IDENTIFICATION OF A NEW CHEMICAL AS A SUBSTITUTE OF AMMONIUM NITRATE (NH_4NO_3) IN MS MEDIA PREPARATION FOR *IN VITRO* REGENERATION OF POTATO (*Solanum tuberosum* L.)

BY

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This Is To Certify That The Thesis Entitled ***"Identification of A New Chemical as a Substitute of Ammonium Nitrate (NH_4NO_3) in MS Media Preparation for In Vitro Regeneration of Potato (Solanum tuberosum L.)"*** *Submitted To The Department Of Biotechnology, Sher-E-Bangla Agricultural University, Dhaka, In Partial Fulfillment Of The Requirements For The Degree Of Master Of Science In Biotechnology Under Agriculture Faculty, Embodies The Results Of A Piece Of Bonafide Research Work Carried Out By* ***MD. ABUL BASHAR,*** *Registration No.* ***09-03494*** *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.

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Dedicated to

My
Beloved Parents and
Family

ABBREVIATIONS AND ACRONYMS

Agril.	:	Agriculture
Biol.	:	Biological
Cm	:	Centimeter
CRD	:	Completely Randomized Design
DMRT	:	Duncan's Multiple Range Test
Conc.	:	Concentration
DAI	:	Days After Inoculation
DAS	:	Days After Sub-culture
et al	:	And others (at elli)
FAO	:	Food and Agricultural Organization
Fig	:	Figure
g/L	:	Gram per litre
BAP	:	6-BenzylAminoPurine
BA	:	Benzyladenine
KIN	:	Kinetine
IAA	:	Indoleacetic acid
IBA	:	Indolebutyric acid
NAA	:	*a*-Napthalene aceticacid
2, 4-D	:	2,4- Dichloro phenoxy acetic acid
Int.	:	International
J.	:	Journal
Mol.	:	Molecular
mg/L	:	Milligram per litre
µM	:	Micromole
MS	:	Murashige and Skoog
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
CV	:	Co-efficient of Variation
^{o}C	:	Degree Celsius
etc.	:	Etcetera
WAI	:	Weeks After Inoculation

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The Author

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BY

MD. ABUL BASHAR[2]

ABSTRACT

An experiment was undertaken at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh during the period of January, 2015 to June, 2016 to identify a new chemical for the substitute of explosive chemical Ammonium nitrate (NH_4NO_3) in MS media composition for *in vitro* regeneration of potato (*Solanum tuberosum* L.). Three potato varieties *viz* Diamant, Cardinal and Asterix were used as an experimental materials. The new chemical was denoted as β chemical for the privacy of the experimental findings. Different concentrations (1, 5. 10, 15 and 20 gmL^{-1}) of β chemical were used for the preparation of stock solution- A, which was finally used for MS media preparation. It was noticed that *in vitro* regeneration of potato has been successfully done with new composition of β chemical in MS media preparation. The shoot length, node number, number of leaf, root length were highest in 5 gmL^{-1} of β chemical at 14, 21 and 30 days after sub-culture (DAS). Although, in some parameters liquid MS standard dose (16.5 gmL^{-1}) gave maximum result but it was statistically non significant with 5 gmL^{-1} of β chemical. All the plantlets were died at higher concentration (15 gmL^{-1} and 20 gmL^{-1}) of β chemical. It may be due to toxic effect of the chemical. The variety Diamant showed best performance in all the traits under studied. It is reveal that ready made MS powder, Liquid MS standard dose and 1 gmL^{-1} of β chemical showed comparatively similar result on most of the parameters under studied. Among the varieties, the Asterix variety showed minimal performance in most of the phenotypic characters under studied. In all the case, Diamant variety showed best performance for all the phenotypic characters under studied. The newly used β chemical is cheap, non explosive and environmental friendly which can be an alternative of destructive chemical Ammonium nitrate (NH_4NO_3) for MS media preparation.

Key words: Ammonium nitrate, new chemical, in vitro, regeneration, potato

1. A thesis paper title presented at research work for thesis for partial fulfillment of MS degree.

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CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important root vegetable crops under the solanaceae family which is cultivated more than 140 countries around the world. It is the fourth most cultivated food crop after wheat, rice and maize (Moeinil *et al.,* 2011). It is originated in South America in sixteenth century, Spanish explorers introduced it in Europe and later it became an important food crop of the world (Khoso, 1988). It is used as a staple food in many countries all over the world but mainly used as a vegetable crop in Bangladesh (Hussain, 1995). The world potato sector is increasing day by day. The world total potato production is estimated at 384,074,114 tones in 2016 (FAOSTAT, 2016). In Bangladesh, 4.75 million hectares of land were cultivated and total production has been estimated at 9,470,000 metric tones during 2016 (BBS, 2016). Although it is contributing a major portion to our vegetable, but its yield is low in Bangladesh (19.07 t/ha) compared with other leading countries such as Japan (41.3 t/ha), Netherlands (42.99 t/ha), USA (38.40 t/ha) and UK (39.64 t/ha) (FAO, 2016). Potato contains starch, vitamins C and B, and minerals. It provides about 20.6% carbohydrates, 2.1% protein, 0.3% fat, 1.1% crude fiber and 0.9% ash. It contains a good amount of essential amino acids like leucine, tryptopan and isoleucine (Khurana and Naik, 2003). Potato also contains a variety of phytonutrients that have antioxidant activity (Steven, 1999; Karim *et al*., 2010).

There are lots of reasons for low yield in potato production such as, use of poor quality seed tubers, inefficient management practices, lack of improved varieties, high post harvest spoliage, fluctuating price of potato in domestic and export market, limited storage facility etc. However, pests, diseases and environmental factors affect the crop from reaching its maximum agricultural potential. Viral diseases have been attributed as the main cause of low yield productivity and the major cause of cultivar decline (Wambugu, 1991).

Potato varieties grown in Bangladesh may be divided into two groups namely, the local or indigenous and the high yielding or modern variety. In the last few decades, several dozens of high yielding varieties (HYV) of potato were brought to Bangladesh and tried experimentally under local conditions before being recommended for general cultivation (Islam *et al.*, 2003). Most of the varieties of potato have been developed through ordinary selection and by conventional breeding which are very prolonged procedure. The regeneration of plants from cell or tissue or other organs culture represent an essential

component of biotechnology and have the potentiality not only to improve the existing cultivars, but also for the generation of novel plants in a comparatively short time compared to conventional breeding process (Khadiga *et al*., 2009). Micro-propagation is used as standard methodology for production of disease free seed potatoes (Hoque *et al*., 2010). Potato is susceptible to several fungus, virus and bacterial diseases and cause heavy economic loss every year during the cultivation and storage period. Researchers showed that viruses can destroy the yield by 40% singly and 90% loss in combination with other viruses (Sidikou *et al*., 2003). Plant regeneration in potato has been progressed a lot in recent years (Shirin *et al.,* 2007). Successful *in vitro* plant regeneration has been achieved from explants of different organs and tissues of potato such as leaf, stem, shoot, node (Garcia and Martinez, 1995; Haque *et al*., 1996), tuber discs and unripe zygotic embryos (Pretova and Dedicova, 1992).

Tissue culture technique has great potentiality which provides quick means of vegetative propagation in potato. It can produces thousands of plants in a year. *In vitro* regeneration of potato has been reported from different explants on readymade MS medium. Modifications of MS medium composition and growth regulators for diseases free good quality plantlets. Production also reported by several workers (Hossain, 1994; Rabbani *et al*., 2001; Zaman *et al*., 2001). Under Bangladesh condition, very few reports are available regarding the effect of varietal and modifications of MS medium composition on plantlets multiplication under *in vitro* condition.

