by using a plastic scale in laminar airflow cabinet at 2^{nd}, 3^{rd} and 4^{th} week from each inoculation.

3.6.18 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

RESULTS AND DISCUSSION

The experiment was conducted to identify any alternate chemical of Ammonium nitrate (NH_4NO_3) for tissue culture media preparation on *in vitro* regeneration of potato (*Solanum tuberosum* L.). Consequently two experiments were conducted under the laboratory condition. The analysis of variance (ANOVA) of the data has been presented in Appendix I-VI. The results have been presented and discussed. Possible interpretations were given under the following parameters:

4.1 Sub-Experiment I: Plantlets regeneration in Diamant variety of potato using different chemical composition of stock solution-1

Normally five different stock solutions were needed for tissue culture media preparation. Composition of each stock solution is given in materials and methods chapter. Stock solution-01 is main consideration of the present study. Hence, variations in preparation of stock solution-01 was made which were used as different treatments of the experiment. The different chemical composition of stock solution-01 generated considerable information regarding plantlet regeneration of potato. The major findings of the experiment are given below:

4.1.1 Effect of treatment on node number of Diamant potato plantlet

The figure-1 represents the effect of various treatment on node development in potato plantlet. Significant difference was noticed among the treatments. It was observed that, the highest number of node (6.00, 8.56 and 12.17) was found in the treatment-07 at 2^{nd}, 3^{rd} and 4^{th} week of plantlet regeneration. The second highest value (10.67) at 4^{th} week was recorded in check treatment-03. The lowest (2.33, 2.83 and 3.06) data was found in the

treatment-02 at 2nd, 3rd and 4th week, respectively. The treatment-07 was prepared by *chemi-iii* and the treatment-02 was made by the *chemi-i*. Hence, it indicated that, the *chemi-i* has negative influence compared to the *chemi-ii* and chemi-*iii* on *in vitro* regeneration of potato. The *chemi-iii* has positive effect on *in vitro* regeneration of potato. Component of stock solution-1 as well as concentration of the components were also important factor for media preparation. It was noticed that, higher dose of *chemi-iii* in treatment-08 was not as response as treatment-07. Finally, for this parameter treatment-07 gave better regeneration potentially among the treatment under studied. It is necessary to mention that, ammonium nitrate is absent in all the treatments except in treatment-03 and 04.

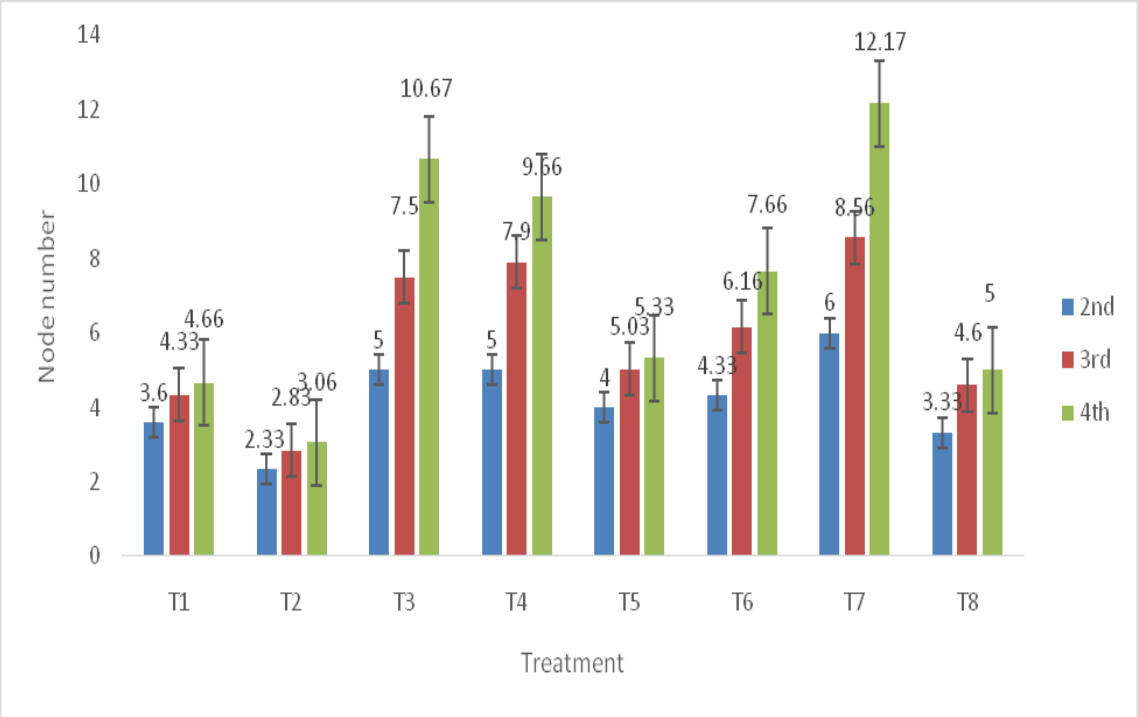

Figure 1. Effect of different treatments on node number in plantlet of Diamant variety at different weeks after regeneration. [Error bar (I) = indicates the standard deviation from mean value.]

4.1.2 Effect of treatment on leaf number of plantlet

Effect of treatments on leaf number of plantlet in Diamant variety is presented in Table 2. Significant variation was observed among the treatments studied. Highest number of leafs (9.00, 11.67 and 12.33) were regenerated in the treatment-07 at 2^{nd}, 3^{rd} and 4^{th} week of explant inoculation, respectively. The check treatment (T_3= MS standard dose) produced the 2^{nd} highest number of leafs (6.66, 10.23 and 12.00) in the plantlet for all the mentioned weeks under investigation. Although significant difference was observed in 2^{nd} and 3^{rd} week of regeneration but in 4^{th} week, there was no statistically different among the two (T_3 and T_7) treatments. The lowest performance was noticed in the treatment-02. The treatment-04 showed very closer response with T_3 and T_7 treatment (Table 2). The leaf number of plantlets for T_4 and T_7 treatment are presented in plate-4. The result indicates that, the *chemi-iii* having 1.00g/L concentration in stock solution-1 showed better response on *in vitro* regeneration of potato.

Table 2: Effect of treatment on leaf number of Diamant potato plantlet at different weeks after regeneration

Sl. No.	Treatment	Leaf number at different week after regeneration		
		2^{nd}	3^{rd}	4^{th}
1	T_1	4.75c ± 1.21	5.66e ± 1.22	6.13b ± 0.70
2	T_2	3.00d ± 1.87	2.50f ± 0.50	2.66c ± 0.32
3	T_3	6.66b ± 0.57	10.23ab ± 0.75	12.00a ± 1.56
4	T_4	6.66b ± 0.57	8.90bc ± 0.36	11.00a ± 1.43
5	T_5	5.33bc ± 0.53	6.50de ± 1.41	7.00b ± 1.00
6	T_6	5.33bc ± 0.53	6.60de ± 1.35	11.67a ± 0.57
7	T_7	9.00a ± 1.86	11.67a ± 1.52	12.33a ± 1.52
8	T_8	5.00c ± 1.34	8.00cd ± 1.00	5.66b ± 2.08
CV	(%)	14.57	11.87	11.24
LSD	(5%)	1.45	1.56	1.68
SD	-	0.48	0.51	0.55

In a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each of other as adjusted by DMRT. Values after ± sign indicate standard deviation from the mean value.