The purpose of the present research is to provide the information on high frequency plantlet production to meet the producer demand as well as morphological performances of different varieties under different modification of MS media composition. *In vitro* propagation for large scale cultivation of econmically important crops like potato, banana, pineapple, stevia, gerbera, chrysenthimum, orchid, flowers, fruits crops etc are commercially practiced in all over the world. Diseases free and quality plantlets production of potato through tissue culture technology has been adopted in our country in last 4 decade. At present, more than 50 different private tissue culture labs has been established in different corner of Bangladesh. Those labs have been successfully producing potato plantlets in every year to fulfil their business objectives. Govt. organizations *viz.* Bangladesh Agricultural Research Institute (BARI), Bangladesh Agricultural Development Corporation (BADC), Rural Development Academy (RDA) also have tissue culture labouratories and they are producing potato plantlets since last 20 years (Hashem *et al.,* 1990). In our country, some universities *viz.* Dhaka University (DU), Bangladesh Agricultural University (BAU), Sher-e-Bangla Agricultural University (SAU),

Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) have been now producing microplants and microtubers for the national seed production program. Some NGOs and Private Companies like Bangladesh Rural Advancement Committee (BRAC), PROSHIKA, North Bengal Agro Farms Ltd., Supreme Seed ltd., Krishibid Seed Ltd., Square Agro Ltd., Lal Teer Seed Ltd. etc. are also producing plantlets and microtubers for large scale cultivation of potato.

Preparation of culture media foundation for any tissue culture work. At least, 17 different macro and micro plant nutrients are used for the preparation of culture media. Most widely used MS media (1962) composition of nutrients is used for rapid micropropagation and meristem culture technique. The recommended dose of nutrient given by Murashige & skoog (1962) has been successfully used for last 50-60 year. Ammonium Nitrate (NH_4NO_3) is an important chemical used as macro nutrient in MS media preparation. The amount of ammonium nitrate per litre is 16.50 gm. It is rich in nitrogen. Near about 35% N present in ammonium nitrate. It is a good source for supply of nitrogen in culture media. But extremely sorry to say that, it has a great disadvantage in human civilization. Ammonium nitrate is an explosive chemical. Heating or ignition causes ammonium nitrate to explode. It is used an oxidizing agent in explosive. It is used for the production of bomb and in many other destractive activities. Melting point of NH_4NO_3 is about 170^0C and it decomposes when heated to about 210^0C. 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. Terrorism is a great problem in our country as well as all over the word. The present Govt. has taken strong initiative to control the terrorism within the country. The Govt. decided to control and check all the raw materials used for the production of bomb or any other destractive materials.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 on going tissue culture program. They used ready made MS powder which is manufactured in abroad. Some of the renowned companies are Duchefa (Netherland), Sigma (USA, Germany), SRL (India) etc. Those ready made MS media are very expensive. For example, 220gm MS powder made by Duchefa company is 8000 taka by which maximum 50 litre MS solution can be prepared. Other company rates price are higher than the mentioned price. 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 practicsed for last 30-40 years in our country. This long time adopted technology is tremendously hampared due to non availability of ammonium nitrate (NH_4NO_3). Hence, an idea was came in mind of our honourable sir Dr. Md. Ekramul Hoque, Professor, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh to develop any alternate chemical of ammonium nitrate (NH_4NO_3) which will be cheap, non destructive, environmental friendly and available in Bangladesh.

Considering the above facts, the present experiment has been undertaken with the following specific **objectives:**

- To find out any alternate chemical for explosive NH_4NO_3 for the preparation of stock solution-A in MS media.
- Identification of suitable concentration of new chemical for MS media preparation
- To study the effect of new chemical on *in vitro* regeneration in potato.
- To study the relative performance of different varieties by the use of new chemical.

CHAPTER II

REVIEW OF LITERATURE

Potato which Bangla name is “Alu” is one of the most leading staple food and most important vegetable crops throughout the Bangladesh as well as all over the whole world. In most cases potato is propagated by using seed tubers. But most of the countries of the world are now applying tissue culture and genetic engineering techniques for the development of this necessary vegetable. Now-a-days many Bangladeshi scientists have carried out their research work in plant genetic engineering to open a new avenue for the betterment of crop. The present study was undertaken to provide information on high frequency of plantlets multiplication in different parameters of three renowned varieties of potato using ready MS powder & development of new MS media composition through *in vitro* regeneration. The literatures, which are most relevant to the present study are reviewed here under the following headings:-

2.1 Concept of tissue culture

This review was prepared as a survey of key articles presenting development, achievements and interconnection of various lines of potato biotechnology research united through the common use of *in vitro* culture techniques. 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 (Vinterhalter *et al*., 2011).

Gong *et al*. (1998) was done an experiment that the establishment of an *in vitro* plant regeneration system in sweet potato which had highly potential importance in sweet potato quality improvement. However, successful transformation cannot be achieved unless efficient plant regeneration has been established. Some plant regeneration procedures through organogenesis and somatic embryogenesis have been described for sweet potato using different explants such as meristem tips, lamina segments, stems, petioles, storage roots, anthers and ovaries. The *in vitro* regeneration of adventitious shoots is an essential base for most methods of genetic transformation (Torregrosa and Bouquet, 1996). Therefore, a protocol to maximize the regeneration of adventitious shoots must be developed before attempting biological transformation using *Agrobacterium* as a vector (Marcotrigiano *et al.,* 1996).

2.2 Concept of potato tissue culture

Propagated by tubers that are used by farmers is known as a conventional method. Potato cultivation with seed tuber is constrained by the accumulation of pathogen, physiological decline and low multiplication rates. Seed tuber is most expensive in potato production. At least 35-40% total cost of potato production is covered by seed tuber and it is very lengthy process. Nowadays plant cell tissue culture techniques are being applied for rapid multiplication of plantlets production of potato. 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 diseases free potato clones combined with conventional multiplication methods has become an integral part of seed production in many countries (Naik and Sarkar, 2000). Callus is an amorphous mass of loosely arranged thin walled undifferentiated parenchyma cells arising from the proliferating cells of parent tissue (Dodds and Roberts, 1990). In plant biology, callus cells are those cells that cover a plant wound. (Murashige and skoog, 1962) reported that the nutritional requirement for optimal growth of *in vitro* tissues may vary with varieties. To induce callus formation, plant tissues are surface sterilized and then plated onto *in vitro* tissue culture medium. Plant hormones such as auxines, cytokinins, gibberellins are supplemented into the medium to initiate callus formation or somatic embryogenesis.

2.3 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 in combinations with BA and NAA in combination with BA for callus induction. The concentration of sucrose was 3% W/V level and the pH of the media was adjusted to 5.7 before the addition of agar 8% W/V. The explants were first dissected out aseptically and then inoculated to the media (with various levels of hormones), then incubated at 27±2ºC in the culture room. 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-Dalone at 3.0 mg L-1. Also NAA and BA with different concentrations produced considerable degrees of callus but the degree of callus was best at

higher concentrations of NAA and BA. 2, 4-D in combination with BA at 2.0 mg L-1 both produced considerable amount of callus. In case of NAA in combination with BA the degree of callus formation was best at concentration 1.0 mg L-1each. So according to the above findings it was concluded that 2,4-D is the best option for induction of callus among the other hormones used in the study. (Khalafalla *et al*., 2010) reported the procedure of plant regeneration from callus culture of potato (*Solanum tuberosum* L.). Calli were induced from 1.0 cm^2 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. Calli were differentiated into shoot-primordia when subcultured on MS medium supplemented with 1.5-5.0 mgL^{-1} of thidiazuron (TDZ) and 2.0-5.0 mgL^{-1} of benzyladenine (BA).

The best result for number of shoot per callus (3.3 ± 0.3) and longest shoot (0.8 ± 0.1) were obtained by using TDZ at 5.0 mgL^{-1} 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 arapid 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. Among the three different explants (nodal segment, sprout and shoot apex) nodal cutting showed the best performance on days to microtuber formation and average weight of microtuber. MS + 6%sucrose + 4 mgL^{-1} kinetin combination of treatment was the best for *in vitro* tuberization among the parameters under study. *In vitro* response and its relationship with different varieties, explants and medium were investigated in potato (*Solanum tuberosum* L.) by (Hossain and Sultana, 2005). Direct *In vitro* regeneration protocol from diverse explant source is a prerequisite for transformation studies. 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. Basal media was Murashige & Skoog and different hormonal combinations of benzyl

adenine and indole acetic acid were supplemented. Statistical analysis showed that explant source had significant effect on direct regeneration and the nodal explants had maximum regeneration. The number of shoots obtained from node was 17.6 from Cardinal followed by Diamont 14.3 and Altamash 9.0. Shoot apices also resulted in shoot regeneration comparatively better than leaf discs and internodal explants but lesser than from nodes. Most suitable medium was MS with 2.0 mgL^{-1} BAP and IAA @ 0.5 mgL^{-1} giving maximum regeneration. It was also observed that interaction of cultivars with explant and media is highly significant at 0.01 % Level of significance.

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). The basal medium consisted of MS salts and vitamins supplemented with 3.0 mgL^{-1} BA, 1.0 mgL^{-1} gibberellic acid, 30 gmL^{-1} sucrose and 7.0 gmL^{-1} phyto-agar. Two full set repeat and one partial set repeat of independent experiments was conducted and all produced similar results. Silver thiosulfate decreased the regeneration frequency and number of shoots per callus. No significant changes were observed with thidiazuron. Regeneration rate of 100% with up to 20 shoots/plantlet per callus was achieved at 2.0 mgL^{-1} IAA with cultivars Desiree, Kennebec, Niska and Lenape (Lowe et al., 1992). These cultivars still showed high regeneration rate (87-98%) on medium with 0.5 mgL^{-1} IAA and good regeneration rates were also achieved by the other three cultivars (48, 50 and 94% for Chieftain, Shepody and Russet Burbank, respectively). With the single medium protocol (0.5% IAA without thiosulfate or thidiazuron), lenape and Niska exhibited a regeneration rate of 98%). 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^{-1}NAA, 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.

2.4 *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 intermodal segment 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. This 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 with 2.5 mgL^{-1} NAA + 2 mgL^{-1} BAP among the combinations. It was also observed that callus was derived from leaf plantlets in a shortest period of time (23.68 days) compared to that from stem (28.96 days).

2.5 Effect of media consistence on micropropagation

In vitro propagation of potato by the serial culture of axillary shoots on separated nodes has been reported by a number of researchers, and since 1950 has become established as an effective mean of rapidly multiplying new or existing cultivars in disease-free conditions (Hussey and Stacey, 1984).

2.6 Effect of silver nitrate on micropropagation

To remove ethylene from potato culture vessels, forced ventilation and the use of some chemical compounds have been reported. Among the different chemicals, AgNO3 has been widely and in most cases the most successfully used one. AgNO3 was also used in order to reduce the occurrence of hyperhydricity in tissue culture of sunflower (Mayor *et al.*, 2003). A major drawback to the *in vitro* propagation systems is that the potato plants are highly sensitive to ethylene, and ethylene accumulation *in vitro* strongly inhibits the growth and development of shoots. It is known that growth of potato plantlets can be distorted by concentrations of ethylene of 0.1 ml or even less (Jackson *et al*., 1987; Sung and Huang, 2000). (Fuentes *et al.*, 2000) demonstrated that the addition of AgNO3 promoted only small modifications of the ionic equilibrium of the medium. This suggests that the effects of this compound are not attributable to any substantial modifications in the levels of available nutrients. However, the mechanism by which AgNO3 can affect potato plantlets development is difficult to elucidate from an experiment of this type.

(Hussey and Stacey, 1981) reported that potato shoots become stoloniferous in tightly closed culture vessels. (Jackson *et al*., 1991) found that shoot height of *Solanum tuberosum* was 64% of that of the control after 14 days of culture in tightly-sealed vessels. They also concluded that accumulated ethylene is responsible for these effects.

Following the recommendations from (Hussey and Stacey, 1981); (Jackson *et al*., 1987); (Sung and Huang, 2000); (Zhang *et al*., 2006) all our micro propagation experiments were established under diffuse ventilation conditions and we tested the effect of AgNO3 added to MS (1962) semi-solid medium. In our hands, after eight weeks of culture, Granola and Arbolona negra plantlets growing on MS medium supplemented with 2 mgL^{-1}-1 AgNO3 show an adequate stem length and a highest leaf area; these plants did not show symptoms of ethylene growth inhibition: epinasty or hiperhidricity.

2.7 Effect of Growth Regulators

(Molla *et al.,* 2011) carried out an experiment 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.

(Rahman *et al.,* 2010) studied on the regeneration ability via callus induction using leaf disc of five tobacco varieties. Explants were cultured on MS medium supplement with different concentrations and combinations of plant grouth regulators. Among the varieties used, the highest percentage of callus induced in 2.0 mgL^{-1} Kinetin and 2.0 mgL^{-1} IAA. Shoots were induced from calli cultured on the same medium. Maximum shoot formation from leaf discs was on medium 2.0 mgL^{-1} Kinetin and 2.0 mgL^{-1} IAA. (Rahman *et al.,* 2010). (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 GA_3 used and no organogenesis was recorded in the basal MS media i.e., T_0 media indicated that both the varieties exhibited fairly high direct plantlet

regeneration, when internodes explants were cultured on T_2 medium i.e. MS+GA_3 (1.0 mgL^{-1}) + IAA (0.01 mgL^{-1})+ Zeatin (2.0 mgL^{-1}). 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 (T_0) i.e.2 and1. Moreover, either increase or decrease in the concentrations of growth regulators declined number of shoots. The replacement of IAA with another auxin i.e. NAA and replacement of zeatin with another cytokinin i.e. BAP increased the number of shoots considerably in both the cultivars in comparison to control. After replacement the growth regulators maximum number of shoot was observed in T_6 but the number was less than T_2 treatment. 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).

This data shows that for direct shoot regeneration IAA and Zeatin was more effective than NAA and BAP. Further, increase in the concentration of IAA i.e. up to 0.015 mgL^{-1} and zeatin up to 3.0 mgL^{-1} was not effective for direct shoot regeneration from internodes. Cytokinins at high concentrations increased chlorophyll content and compactness of the tissues. The higher levels of cytokinins might have resulted in chlorophyll development rather than shoot regeneration.

2.8 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 represent the number of roots per explant were obtained on all tested media compositions except in T_0 which was lack of plant growth hormones. The highest number of roots i.e., 28 per explant was recorded in variety on media composition in which MS media was supplemented with GA_3 (1.0 mgL^{-1})+IAA (0.01 mgL^{-1})+ Zeatin (2.0 mgL^{-1}). Variety K. CH_3 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 T_0 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.