T_1 = Stock solution-01 having 1.00 gm/L of *chemi-i*

T_2 = Stock solution-01 having 5.00 gm/L of *chemi-i*

T_3 = Stock solution-01 as recommended by Murashige & Skoog (1962)
T_4 = Stock solution-01 as recommended by Murashige & Skoog (1962) but has different concentration of each component
T_5 = Stock solution-01 having 1.00 mg/L of *Chemi-ii*
T_6 = Stock solution-01 having 5.00 gm/L of *Chemi-ii*
T_7 = Stock solution-01 having 1.00 gm/L of *chemi-iii*
T_8 = Stock solution-01 having 5.00 gm/L of *chemi-iii*

Plate 5: Leaf number of plantlet in Diamant variety of potato on T_4 and T_7 treatment at 4th week after regeneration

4.1.3 Shoot length of plantlet of Diamant variety of potato

Remarkable variation was noticed for the parameter of shoot length among the different treatments. The maximum shoot lengths 6.03, 10.73 and 9.43cm were generated in the treatment-07 at 2nd, 3rd and 4th week, respectively (Table 3).

The check treatment (T_3) showed second maximum data at 2nd and 3rd week of regeneration (Table 3 and plate 4). But in the 4th week the second highest shoot length (9.00cm) was found in treatment-04. Lowest (2.33, 2.43 and 1.93cm) shoot length was observed in treatment-02 in all the period. In respect of this trait the *Chemi-i* and *Chemi-ii* showed lower performance than *Chemi-iii* for all the concentration on stock solution-01 preparation.

Table 3: Shoot length of potato plantlet on Diamant variety from various treatments at different weeks after regeneration

Sl. No.	Treatment	Shoot length at different week after regeneration		
		2nd	3rd	4th
1	T_1	2.63c ± 0.70	3.66b ± 0.76	3.63cd ± 0.15
2	T_2	2.33c ± 0.66	2.43e ± 0.58	1.93d ± 0.92
3	T_3	5.73a ± 0.64	8.00b ± 0.50	6.16bc ± 4.48
4	T_4	5.40a ± 0.85	7.76b ± 0.55	9.00ab ± 0.50
5	T_5	3.16bc ± 0.76	4.83cd ± 1.04	6.33bc ± 1.52
6	T_6	4.10b ± 0.90	5.63c ± 1.40	5.33c ± 1.51
7	T_7	6.03a ± 0.15	10.73a ± 0.25	9.43a ± 1.50
8	T_8	4.16b ± 0.35	5.33c ± 1.14	5.56c ± 1.45
CV	(%)	16.27	11.47	28.23
LSD	(5%)	1.19	1.21	2.93
SD	-	0.39	0.40	0.96

In a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each of other as adjusted by DMRT. Values after ± sign indicate standard deviation from the mean value. LSD (0.05) = Least significant difference CV = Coefficient of variation

T_1 = Stock solution-01 having 1.00 gm/L of *chemi-i*

T_2 = Stock solution-01 having 5.00 gm/L of *chemi-i*

T_3 = Stock solution-01 as recommended by Murashige & Skoog (1962)

T_4 = Stock solution-01 as recommended by Murashige & Skoog (1962) but has different concentration of each component

T_5 = Stock solution-01 having 1.00 mg/L of *Chemi-ii*

T_6 = Stock solution-01 having 5.00 gm/L of *Chemi-ii*

T_7 = Stock solution-01 having 1.00 gm/L of *chemi-iii*

T_8 = Stock solution-01 having 5.00 gm/L of *chemi-iii*

Plate 6: Shoot length of plantlet in Diamant variety of potato in T_3 and T_7 treatments at 3rd week after regeneration

4.1.4 Effect of treatment on root number of plantlet in Diamant variety of potato

The root number under all the treatments are represented in figure-02. Although, the treatment-07 gave little higher value (7.66) than treatment-03 but statistically non-significant data was recorded among the treatment-03 and 07 for all the experimental period (2^{nd}, 3^{rd} and 4^{th} week). It means that, both the treatments showed better response for this parameter. The lowest value (2.00) was observed in treatment-02 for all the experimental period. The treatment-05 and 06 gave moderate number of root (3.00 and 5.33) the experimental period. Ammonium nitrate free treatments were T_1, T_2, T_5, T_6, T_7 and T_8. Among them T_7 showed best response. It showed similar performance with check treatment-T_3 which has the composition of ammonium nitrate. It proved that, potato plantlet can be regenerate with different media composition in absence of ammonium nitrate.

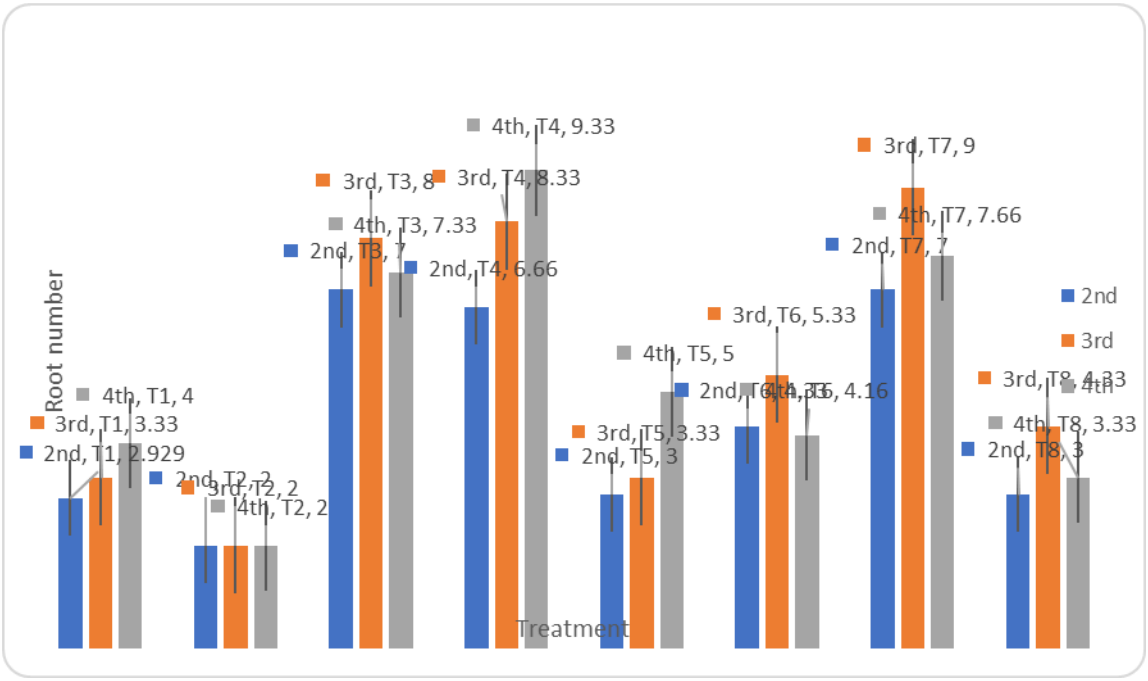

Figure 2. Effect of treatment on root number in potato plantlet of Diamant variety at different weeks after regeneration. [Error bar (I) = indicates the standard deviation from mean value.]

4.1.5 Root length of plantlet in Diamant variety of potato under different treatments

The treatment-04 which has same component of MS standard media but different concentration showed best result on root length in the plantlet of Diamant variety. It was the highest (7.73cm) in 4th week after regeneration (Plate 5). The second highest (7.66cm) data was show in check treatment (T_3) and there was no statistical difference between the treatment-04 and Treatment-03 (Table 4 and Plate 5 & 6). The lowest performance was observed in treatment-02 in all the weeks during the experimental period. The treatment T_6 and T_7 showed better response as compared to T_1, T_2, T_5 and T_6 treatments. It indicated that *chemi-iii* is more suitable than *chemi-i* and *chemi-ii* for this trait in plantlet regeneration. Plantlet regeneration from all the treatments are mentioned in Plate 7. It indicated that, the plantlet of treatment-03, 04 and 07 have normal growth and development at 3rd week after regeneration.

Table 4: Root length of potato plantlet of Diamant variety from various treatments at different weeks after regeneration

Sl. No.	Treatment	Leaf number at different weeks after regeneration		
		2nd	3rd	4th
1	T_1	1.47bc ± 0.513	0.60g ± 0.265	0.66e ± 0.289
2	T_2	0.90c ± 0.361	0.33g ± 0.153	0.36e ± 0.058
3	T_3	3.50a ± 1.000	7.60a ± 0.361	7.66a ± 0.321
4	T_4	3.20a ± 0.265	6.90b ± 0.361	7.73a ±0.643
5	T_5	1.63bc ± 0.416	2.66e ± 0.289	2.16d ± 0.764
6	T_6	2.06b ± 0.379	3.50d ± 0.500	3.40c ± 0.520
7	T_7	2.13b ± 0.153	4.83c ± 0.764	4.66b ± 0.289
8	T_8	0.93c	1.83f ± 0.289	1.86d ± 0.351
CV	(%)	26.18	10.51	13.61
LSD	(5%)	0.908	0.650	0.850
SD	-	0.299	0.214	0.280

In a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each of other as adjusted by DMRT. Values after ± sign indicate standard deviation from the mean value. LSD (0.05) = Least significant difference CV = Coefficient of variation

T_1 = Stock solution-01 having 1.00 gm/L of *chemi-i*

T_2 = Stock solution-01 having 5.00 gm/L of *chemi-i*

T_3 = Stock solution-01 as recommended by Murashige & Skoog (1962)

T_4 = Stock solution-01 as recommended by Murashige & Skoog (1962) but has different concentration of each component

T_5 = Stock solution-01 having 1.00 mg/L of *Chemi-ii*

T_6 = Stock solution-01 having 5.00 gm/L of *Chemi-ii*

T_7 = Stock solution-01 having 1.00 gm/L of *Chemi-iii*

T_8 = Stock solution-01 having 5.00 gm/L of *Chemi-iii*

Plate 7: Root length of plantlet in Diamant variety of potato in treatment T_4 at 4th week after regeneration

Plate 8: Root length (cm) of plantlet in Diamant variety of potato in treatment T_3 at 4th week after regeneration

Plantlet of Diamant variety in different treatments

Plantlet regeneration from potato plant has 50 years back technology in all over the world. Meristem derived virus free plantlet production method has been practiced for commercial potato production in last four decades. MS (1962) media composition is used for the tissue culture of potato. Ammonium nitrate is one of the important component of stock solution-01 in MS media. Unlimited literature is available in this regard. But here the present concern is to develop a new media composition where ammonium nitrate will be absent. In the present study 8 different treatments were made to find out the best combination of stock soluation-1 which has no NH_4NO_3. Significant variation was noticed among the treatments. The overall regeneration potentiality of those treatments at 3rd week was presented in plate no 8.

Plate 9: Plantlet of Diamant variety of potato in different treatments at 3rd week after regeneration

Bashar *et. al.* (2021) reported that tissue culture is an important biotechnological aspect for fast multiplication and regeneration of plantlets

focusing disease free plant. Ammonium nitrate (NH_4NO_3), an important ingredient of tissue culture medium is used as a major salt but it is explosive in nature and risky which restricted its use in cell culture. This study was carried out to find out the efficient substitute of NH_4NO_3 with urea for three potato varieties following completely Randomized Design (CRD). According to their hypothesis, they used urea as a major salt in stock solution-1 for the preparation of tissue culture medium instead of NH_4NO_3. Four different media composition (T1=company recommended dose, Ready MS powder-Dutchefa, Netherlands, T2=16.50 gm/L of ammonium nitrate-MS standard dose, T3= 1gm/L of urea and T4= 5gm/L) were used. T4 (5 gm/L of urea) showed best efficient on *in vitro* regeneration with maximum shoot and leaf numbers with healthy and robust plantlets. Findings presented the urea as an efficient substitute of explosive chemical NH_4NO_3 and it is the first reported works in plant tissue culture. Their results indicated the urea as a safe and efficient substitute for potato tissue culture and plantlet regeneration. The result also showed the appropriate concentration of urea for tissue culture media. Urea as an substitution of formulation of stock solution-1 is new invention in our present study.

Wakil (2019) conducted an experiment at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), from, 2018 to 2019 to develop a substitute of explosive chemical Ammonium nitrate (NH_4NO_3) in MS media for *in vitro* regeneration of potato variety Asterix. A 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 gm/L) of α chemical which have twice dose of other component in stock solution-I for the preparation of tissue culture medium was used as treatment. It was noticed that, *in vitro* regeneration of potato had been successfully done with α-chemical in new composition of tissue culture medium. The shoot length, node number,

number of leaf, root length were the 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 different 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 gm/L 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.

Hena (2017) reported on successful *in vitro* regeneration of potato without ammonium free stock solution-01. She used the Asterix variety of potato for rapid plantlet regeneration. She mentioned that the regenerated plantlets were comparatively weaker than the plantlets regenerated in MS (1962) medium in respect of leaf number, node number, shoot length and root length.

4.2 Sub-Experiment II: Plantlets regeneration in Asterix variety of potato using different chemical composition of stock solution-1

Potato is an important vegetable and cash crop in our country. More than eighty varieties were cultivated in different areas of Bangladesh. Among them Diamant and Asterix variety are more popular to the farmer due to their yield and other agronomic traits. Hence, we choice this two variety as experimental material. The *in vitro* regeneration of Asterix variety was carried out on different composition of stock solution-01. Each of composition in stock solution-01 was treated as separate treatment. The experimental findings were presenting the following sub-heading.

4.2.1 Effect of different treatments on number of node in potato plantlet of Asterix variety

The figure-3 represent the regeneration potentiality of different treatments in Asterix variety of potato. It was noticed that, the treatment-04 regenerated highest number (6.33 and 1167) of node in 2nd and 4th week after regeneration. But in 3rd week highest number of node (8.33) was found in check treatment (T_3). Although check treatment (T_3) gave second highest data in 2nd and 4th week but it was statistically non significant with treatment-04. The lowest node number (2.00 and 1.83) was produced by the treatment-02. The treatment-01 also has the poor performer for this trait under study. Hence, it is clear that, the chemi-01 has negative effect on media composition. Probable causes of the chemi-01 may be react with other component of stock solution-01 and produced toxic substance for the growth and cell division of explant. The treatment-07 which showed good response in plantlet regeneration in Diamant variety at sub-experiment-01. Here, the same treatment showed moderate response in respect of this parameter. It is may be due to genotypic potentiality of regeneration.