2.9 Effect of Growth Regulators on Shoot induction

The provision of a steady supply of indexed planting materials through *in vitro* culture appears economically and technologically feasible. Although, there are many reports on potato micropropagation (Yousef *et al*., 2011); (Badoni and Chauhan, 2009); (Rahman *et al.,* 2010). It is a well known fact that the regeneration potential of micropropagated plants is genotype dependent (Abe and Futsuhara, 1986). However, 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 micropropagation.

(Modarres and Moeini, 2003) conducted an experiment with solid MS medium with 0.25 mgL^{-1} of GA_3 and 0.01 mgL^{-1} of NAA where he observed significant variation between different P^H levels in respect of it's ability to induction of rooting and shooting in plantlets regenerated from the single node of two potato varieties after subjecting them with thermotherapy. Overall P^H 5.5 was the best for all the traits. Low and high level of P^H from that 5.5 were found to reduce the growth and rooting of single nodes. The reduction was more pronounced at low levels than high levels P^H.

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} GA_3. Between two varieties, namely Lal Pakri and Jam Alu, the former 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+GA_3 (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. Similarly, plantlet height was observed maximum on T_2 medium i.e 8.4 in K. CH_3 Cultivar and 7.9 in K. Jyoti. K. CH_3 consistently responded the best to the all studied growth traits on T_2. Although K. Jyoti also revealed same pattern to growth parameters but lesser than K. CH_3. Genotypes were found detrimental for *in vitro* growth responses. (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 GA_3 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 GA_3 was applied. The number of nodes was no significantly by any of GA_3 concentrations used. 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} GA_3 /L. Highest number of shoot (3.11) was obtained with the high concentration of GA_3. 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 GA_3 for two weeks and then transferred to shooting medium with 2.5 mgL^{-1} ZR (Zel *et al*., 1999).

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.

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.

CHAPTER III
MATERIALS AND METHODS

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 in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU) Sher e-Bangla Nagar, Dhaka-1207 from the period of January, 2015 to June, 2016.

3.2 Experimental materials

Potato is a model plant for tissue culture research. Node & shoot tip of potato was used as a experimental material for *in vitro* regeneration of potato. Three potato varieties *viz* Cardinal, Diamont and Asterix were used for this experiment. All the materials were collected from Tuber Crop Research Center (TCRC), Bangladesh Agricultural Research Institute (BARI), Gazipur. Data were recorded on number of node, length of shoot (cm), number of shoot, number of leaf, number of root and length of root (cm) per plantlet. Observation was done at 7, 14and 21& 30 days after transplant of explant in media.

3.3 Experimental Procedure

As a substitute of NH_4NO_3 we used a new chemical. The name, formula of the chemical is secrete for all upto patenting this technology. For the presentation of the result we denoted the name of the β chemical as chemical. The whole thesis it will be known as β chemical.
Two sub-experiments were carried out to fulfill the mentioned objectives:

Sub-Experiment I: *In vitro* plantlets regeneration of potato varieties using β chemical as a substitute of NH_4NO_3

Sub-Experiment II : Comparative performance of different formulations of MS media for *in vitro* regeneration of potato

3.4 Laboratory preparation

Laboratory preparation was started in January 2015 by collecting chemical and instruments and presented in Table 1.

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

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

3.5 Mathametical calculation for initiation of the new experiment

NH_4NO_3 supplies "N" (Nitrogen) in culture media which is uptaken by explant as NH_4^+ form. Hence, amount of "N" need to be calculated in NH_4NO_3.
It can be done by the following way:

Formula of ammonium nitrate is NH_4NO_3

Molecular weight of ammonium nitrate= 14+1×4+14+16×3 = 80

Hence,

80 gm NH_4NO_3 containd = 28 gm N

100 " " " $\frac{28 \text{ X } 100}{80}$

= 35 gm

Standard dose of NH_4NO_3 in one litre medium is = 16.50 gm/L

Hence, amount "N" is present in 16.50 gm of NH_4NO_3 is needed to be measused to findout. It can be done by following way.

100 gm NH_4NO_3 contains=34 gm N (nitrogen)

16.50 " " $\frac{35X16.50}{100}$

= 5.775 gm

=5.78 gm

i.e. Ammonium nitrate supplieds 5.78 gm N in one litre of medium.

Our target is to use more or less equal amount of "N" (nitrogen) which is supplied by ammonium nitrate in the medium. Hence, we make the calculation for β chemical as followes. The newly used β chemical has 46% Nitrogen (N)

46 gm Nitrogen present in =100 gm of β chemical

5.78 " " $\frac{100X5.78}{46}$

=12.565 gm = 12.57 gm of β chemical

It seems that, 12.57 gm of β chemical is needed for 1 litre of MS medium preparation.Now, we have an idea to design an experiment with new β chemical.

3.6 Stock solutions preparation

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

All the chemicals used for stock solution is highly purified and labeled as plant tissue culture tested grade. The chemicals are dissolved in double distilled water or highly purified de-ionized water. Each chemical are added according to the list of ingredient presented in Appendix-1

3.6. 1 Macronutrients stock solution (stock A)

3.6.1. 1 Stock solution –A for MS liquid (MSL) medium

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

3.6.1.2 Modification of MS medium by β chemical

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

3.6.1.2. I Modification –1 of stock –A solution

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

3.6.1. 2. II Modification –2 of stock –A solution

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

3.6.1. 2. III Modification –3 of stock –A solution

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

3.6.1. 2. IV Modification –4 of stock –A solution

The modification-4 of stock solution-I is same as standard MSL. Here we used 15 gmL^{-1} β chemical for replacement of NH_4NO_3.

3.6.1.2.V Modification –5 of stock –A solution

The modification-5 of stock solution-A is same as MSL. Here we changed the concentration of β chemical as 20 gmL^{-1} as a substitute of NH_4NO_3

3.6.2 Micronutrients stock solution (stock B)

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 C)

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

3.6.4 Vitamins stock solution (stock D)

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

3.7 The treatment combinations of sub-experiment –I

3.7.1 Sub-experiment I: *In vitro* plantlets regeneration of potato varieties using β chemical as a substitute of NH_4NO_3

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

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

T_1= 16.50 gm/L of ammonium nitrate (MS standard dose in stock solution-A)
T_2= 1 gm of β chemical/litre in stock solution- A
T_3= 5 gm of β chemical/litre in stock solution- A
T_4=10 gm of β chemical/litre in stock solution- A
T_5=15 gm of β chemical/litre in stock solution- A
T_6=20 gm of β chemical/litre in stock solution- A

3.8 The treatment combinations of sub-experiment -II

3.8.1 Sub-experiment II : Comparative performance of different MS media for *in vitro* regeneration of potato

T_1= Ready made MS powder (Dutchefa, Netherland)
T_2= 16.50 gm/L of ammonium nitrate (MS standard dose in liquid)
T_3= 1 gm of β chemical/Litre (Newly used β chemical in MS media)
T_4= 5 gm of β chemical/Litre (Newly used β chemical in MS media)

3.9 Different Stock solutions for MS media preparation

Plate 1: Standard dose of Stock solution-A (16.50 gmL^{-1} of NH_4NO_3) and other stock solution B, C, D and E for MS media preparation

Plate 2: Modification -1 of Stock solution-A (1 gmL^{-1} of β chemical) and other stock solution B, C, D and E for MS media preparation

Plate 3: Modification -2 of Stock solution-A (5 gmL^{-1} of β chemical) and other stock solution B, C, D and E for MS media preparation

Plate 4: Modification -3 of Stock solution-A (10 gmL^{-1} of β chemical) and other stock solution B, C, D and E for MS media preparation