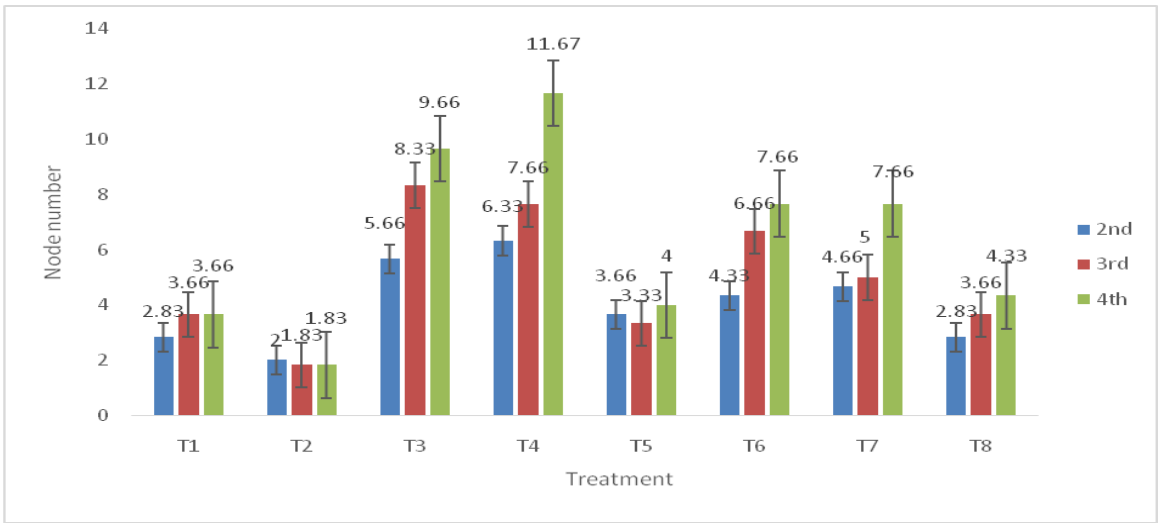

Figure 3. Effect of the treatment on number of node at different week in potato plantlet of Asterix variety. [Error bar (I) = indicates the standard deviation from mean value.]

4.2.2 Leaf number of potato plantlet in Asterix variety

The effect of various treatment on leaf number of potato plantlet in Asterix variety was given in Table-05. The maximum number of leaf (9.66, 10.67 and 12.33) were generated in check treatment-03 and it was second highest (9.33 and 12.00) in treatment-04 for all the experimental period (Plate 8 and 9). The lowest number of leaf (2.66 and 2.33) was recorded in treatment-02. The treatment-07 regenerated 7.33 and 9.33 number of leaf at 2nd, 3rd and 4th week of regeneration. For this parameter treatment-04 showed best response. The composition of T_4 was same as component of check treatment (T_3) but concentration was different. It proved that, some modification of MS (1962) standard dose for stock solution-1 preparation has positive relation on growth and development of explant.

Table 5: Leaf number of different potato plantlet in Asterix variety from the treatments at different week of regeneration

Sl. No.	Treatment	Leaf number at different week of regeneration		
		2nd	3rd	4th
1	T_1	3.66d ± 0.54	4.66d ± 0.53	4.66d ± 0.56
2	T_2	2.66d ± 0.51	2.33f ± 0.47	2.33e ± 0.32
3	T_3	9.66a ± 0.82	10.67a ± 0.57	12.33a ± 0.58
4	T_4	9.33a ± 0.84	9.33b ± 0.87	12.00a ± 1.75
5	T_5	5.33c ± 0.75	5.00d ± 1.23	5.33d ± 0.57
6	T_6	5.00c ± 1.21	7.00c ± 1.34	8.00c ± 1.00
7	T_7	7.33b ± 0.57	7.33c ± 0.56	9.33b ± 0.57
8	T_8	2.66d ± 0.20	4.00e ± 0.33	4.66d ± 0.56
CV	(%)	11.55	5.62	8.68
LSD	(%)	1.15	0.61	1.11
SD	-	0.38	0.204	0.36

In a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each of other as adjusted by DMRT. Values after ± sign indicate standard deviation from the mean value. LSD (0.05) = Least significant difference CV = Coefficient of variation

T_1 = Stock solution-01 having 1.00 gm/L of *chemi-i*

T_2 = Stock solution-01 having 5.00 gm/L of *chemi-i*

T_3 = Stock solution-01 as recommended by Murashige & Skoog (1962)

T_4 = Stock solution-01 as recommended by Murashige & Skoog (1962) but has different concentration of each component

T_5 = Stock solution-01 having 1.00 mg/L of *Chemi-ii*

T_6 = Stock solution-01 having 5.00 gm/L of *Chemi-ii*

T_7 = Stock solution-01 having 1.00 gm/L of *chemi-iii*

T_8 = Stock solution-01 having 5.00 gm/L of *chemi-iii*

4.2.3 Shoot length of potato plantlet in Asterix variety

The effect of treatment on shoot length production was presented in Table-6. It was observed that, the highest shoot length (6.96 and 12.40 cm) was regenerated in treatment-04 at 2nd and 4th week of explant transformation to the media. In 3rd week highest (8.83cm) shoot length was recorded in T_3 and it was statistically similar (8.33a cm) with treatment-04. The result reveal that, the treatment-03 and 04 was equally good for the trait development in potato plantlet production (Plate 8 and 9). The lowest shoot length (1.93, 2.16 and 2.50 cm) was found in treatment-02. The moderate shoot length (5.63, 6.50 and 7.66 cm) was produced by the treatment-07. The *chemi-i* showed poor responsed for this parameter. The *chemi-ii* and *chemi-iii* showed intermediate regeneration potentiality for the trait under studied.

Table 6: Shoot length of potato plantlet of Asterix variety from various treatment at different week of regeneration

Sl. No.	Treatment	Shoot length (cm) at different week of regeneration		
		2nd	3rd	4th
1	T_1	3.20e ± 0.26	3.33d ± 0.57	4.33e ± 0.26
2	T_2	1.93f ± 0.11	2.16e ± 0.28	2.50g ± 0.50
3	T_3	5.23bc ± 0.25	8.83a ± 0.78	10.57b ± 0.40
4	T_4	6.96a ± 0.40	8.33a ± 0.28	12.40a ± 0.17
5	T_5	4.56d ± 0.60	4.66c ± 1.15	5.86d ± 0.32
6	T_6	4.93cd ± 0.40	5.33c ± 0.57	5.50d ± 0.50
7	T_7	5.63b ± 0.15	6.50b ± 0.50	7.66c ± 0.28
8	T_8	2.90e ± 0.10	2.83de ± 0.28	3.66f ± 0.25
CV	(%)	6.50	10.93	5.22
LSD	(%)	0.50	0.93	0.59
SD	-	0.16	0.30	0.19

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T_1 = Stock solution-01 having 1.00 gm/L of *chemi-i*

T_2 = Stock solution-01 having 5.00 gm/L of *chemi-i*

T_3 = Stock solution-01 as recommended by Murashige & Skoog (1962)

T_4 = Stock solution-01 as recommended by Murashige & Skoog (1962) but has different concentration of each component

T_5 = Stock solution-01 having 1.00 mg/L of *Chemi-ii*

T_6 = Stock solution-01 having 5.00 gm/L of *Chemi-ii*

T_7 = Stock solution-01 having 1.00 gm/L of *chemi-iii*

T_8 = Stock solution-01 having 5.00 gm/L of *chemi-iii*

Plate 10: Maximum number of leaf and highest shoot length of plantlet in Asterix variety of potato in the treatment T_4 at 4th week after regeneration.

Plate 11: Leaf number and shoot length of plantlet in Asterix variety of potato at the treatment T_3 at 4th week after regeneration.

4.2.4 Root number of potato plantlet in Asterix variety

The treatment effect was presented in figure-4. The maximum number of roots (11.67) was found in the check treatment (T_3) at 2nd and 4th week after regeneration. On the other hand, maximum number of root (10.00) was found in Treatment-04 at 3rd week of inoculation. Statistically, similar value was noticed for 2nd and 3rd week of regeneration among the two treatment (T_3 and T_4). Its indicated that, the treatment-03 and treatment-04 regenerated similar number of root in plantlet of potato. Hence, the modification of MS (1962) media composition for stock solution-01 has positive effect of potato micropropagation. The minimum number of roots (2.33, 1.66 and 1.83) were observed in treatment-02 at all the week of experimental period. The 3rd highest number of roots (5.33, 7.33 and 9.00) was recorded in treatment-07.