Plate 5: Modification -4 of Stock solution-A (15 gmL^{-1} of β chemical) and other stock solution B, C, D and E for MS media preparation

Plate 6: Modification -5 of Stock solution-A (20 gmL^{-1} of β chemical) and other stock solution B, C, D and E for MS media preparation

Plate 8: Measuring composition of different stock solutions

3.10 Other stock solutions preparation

3.10.1 Preparation of IN NaOH

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

3.10.2 Preparation of IN HCl

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

3.10.3 Preparation of 70% Ethanol

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

3.10.4 Preparation of 10% NaOCl

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

3.10.5 Preparation of 5% NaOCl

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

3.11 MS Media preparation from ready made MS powder

To prepared one liter of MS medium, the following steps were followed:

1. 700 ml double distilled water was taken into 1000 ml beaker
2. 5 gm of MS powder and 30 gm of sucrose was added and gently stirred to dissolved ingredients completely with the help of a hot plate magnetic stirrer.
3. The whole mixture was then made up to 1 liter with further addition of double distilled water.
4. pH of the medium was adjusted to 5.80±0.1 by pH meter with the addition of 1 N NaOH or 0.1 N HCl whichever was necessary.
5. Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar

3.12 MS Media preparation from Stock Solution

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

1. 700 ml double distilled water was taken into 1000 ml beaker
2. 100 mL of Stock solution- A (MSL-A) 10 mL of stock solution- B,10 mL of stock solution- C,10 mL of stock solution- D + E 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 double distilled water.
4. pH of the medium was adjusted to 5.80±0.1 by pH meter with the addition of 1 N NaOH or 0.1 N HCl whichever was necessary.

5. Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.
6. According to treatment the modification of stock solution-A were taken.

3.13 Agar

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

3.14. Sterilization

3.14.1 Sterilization of culture media

One liter of MS medium were divided into two 1 liter conical flasks and capped with aluminium foil. Then the conical flasks were autoclaved at 15 psi pressure at 121^0C for 20 minutes. The medium was then transfer into the culture room and cooled at 24^0C temperature. The media was aliquot fixed volume into culture vial. After dispensing the vial were covered with thin polythene cap and marked with different codes with the help of a permanent glass marker to indicate specific treatment.

3.14.2 Sterilization of glassware and instruments

All types of glassware instrument was washed properly by liquid detergent, cleaned with running tap water and finally washed with distilled water and dried in automatic drying oven. Glassware, culture vessels, beakers, petridishes, pipettes, slides, plastic caps, other instruments such as forceps, needles, scissor, spatula, surgical blades, brush, cotton, instrument stand were sterilized in an autoclave at a temperature of 121°C for 20 minutes at 15 psi pressure.

3.14.3 Sterilization of culture room and transfer area

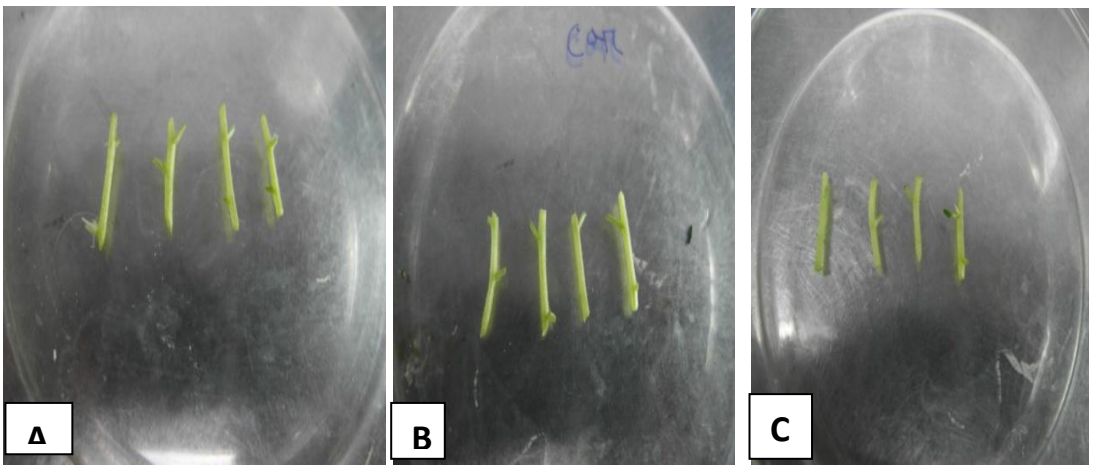
At the beginning, the culture room was spray with formaldehyde and then the room was kept closed for one day. Then the room was cleaned through gently washing the floors walls and rakes with a detergent. This is followed by careful wiping them with 70% ethanol. This process of sterilization of culture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

3.15 Sterilization of Laminar Air Flow Cabinet

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

3.16 Preparation of explants

The sprouts of potato were used as explants. The sprouts were separated from the potato and washed thoroughly with double distilled water into laminar airflow cabinet for surface sterilization, potato sprouts were first sterilized with 70% (v/v) ethanol for one minute. The sprouts were then rinsed twice with sterile distilled water. Afterwards the sprouts were surface sterilized by immersing in 0.1% $HgCl_2$ solution containing three drops of tween-20 solution and then finally rinsed and washed four times with sterilized distilled water. The surface sterilized disinfected sprouts were then cut into small segments and kept under sterilized distilled water into sterilized petridishes to make the sprout alive (Plate 9). Then the explants were ready for inoculation.



A. Diamant **B. Cardinal** **C. Asterix**

Plate 9. Explants preparation for *in vitro* regeneration of potato

Plate 10: Sub-culturing & transferring Inoculation of explants in culture media

3.17 Inoculation of culture

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

3.18 Sub-culture of the regenerated shoot for shoot induction

The regenerated plantlets were sub-cultured after 4 week of inoculation. The shoot was cut into small pieces and placed on prepared sterilized MS medium. The sub-cultured vials were then inoculated at 25 ± 1^{0}C with 16 h photoperiod. Repeated subculture was attended at regular interval of 28 days. The observations and data collection were noted regularly.

3.19 Subculture of the regenerated shoot for root induction

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

3.20 Experimental design

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

3.21 Data collection

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

3.21.1 Length of shoot

The length of shoot was recorded by using a plastic scale in laminar airflow cabinet at 7, 14, 21 & 30 DAS from each subculture.

3.21.2 Number of shoot

Total number of shoot was recorded by visual observation at 7, 14, 21 & 30 days after sub-culture (DAS) from each subculture. The mean value of the data provided the number of shoot.

3.21.3 Number of node

Total number of internode was recorded by visual observation at 7, 14, 21 & 30 days after sub-culture (DAS) from each subculture. The mean value of the data provided the number of internode.

3.21.4 Number of leaves per plant

Total number of leaves per plant was recorded by visual observation at 7, 14, 21 & 30 days after sub-culture (DAS) from each subculture.

3.21.5 Length of root

The length of root was recorded by using a plastic scale in laminar airflow cabinet at 7, 14, 21 & 30 DAI days after sub-culture (DAS).

3.21.6 Number of root

Total number of root was recorded by visual observation at 7, 14, 21 & 30 days after sub-culture (DAS) from each subculture. The mean value of the data provided the number of root.

3.22 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 1% level of probability. LSD was also calculated to compare the differences between two treatment means.

CHAPTER IV

RESULTS AND DISCUSSION

The present experiment was conducted to identify any alternate chemical Ammonium nitrate (NH_4NO_3) for tissue culture in MS media preparation for *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 II-XIII. The results have been presented and discussed and possible interpretations were given experiment wise under the following parameters:-

4.I.1 Sub-Experiment I: *In vitro* plantlets regeneration of potato varieties using β chemical as a substitute of NH_4NO_3

4.I.1.1 Effect of potato variety on shoot length

Significant variation was observed among the varieties in respect of the shoot length of potato plantlets at different days after sub-culture (DAS). It was presented in Fig 1. The maximum length of shoot (2.93 cm, 3.61cm, 5.22 cm and 5.37 cm) were recorded in Diamant variety (V_1) which was statistically different from all other varieties at 7, 14, 21 and 30 DAS respectively and minimum shoot length was recorded in Asterix variety.

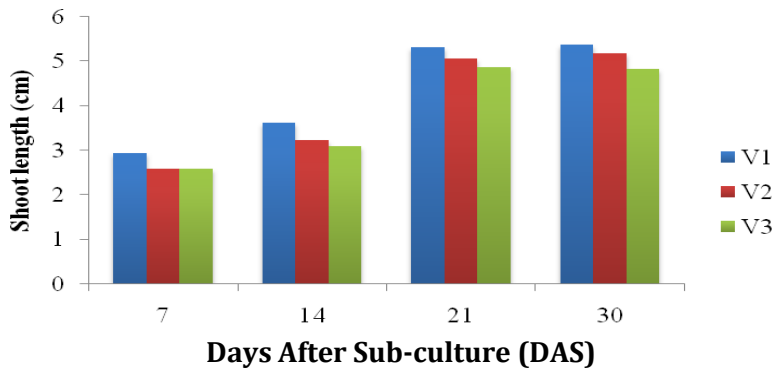
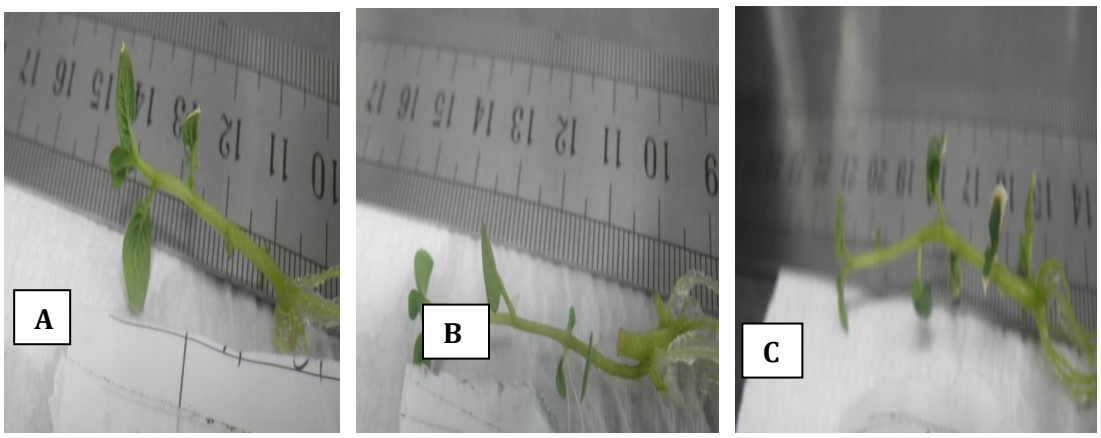


Fig 1. Shoot length of potato plantlets at different days after sub-culture (DAS)
LSD $_{(0.01)}$ = 0.13, 0.13, 0.17 and 0.12 at 7, 14, 21 and 30 DAS respectively)
V_1- Diamant Variety V_2- Cardinal variety V_3- Asterix Variety

4.I.1.2 Effect of treatment on shoot length (cm)

The effect of treatment in shoot length is presented in Table 02. The maximum shoot length (7.52cm) was found in 5 gmL^{-1} of β chemical (T_3) which was statistically different from all other treatments at 21 days after sub-culture.

Highest length (8.22 cm) was also noticed at 30 days after sub-culture (DAS) from the same treatment. The required plantlet of the treatment 15 gmL^{-1} & 20 gmL^{-1} of β chemical showed yellow color and those plantlets were died within 30 days after sub-culture. A comparison between different treatment was given in plate 11. The robust plantlet was observed in 5 gmL^{-1} of β chemical (Plate 11). It might be due to toxic effect of urea as higher concentration. Our investigation is first on the use of urea as a major salt in tissue culture medium. Hence, reference related to β chemical in culture media preparation is quite unavailable. Here, we mentioned few references of β chemical used in field condition for potato production. Oliveira *et al.* (2000) reported that maximum stem elongation was reached quickly with double density and had the tendency to keep constant at the highest (3%) and lowest (1%) nitrogen levels 70 days after planting. The rate of leaf appearance increased drastically due to more branching caused by high nitrogen level, and increased above ground dry matter per plant. Rizk *et al.* (2013) reveal that Urea as foliar spraying resulted the vigor potato plant, i.e. the tallest plants and that carried largest number, fresh and dry weight of leaves and stems. Moreover, the better plant growth was recorded with that plants received the higher urea level, i.e. 3%. the application of urea within 2 – 3 % as foliar spraying, had an increase in tuber yield.



A: Diamant variety B: Cardinal variety C: Asterix Variety

Plate 11: Measuring of shoot length at 14 days after sub-culture (DAS) in different potato varieties

Table 2. Effect of treatments on shoot length (cm) of potato plantlets at different days after sub-culture (DAS)

Treatment	Shoot length (cm) at different days after sub-culture (DAS)					
	7 DAS	14 DAS	21 DAS	30 DAS	Morphological appearance	
					14 DAS	21 DAS
T_1	3.51a	4.17ab	7.20 b	7.80 b	Greenish	alive
T_2	3.10b	4.01 a	7.18 b	7.53 c	Greenish	alive
T_3	3.23 b	4.36 b	7.52 a	8.22 a	Greenish	alive
T_4	2.73c	3.66 c	6.84 c	7.16 d	Greenish	alive
T_5	2.08d	2.08 d	1.73 d	0.00 e	Brownish	died
T_6	1.59e	1.59 e	0.00 e	0.00 e	Yellowish	died
LSD (0.01)	0.19	0.19	0.24	0.17	--	--
CV (%)	5.53	4.49	3.71	2.52	--	--