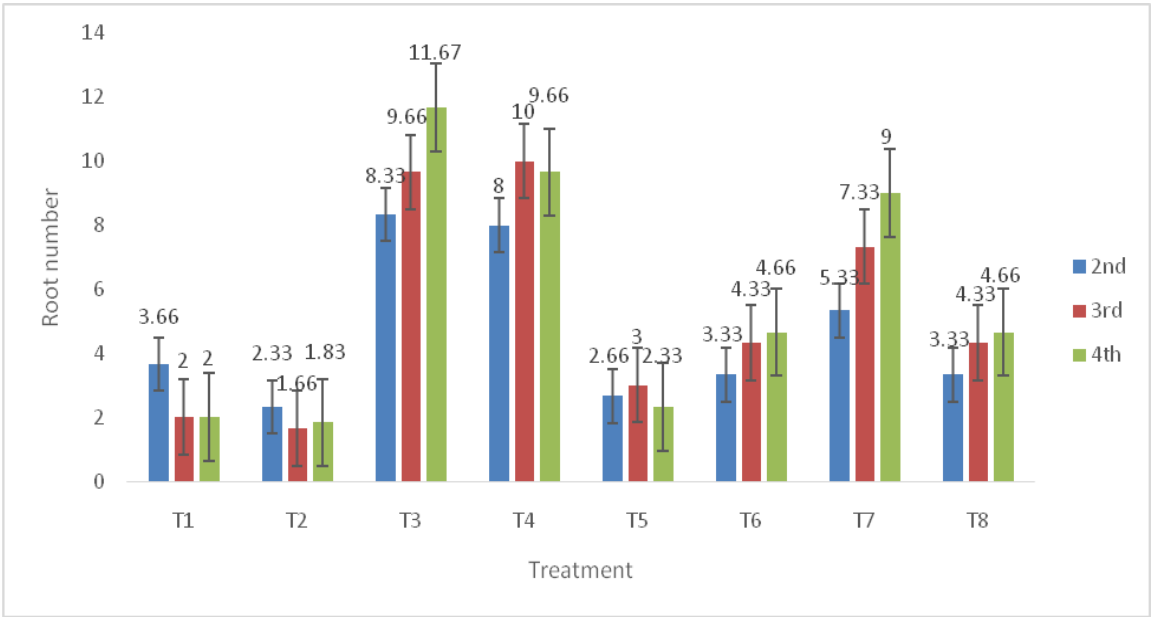

Figure 4. Effect of different treatment on number of roots in potato plantlet of Asterix variety at different week of regeneration. [Error bar (I) = indicates the standard deviation from mean value.]

4.2.5 Root length of potato plantlet in different treatment

The root length was presented in Table 7. Significant variation was observed for this parameter among the different treatments. The highest length of roots (5.16, 6.56 and 8.73 cm) were noticed in the treatment-04 (Plate 10). And the second highest (4.66, 5.73 and 7.83 cm) root length was found in check treatment (T_3) at all the experimental period. The lowest length of roots (1.13, 1.26 and 1.26 cm) was recorded in the treatment-02. The 3rd highest length of root (4.96, 5.00 and 5.50 cm) were found in treatment-07. Three alternate chemical of ammonium nitrate were used to find out any suitable combination to prepare tissue culture media for potato plantlet regeneration. From the above finding was reveal that, the *chemi-i* and *chemi-ii* has toxic and negative effect on tissue culture media preparation. The *chemi-iii* has significant positive role on culture media preparation. Although, treatment made from *chemi-iii* was not perform best on plantlet regeneration in Asterix variety but it has the capacity to regenerate plantlet in moderate level in both the varieties- Diamant and Asterix. Plantlet regeneration from 8 treatments were shown in plate 11. It showed that, the check treatment (T_3) performed well at 3rd week after regeneration.

Table 7: Root length of potato plantlet in Asterix variety from various treatments at different week of regeneration

Sl. No.	Treatment	Root length (cm) at different week of regeneration		
		2nd	3rd	4th
1	T_1	2.26c ± 0.25	2.20f ± 0.10f	2.06e ± 0.05
2	T_2	1.13d ± 0.15	1.26g ± 0.20	1.26f ± 0.252
3	T_3	4.66a ± 0.57	5.73b ± 0.46	7.83b ± 0.28
4	T_4	5.16a ± 0.76	6.56a ± 0.40	8.73a ± 0.46
5	T_5	1.26d ± 0.20	1.56g ± 0.11	1.43f ± 0.11
6	T_6	3.30b ± 0.60	3.83d ± 0.28	4.46d ± 0.15
7	T_7	4.96a ± 0.41	5.00c ± 0.50	5.50c ± 0.50
8	T_8	2.16C ± 0.28	3.16e ± 0.28	4.23d ± 0.25
CV	(%)	14.59	9.35	6.72
LSD	(5%)	0.79	0.59	0.53
SD	-	0.26	0.19	0.17

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T_1 = Stock solution-01 having 1.00 gm/L of *chemi-i*

T_2 = Stock solution-01 having 5.00 gm/L of *chemi-i*

T_3 = Stock solution-01 as recommended by Murashige & Skoog (1962)

T_4 = Stock solution-01 as recommended by Murashige & Skoog (1962) but has different concentration of each component

T_5 = Stock solution-01 having 1.00 mg/L of *Chemi-ii*

T_6 = Stock solution-01 having 5.00 gm/L of *Chemi-ii*

T_7 = Stock solution-01 having 1.00 gm/L of *chemi-iii*

T_8 = Stock solution-01 having 5.00 gm/L of *chemi-iii*

Plate 12: Root length of plantlet in Asterix variety of potato of the treatment T_4 at 4th week after regeneration

Plate 13: Plantlet of Asterix variety of potato in different treatments at 3rd week of regeneration

4.2.6 Physical appearance of media

Stock soluation-01 was made with three different chemicals *viz chemi-i*, *chemi-ii* and *chemi-iii*. Six different treatments *viz* T_1, T_2, T_5, T_6, T_7 and T_8 were made with those chemicals having different combination and concentration. Those are alternate chemicals of ammonium nitrate for stock soluation-01 preparation. The newly used chemicals reacted with other component of stock solution-01 and showed some negative and toxic effects on media. The morphological change and phenotypic variation of each of the above treatment are presented in Table-08. It was noted that, in 1^{st} week of time, all the media of total eight treatment were same. There was no change of media, in respect of appearance with check treatment.

Due to course of time, media colour and physical appearance were converted to brown, off white, yellow and milky like structure etc. In 2^{nd} week the media of the treatments T_1, T_2, T_5, T_6 and T_8 showed brown colour. The light brown colour was found in T_7 treatment. Off white, brown, deep brown colour developed on the media in the treatment- T_1, T_2, T_5, T_6 and T_8 in 3^{rd} week. The media of T_7 showed light yellow to yellow colour (Plate 12).

Huge variation were recorded in 4^{th} week of regeneration. Such as milk layer formation on the upper portion of media in the treatments T_1, T_2, T_3, T_5, T_6 and T_8. Curd formation and looking contamination like structure of media was found in the treatments T_1, T_2, T_5, T_6 and T_8 at 3^{rd} week of regeneration (Plate 13). Root of explants was not visible due to deep brown and discoloration of media (Plate 13).