T_1= 16.50 gm/L of Ammonium nitrate T_2= 1 gm of β chemical/litre

T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre

T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

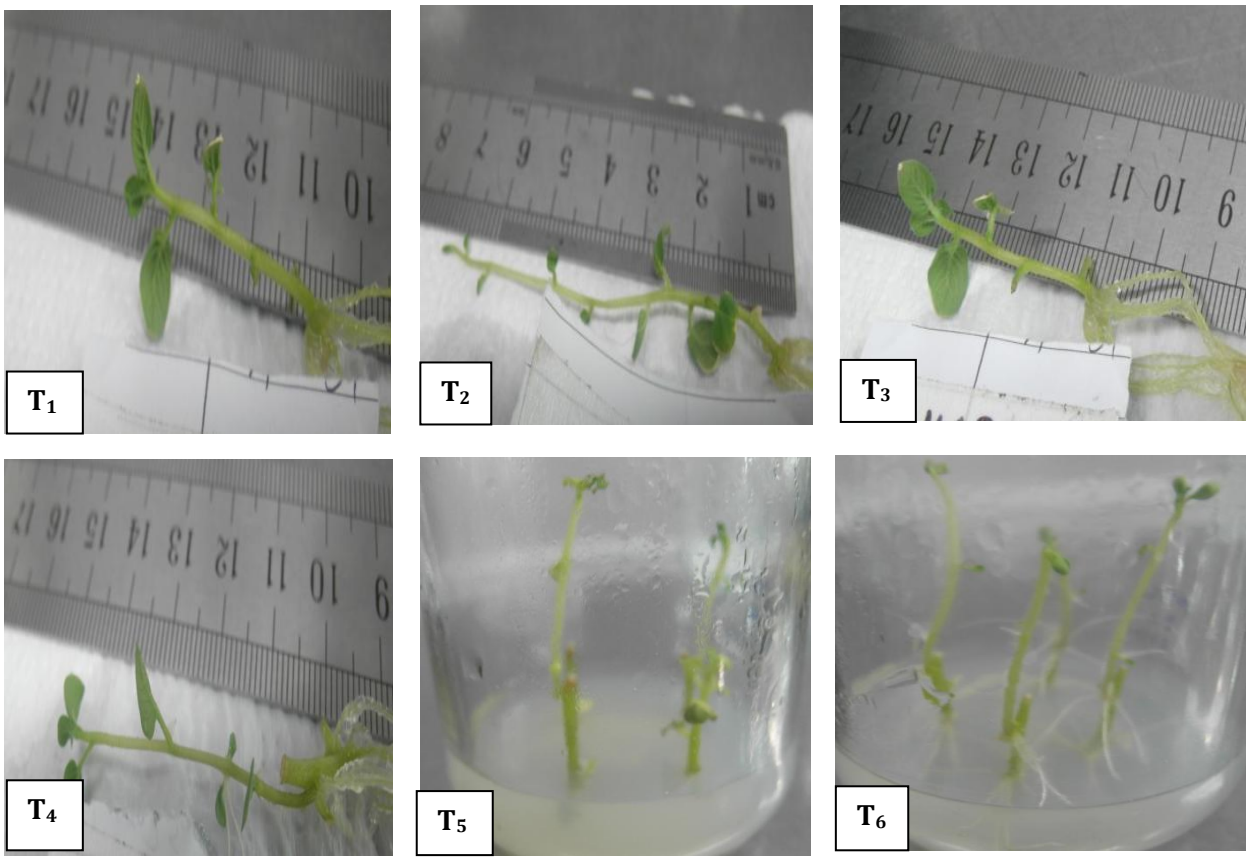


Plate 12: Shoot length of different treatments at 14 days after sub-culture (DAS)

4.I.1.3 Combined effect of variety and treatment on Shoot length (cm)

Interaction effect of different varieties and different treatments in length of shoot (cm) of potato plantlet at different days after sub-culture (DAS) was presented in Table 3. The highest length of shoot (3.73 cm) was found in combination of Diamant variety with MS standard dose (16.50 gmL^{-1} of NH_4NO_3, V_1T_1) which was statistically different similar with 1 gmL^{-1} and 5 gmL^{-1} of β chemical treatments at 7 DAS.

On the other hand, the lowest length of shoot (1.47 cm) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically similar to combination of Cardinal variety with 20 gmL^{-1} of β chemical (V_2T_6) & combination of Diamant variety with 20 gmL^{-1} of β chemical (V_1T_6). The highest length of shoot (4.93 cm) was found in combination of Diamant variety with 1 gmL^{-1} of β chemical (V_1T_2) which was statistically similar with MS standard dose and dissimilar with all other combinations at 14 DAS. While the lowest length of shoot (1.47 cm) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically similar to Cardinal variety with 20 gmL^{-1} of β chemical (V_2T_6) & Diamant variety with 20 gmL^{-1} of β chemical (V_1T_6) but different from all other combinations at 14 Days after sub-culture (DAS). The morphological appearance of plantlets in the combination of varieties with 15 gmL^{-1} and 20 gmL^{-1} of β chemical were turning to brownish and yellowish. The maximum shoot length (7.70 cm) was found in combination of Diamant variety with 1 gmL^{-1} of β chemical treatment (V_1T_2) which was statistically non significant with MS standard dose and 5 gmL^{-1} of β chemical dose. It was statistically different from all other combinations at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the combination of varieties (Diamant, Cardinal & Asterix) with 15 gmL^{-1} and 20 gmL^{-1} of β chemical treatment in (V_1T_5 ,V_1T_6 , V_2T_5 , V_2T_6, V_3T_5, V_3T_6) interactions. Similar result also noticed at 30 days after sub-culture (DAS), It may be due to toxic effect of β chemical in MS media concentration (Table 3 & Plate 12).

Table 3. Combined effect of variety and treatment on shoot length (cm) of potato plantlets at different days after sub-culture (DAS)

Treatment	Shoot length (cm) at different days after sub-culture (DAS)				
	7 DAS	14 DAS	21 DAS	30 DAS	Morphological performance at 21DAS
V_1T_1	3.73 a	4.67 ab	7.60 a	8.20 b	Greenish
V_1T_2	3.50 ab	4.93 a	7.70 a	8.50 a	Greenish
V_1T_3	3.50 ab	4.40 bc	7.40 ac	8.00 bc	Greenish
V_1T_4	2.87 d-f	3.67 ef	7.10 b-e	7.50 ef	Greenish
V_1T_5	2.27 gh	2.27 g	2.10 g	0.00 h	Died
V_1T_6	1.73 ij	1.73 hi	0.00 i	0.00 h	Died
V_2T_1	3.10 cd	3.97 de	7.30 a-d	7.90 cd	Greenish
V_2T_2	3.30 bc	4.20 cd	7.50 ab	8.20 b	Greenish
V_2T_3	3.00 c-e	3.87 ef	7.03 c-e	7.70 de	Greenish
V_2T_4	2.57 fg	3.70 ef	6.90 d-f	7.23 f	Greenish
V_2T_5	2.03 hi	2.03 gh	1.60 h	0.00 h	Died
V_2T_6	1.57 j	1.57 i	0.00 i	0.00 h	Died
V_3T_1	3.10 cd	3.87 ef	6.70 ef	7.30 f	Greenish
V_3T_2	3.50 ab	3.93 de	7.37 a-c	7.97 b-d	Greenish
V_3T_3	2.80 d-f	3.77 ef	7.10 b-e	6.90 g	Greenish
V_3T_4	2.77 ef	3.60 f	6.53 f	6.73 g	Greenish
V_3T_5	1.93 i	1.93 h	1.50 h	0.00 h	Yellowish,Brownish
V_3T_6	1.47 j	1.47 i	0.00 i	0.00 h	Brownish,Yellowish
LSD (0.01)	0.33	0.33	0.42	0.29	--
CV (%)	5.53	4.49	3.71	2.52	--

V_1- Diamant Variety, V_2- Cardinal variety and V_3- Asterix Variety

T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre

T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre

T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

4.I.2.1 Effect of variety on Shoot number

Shoot number of three (3) varieties showed statistically similar result at 7 DAS which was presented in Fig 2. The variation of shoot number was noticed at 14, 21 and 30 days after sub-culture (DAS) and the three (3) varieties under studied. The Diamant variety showed highest number (1.35, 1.17, 1.33) at 14, 21 and 30 days after sub-culture (DAS) respectively. On the other hand, the lowest shoot number (1.22, 1.01, 1.29) were recorded in Asterix variety (V_3) which were statistically different from Cardinal variety (V_2) at 14,21 and 30 days after sub-culture (DAS) respectively.