Table 8: Physical appearance of media in different treatments

Treatment	1st week	2nd week	3rd week	4th week
T_1	Normal	Brown	Brown, Off white, Deep brown, curd like structure	1. Deep brown & hazy 2. Root not visible due to discoloration of media 3. Yellowish colour in the attachment point of explant 4. Milky layer formation on the upper portion of media 5. Curd formation and looking like contamination of media
T_2	Normal	Brown		
T_5				
T_6				
T_8				
T_7	Normal	Light Yellow	Yellow colour at the bottom of explant	Light yellow, yellow and contamination like structure
T_3 T_4	Normal	Normal	Normal	Normal

T_1 = Stock solution-01 having 1.00 gm/L of *chemi-i*
T_2 = Stock solution-01 having 5.00 gm/L of *chemi-i*
T_3 = Stock solution-01 as recommended by Murashige & Skoog (1962)
T_4 = Stock solution-01 as recommended by Murashige & Skoog (1962) but has different concentration of each component
T_5 = Stock solution-01 having 1.00 mg/L of *Chemi-ii*
T_6 = Stock solution-01 having 5.00 gm/L of *Chemi-ii*
T_7 = Stock solution-01 having 1.00 gm/L of *chemi-iii*
T_8 = Stock solution-01 having 5.00 gm/L of *chemi-iii*

4.2.7 Abnormalities of explant and plantlet

Different variation and morphological abnormalities as presented in Table 9. Abnormalities and different variation was noticed on the morphology of explants as well as newly regenerated plantlet in the treatment T_1, T_2, T_5, T_6 and T_8. Shoot initiation and shape was normal in 1st week of explant inoculation. Variation was started within 2nd week of time. Some of the abnormalities of 2nd week duration were as follows- slow growth of plantlet, less root formation, week and thin plantlet. It was common for the treatment T_1, T_2, T_5, T_6 and T_8 (Plate 15). The plantlet regenerated form T_7 was thin and media was light yellow colour (Plate 16 (a) 16 (b).

It was observed that, the growth and development of plantlet was stop within 3rd week. Small leaf formation, dwarf size and contamination like structure found in the treatments T_1, T_5, and T_6 at 3rd week of regeneration (Plate 17). Black layer formation at root tip and root growth also stop. The above morphology was noticed in the treatment T_1, T_2, T_5, T_6 and T_8 (Plate 17).

The plantlet regenerated from T_7 were comparatively weak and tall but not abnormal as other treatment. The plantlet of the treatment T_1, T_2, T_5, T_6 and T_8 were started to die within 4^{th} week of time. Growth totally stop and various abnormalities were recorded of those plantlet (Plate 18).

The plantlet regenerated from T_7 were tall, thin and showing logging due to less amount of root development. The plantlet regenerated from T_4 were comparatively weak than T_3 treatment in all the period under investigation. The plantlet of check treatment (T_3) were healthy, rubust and well growth condition in all over the experimental period. Hence, it was concluded that, the media combination T_1, T_2, T_5, T_6 and T_8 were not suitable for plantlet regeneration. The compound of T_7 has some positive effect on plantlet regeneration but not as standard as check treatment (T_3).

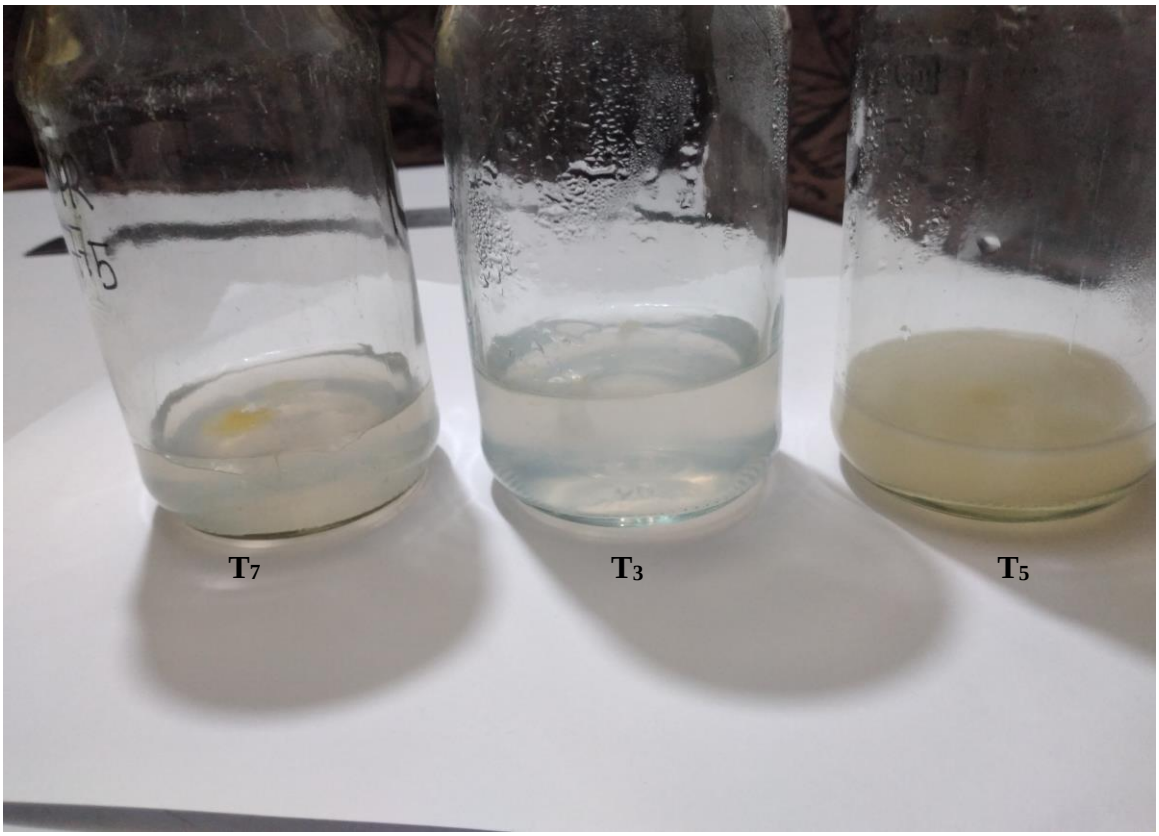


Plate 14: Normal and discolouration of media in T_3, T_5 and T_7 treatment

Plate 15: Curd colour of media in the root zone of plantlet in Asterix variety of potato with the treatments T_6 and T_7 at 3rd week after regeneration

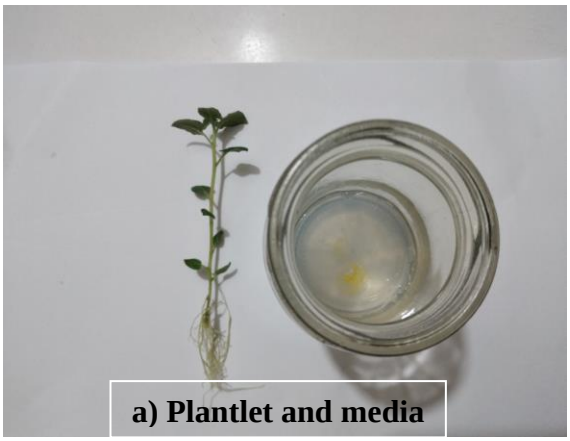
Plate 16: Abnormal plantlet and discolouration of media in the treatments T_2 or T_5 or T_6 or T_8

Table 9: Morphological appearance of explant and plantlet in different treatment for both the variety (Asterix & Diamant)

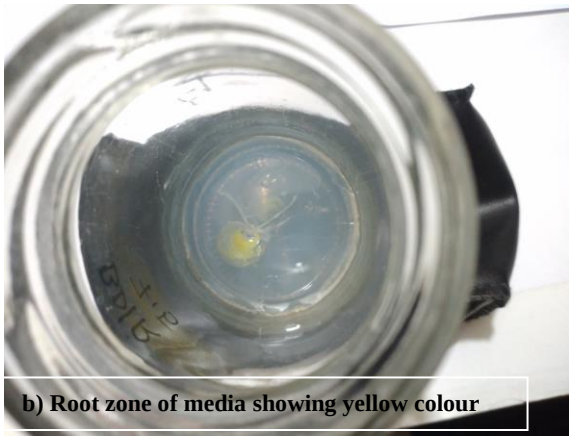
Treatment	1st week	2nd week	3rd week	4th week
T_1 T_2 T_5 T_6 T_8	Normal	1. Slow growth of plantlet 2. Less root initiation 3. Weak and thin plantlet 4. Small leaf formation	1. Growth and development stop 2. Dwarf and short plantlet 3. Brownish and abnormal plantlet development 4. Root development stop 5. Black layer formation at the root tip region 6. Root formation in the upper node	1. Deform leaf and thin plantlet 2. Growth totally stop 3. Plantlet start to die 4. Thin, weak and abnormal plantlet formation 5. Root formation in most of the upper node 6. Leaf are small in size
T_7	Normal	Normal growth	1. Tall & thin plantlet 2. Less root growth 3. Comparatively weak plantlet	1. Tall & week plantlet 2. Very thin stem 3. Root at upper node 4. Logging formation
T_3 T_4	Normal growth	Normal development	1. Robust and excellent plantlet	1. Normal growth 2. Well developed plantlet

T_1 = Stock solution-01 having 1.00 gm/L of *chemi-i*
T_2 = Stock solution-01 having 5.00 gm/L of *chemi-i*
T_3 = Stock solution-01 as recommended by Murashige & Skoog (1962)
T_4 = Stock solution-01 as recommended by Murashige & Skoog (1962) but has different concentration of each component
T_5 = Stock solution-01 having 1.00 mg/L of *Chemi-ii*
T_6 = Stock solution-01 having 5.00 gm/L of *Chemi-ii*
T_7 = Stock solution-01 having 1.00 gm/L of *chemi-iii*
T_8 = Stock solution-01 having 5.00 gm/L of *chemi-iii*

Plate 17: Abnormal plantlet regeneration in T_2 treatment of the Asterix variety of potato at 2nd week of regeneration

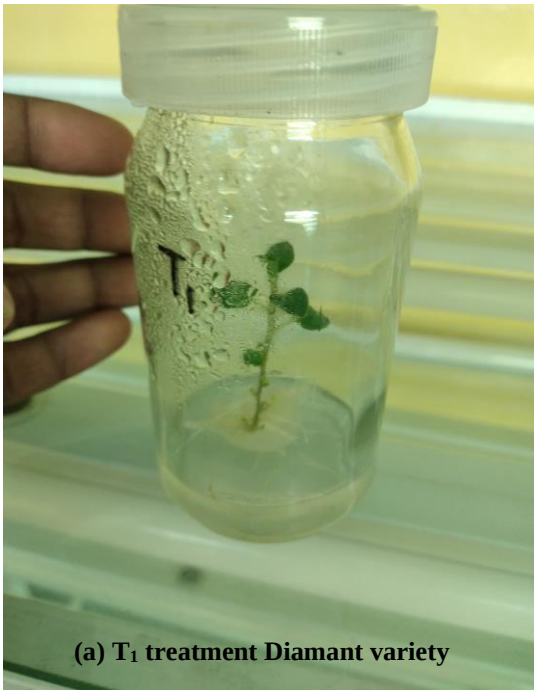


a) Plantlet and media



b) Root zone of media showing yellow colour

Plate 18: Plantlet of Diamant variety of potato in the treatment T_7 showing yellow colour in the root zone of the media



(a) T_1 treatment Diamant variety

(b) T_5 treatment Asterix variety

Plate 19: Contamination like symptom in the root zone of plantlet in the treatment T_1 at 3rd week of regeneration

(a) contaminated root zone

(b) Off white coloured media

Plate 20: Brownish and off white colour of media in the treatments T_5, T_6, and T_7

CHAPTER V

SUMMARY AND CONCLUSION

The experiment was carried out at North Bengal Agro Farms Ltd., Thakurgaon, Bangladesh, during the period of January, 2020 to September, 2020. Tissue culture medium is consist of more than 17 different plant nutrients. Those are categories on major salt, minor salt, vitamin, hormone and others. Stock solution-01 has the component of major salt. The destructive chemical ammonium nitrate is one important component of stock solution-01. The key objective of this experiment is to find out any alternate of ammonium nitrate for the preparation of stock solution-01. Three alternate chemicals were used for the preparation of stock solution -01. Eight different treatments were designed with three chemicals by applying different concentration and combination for the preparation of Stock solution-01. Two potato varieties viz. Diamant and Asterix were used for large scale regeneration in new tissue culture media under two sub-experiments. The major findings of the each experiment was given below.

Sub-experiment-1: Plantlet regeneration in Diamant variety of potato using new stock solution-1.

Significant difference was notice among the treatments. The highest (6.00, 8.56 and 12.17) number of node was found in the treatment-07 at 2^{nd}, 3^{rd} and 4^{th} week of plantlet regeneration. The second highest value (10.67) at 4^{th} week was recorded in check treatment-03. The lowest (2.33, 2.83 and 3.06) data was found in the treatment-02. Highest number of leaf (9.00, 11.67 and 12.33) was regenerated in the treatment-07 at all the experimental period. Although, significant different was observed with the treatment-03 at 2^{nd} and 3^{rd} week of regeneration but in 4^{th} week there was no significant difference among the two (T_3 and T_7) treatment. Remarkable variation was noticed for

the parameter of shoot length among the different treatments. The maximum shoot length 6.03, 10.73 and 9.43cm was generated in the treatment-07 at 2nd, 3rd and 4th week respectively. The check treatment (T_3) showed second highest data at 2nd and 3rd week of regeneration but statistically non-significant data was recorded among the treatment-03 and 07. The treatment T_7 showed similar performance with check treatment-T_3 which has the composition of ammonium nitrate. It indicated that, potato plantlet can be regenerate with another media composition in absence of ammonium nitrate. The treatment-04 which has same component of MS standard media but different concentration showed best result on root length in the plantlet of Diamant variety. The treatment T_6 and T_7 showed better responsed as compare to T_1, T_2, T_5 and T_6 treatment. Its indicated that, the *chemi-iii* is more suitable than *chemi-i* and *chemi-ii* for this trait of plantlet regeneration.

Sub-experiment-2: Plantlet regeneration in Asterix variety of potato using new stock solution -01.

The treatment-04 regenerated highest number (6.33 and 1167) of node in 2nd and 4th week of regeneration and the check treatment (T_3) gave second highest data but it was statistically non-significant with treatment-04. The maximum number of leaf (9.66, 10.67 and 12.33) were generated in check treatment-03 and it was the second highest (9.33 and 12.00) in treatment-04 for all the experimental period. The lowest number of leaf (2.66 and 2.33) was recorded in treatment-02. The highest shoot length (6.96 and 12.40 cm) was regenerated in treatment-04 at 2nd and 4th week of explant transformation to the media. The lowest shoot length (1.93, 2.16 and 2.50 cm) was found in treatment-02. The highest length of roots (5.16, 6.56 and 8.73 cm) were noticed in the treatment-04. Although, treatment made from *chemi-iii* was not perform best on plantlet regeneration in Asterix variety but it has the

capacity to regenerate plantlet in moderate level in both the varieties- Diamant and Asterix.

The newly used chemicals reacted with other components of stock solution-01 and showed some negative and toxic effect on explants, plantlet and media. The morphological change and phenotypic variation increased day by day. Media colour and physical appearance were converted to brown, off white, yellow and milky like structure in the treatment of T_1, T_2, T_5, T_6 and T_8 at 2^{nd} week of culture. The media of T_7 showed light yellow to deep yellow colour.

Huge abnormalities were recorded in 4^{th} week. Such as milk layer formation on the upper portion of media in the treatments T_1, T_2, T_3, T_5, T_6 and T_8. Curd formation and looking like contamination of media. Root of explants was not visible due to deep brown and discoloration of media. The growth and development of plantlet was stopped within 3^{rd} week. Small leaf formation, dwarf size and thin plantlet were noticed. Black layer formation at root tip and root growth also stopped. The above morphology was noticed in the treatments T_1, T_2, T_5, T_6 and T_8. The plantlet of the treatments T_1, T_2, T_5, T_6 and T_8 started to die within 4^{th} week. The plantlet regenerated from T_7 were tall, thin and showing lodging due to less root development. The plantlets regenerated from T_4 were comparatively weaker than T_3 treatment throughout the period under investigation. The plantlet of check treatment (T_3) were healthy, rubust and well growth in all over the experimental period. Hence, it was concluded that, the media combination of T_1, T_2, T_5, T_6 and T_8 were not suitable for plantlet regeneration. The composition of T_7 has some positive effect on plantlet regeneration but not as standard as check treatment (T_3).

CHAPTER VI

RECOMMENDATION

The present research finding reveals that ammonium nitrate free stock solution-01 for the preparation of tissue culture media has the ability to regenerate potato plantlet but the media has some abnormalities and has some toxic effect on plantlet. Hence, further investigation may be carried out on the following points:

1. More investigation is needed to validate the present finding of the mentioned *chemi-ii* and *chemi-iii* for preparation of stock solution-1
2. The new nitrogenous chemical can be used as a alternate of ammonium nitrate.
3. Regeneration ability of *chemi-iii* should be validated with other varieties of potato.
4. Regenerated plantlet from *chemi-iii* should be sub-cultured on the new composition of media for further validation.
5. The newly formulated stock solution-01 should be used with some other crops for *in vitro* regeneration.

CHAPTER VII

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APPENDICES

Appendix i: Analysis of variance for different traits of diamant variety of potato in second week of growth

Source of Variation	DF	Mean Squares				
		Node no.	Leaf no.	Shoot Length	Root no.	Root Length
Rep	2	0.873	0.383	0.372	1.654	0.072
Treatment	7	3.928**	9.303**	6.078	13.046**	2.774**
Error	14	0.654	0.694	0.466	1.389	0.269

Table: Coefficient of variation (CV) and standard deviation (SD) of different traits of diamant variety of potato at second week of growth.

Parameters	CV (%)
Node no.	19.25
Leaf no.	14.57
Shoot Length	16.27
Root no.	26.24
Root Length	26.18

Appendix ii: Analysis of variance for different traits of diamant variety of potato in third week of growth

Source of Variation	DF	Mean Squares				
		Node no.	Leaf no.	Shoot Length	Root no.	Root Length
Rep	2	1.675	4.868	2.304	2.792	0.388
Treatment	7	12.014**	24.520**	21.262**	21.232**	22.307**
Error	14	0.307	0.795	0.481	1.125	0.138

Table: Coefficient of variation (CV) and standard deviation (SD) of different traits of diamant variety of potato at third week of growth.

Parameters	CV (%)
Node no.	9.45
Leaf no.	11.87
Shoot Length	11.47
Root no.	19.43
Root Length	10.51

Appendix iii: Analysis of variance for different traits of diamant variety of potato in fourth week of growth

Source of Variation	DF	Mean Squares				
		Node no.	Leaf no.	Shoot Length	Root no.	Root Length
Rep	2	5.745	4.363	10.624	1.323	0.013
Treatment	7	32.042**	39.901**	18.708**	18.772**	25.247**
Error	14	0.645	0.925	2.799	0.442	0.236

Table: Coefficient of variation (CV) and standard deviation (SD) of different traits of diamant variety of potato at fourth week of growth.

Parameters	CV (%)
Node no.	11.04
Leaf no.	11.24
Shoot Length	28.23
Root no.	12.42
Root Length	13.61

Appendix iv: Analysis of variance for different traits of asterix variety of potato in second week of growth

Source of Variation	DF	Mean Squares				
		Node no.	Leaf no.	Shoot Length	Root no.	Root Length
Rep	2	0.792	0.292	0.292	0.500	0.225
Treatment	7	6.685**	23.470**	8.094**	16.708**	8.161**
Error	14	0.149	0.435	0.083	0.262	0.207

Table: Coefficient of variation (CV) and standard deviation (SD) of different traits of asterix variety of potato at second week of growth.

Parameters	CV (%)
Node no.	9.54
Leaf no.	11.55
Shoot Length	6.50
Root no.	11.07
Root Length	14.59

Appendix v: Analysis of variance for different traits of asterix variety of potato in third week of growth

Source of Variation	DF	Mean Squares				
		Node no.	Leaf no.	Shoot Length	Root no.	Root Length
Rep	2	0.760	3.792	0.594	0.073	0.038
Treatment	7	16.010**	23.661**	18.548**	32.804**	11.596
Error	14	0.332	0.125	0.284	0.335	0.117

Table: Coefficient of variation (CV) and standard deviation (SD) of different traits of asterix variety of potato at third week of growth.

Parameters	CV (%)
Node no.	**11.47**
Leaf no.	5.62
Shoot Length	10.15
Root no.	10.93
Root Length	9.35

Appendix vi: Analysis of variance for different traits of asterix variety of potato in fourth week of growth

Source of Variation	DF	Mean Squares				
		Node no.	Leaf no.	Shoot Length	Root no.	Root Length
Rep	2	0.594	1.167	0.226	1.073	0.065
Treatment	7	34.249**	40.476**	35.486**	44.713**	25.280**
Error	14	0.356	0.405	0.117	0.240	0.093

Table: Coefficient of variation (CV) and standard deviation (SD) of different traits of asterix variety of potato at fourth week of growth.

Parameters	CV (%)
Node no.	**9.45**
Leaf no.	8.68
Shoot Length	5.22
Root no.	8.54
Root Length	6.72

Appendix vii: Murashige & Skoog (1962) tissue culture medium composition

Components	Standard mg/L	Actual weight (gm)	Final volume of stock (mL)	Volume of the stock per litre of medium (mL)
Macroelements				
NH_4NO_3	1650	33	1000	50
KNO_3	1900	38		
$CaCl_2 \cdot 2H_2O$[a]	330	6.6		
$MgSO_4 \cdot 7H_2O$	370	7.4		
KH_2PO_4	170	3.4		
$FeSO_4 \cdot 7H_2O$[b]	27.90	1.395	500	10
Na_2EDTA[b]	37.30	1.865		
Microelements				
H_3BO_3	6.20	0.31	500	10
$MnSO_4 \cdot 4H_2O$	22.30	1.115		
KI	0.83	0.0415		
$ZnSO_4 \cdot 7H_2O$	8.60	0.43		
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.0125		
$CoCl_2 \cdot 6H_2O$	0.025	0.00125		
$CuSO_4$ $5H_2O$	0.025	0.00125		
Organic components				
Nicotinic acid	0.5	0.025	500	10
Pyridoxine–HCl	0.5	0.025		
Thiamine–HCl	0.1	0.005		
Glycine	2.0	0.1		
Myo-Inositol	100	5.0		
Sucrose	20,000–30,000	20–30	Added directly	
Agar[c]	7000–8000	7–8	Added directly	
pH	5.8–5.9			