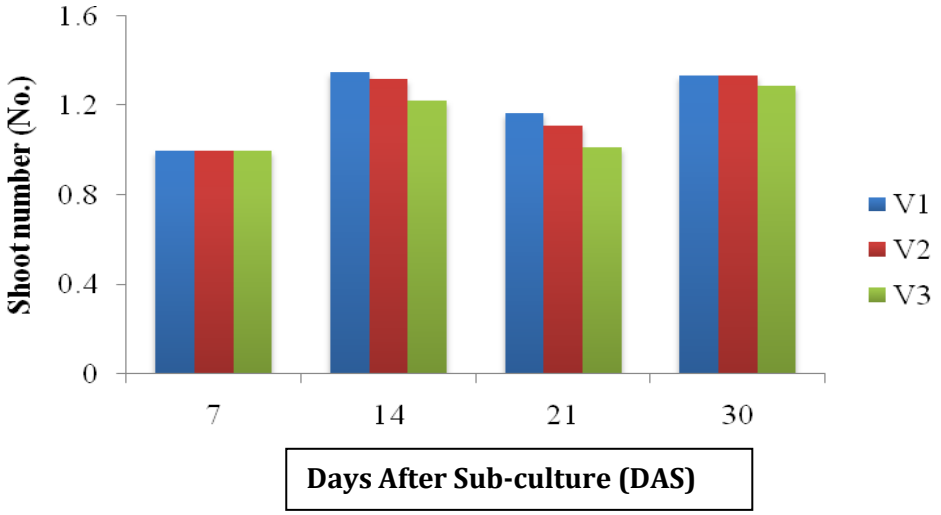


Figure 2. Effect of variety on the shoot number (no.) of potato plantlets at different days after sub-culture (DAS)

(LSD $_{(0.01)}$ =NS, 0.04, 0.05 and NS at 7, 14, 21 and 30 DAS respectively)
V_1- Diamant Variety V_2- Cardinal Variety V_3- Asterix Variety

4.I.2.2 Effect of treatments on the number of shoots

All the treatments showed only one shoot (1) at 7 days after sub-culture (DAS). At 14 days after sub-culture (DAS), the maximum shoot number (2.03) was recorded in 5 gmL^{-1} of β chemical treatment (T_2) followed by MS standard dose (1.80) which was statistically different from all other treatments. On the other hand, the minimum shoot number (0.94) was recorded in 20 gmL^{-1} of β chemical treatment (T_6) which was statistically different from all other treatments. The result found (1.00) in 5 gmL^{-1} of β chemical treatment (T_3) was statistically similar (1.00) to 15 gmL^{-1} of β chemical treatment (T_5) but different from all other treatments. The morphological appearance of plantlets in the treatment (T_5) with 15 gmL^{-1} and treatment (T_6) with 20 gmL^{-1} of β chemical were turning to brownish and yellowish color. The maximum shoot

number (2.18) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the treatment of 15 gmL^{-1} and 20 gmL^{-1} of β chemical. Similar result also noticed at 30 days after sub-culture (DAS), It may be due to toxic effect of β chemical (Table 4).

Table 4. Effect of treatments on the shoot number (no.) of potato plantlets at different days after sub-culture (DAS)

Treatment	Shoot number (no.) at different days after sub-culture (DAS)				
	7 DAS	14 DAS	21 DAS	30 DAS	Morphological performance at 21 days
T_1	1.00	1.80 b	1.69 b	2.00 a	Greenish
T_2	1.00	1.00 c	1.00 c	1.99 a	Greenish
T_3	1.00	2.03 a	2.18 c	2.11 a	Greenish
T_4	1.00	1.00 c	1.00 c	1.92 a	Greenish
T_5	1.00	1.00 c	1.00 c	0.00 b	Brownish
T_6	1.00	0.94 c	0.00 d	0.00 b	Yellowish
LSD $_{(0.01)}$	NS	0.06	0.07	0.09	--
CV (%)	0	3.79	4.77	5.25	--

T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre
T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre
T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

4.I.2.3 Combined effect of variety and treatment on shoot number

Interaction effect of different varieties and different concentrations of β chemical in number of shoot (no.) of potato plantlets at different days after sub-culture (DAS) was presented in Table 5. There was no significant variation among the different treatments at 7 DAS. The highest number of shoot (2.10) was found in combination of Diamant variety with 5 gmL^{-1} of β chemical (V_1T_2) which was statistically different from all other combinations at 14 DAS, While the lowest number of shoot (0.87) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically different from combination of Cardinal variety with 20 gmL^{-1} of β chemical (V_2T_6 ,0.97) & combination of Diamant variety with 20 gmL^{-1} of β chemical (V_1T_6 ,1.00) from all other combinations at 14 Days after sub-culture (DAS). The maximum shoot number (2.00) was found in combination of Diamant variety with 1 gmL^{-1} of β chemical treatment (V_1T_2) which was statistically different from all other combinations at 21 days after sub-culture (DAS).

Table 5. Combined effect of variety and treatment on shoot number (no.) of potato plantlets at different days after sub-culture (DAS)…..Cont.

Treatment	Shoot number (no.) at different days after sub-culture(DAS)				
	7 DAS	14 DAS	21 DAS	30 DAS	Morphological performance at 21 DAS
V_1T_1	1.00	2.00 b	2.00 a	2.00 a	Greenish
V_1T_2	1.00	2.10 a	2.00 a	2.00 a	Greenish
V_1T_3	1.00	1.00 d	1.00 d	2.00 a	Greenish
V_1T_4	1.00	1.00 d	1.00 d	2.00 a	Greenish
V_1T_5	1.00	1.00 d	1.00 d	0.00 c	Yellowish, Brownish
V_1T_6	1.00	1.00 d	0.00 e	0.00 c	Brownish, Yellowish
V_2T_1	1.00	1.93 b	1.67 b	2.00 a	Greenish
V_2T_2	1.00	2.00 b	2.00 a	2.00 a	Greenish
V_2T_3	1.00	1.00 d	1.00 d	2.00 a	Greenish
V_2T_4	1.00	1.00 d	1.00 d	2.00 a	Greenish
V_2T_5	1.00	1.00 d	1.00 d	0.00 c	Yellowish, Brownish
V_2T_6	1.00	0.97 d	0.00 e	0.00 c	Brownish, Yellowish
V_3T_1	1.00	1.47 c	1.40 c	2.00 a	Greenish
V_3T_2	1.00	2.00 b	1.67 b	2.00 a	Greenish
V_3T_3	1.00	1.00 d	1.00 d	1.97 a	Greenish
V_3T_4	1.00	1.00 d	1.00 d	1.77 b	Greenish
V_3T_5	1.00	1.00 d	1.00 d	0.00 c	Yellowish, Brownish
V_3T_6	1.00	0.87 e	0.00 e	0.00 c	Brownish, Yellowish
LSD (0.01)	NS	0.10	0.12	0.16	--
CV (%)	0	3.79	4.77	5.25	--

V_1- Diamant Variety, V_2- Cardinal variety and V_3- Asterix Variety

T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre

T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre

T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

4.I.3.1 Effect of variety on leaf number

Significant variation was observed among the varieties in respect of the leaf number (no.) of potato plantlets at different days after sub-culture (DAS). It was presented in Fig-3. The maximum leaf number (2.92) was recorded in Diamant variety (V_1) which was statistically significant to all other varieties at 7 DAS. Similar result (6.56, 9.00, 9.06) was also observed at 14, 21, 30 DAS respectively in Diamant variety (V_1). On the other hand,the minimum leaf number (1.08, 3.28, 0.00, 0.00) was recorded in Asterix variety (V_3) which was statistically different from Cardinal variety (V_2) at 7, 14, 21 and 30 days after sub-culture (DAS) respectively (Plate 13 and 14).

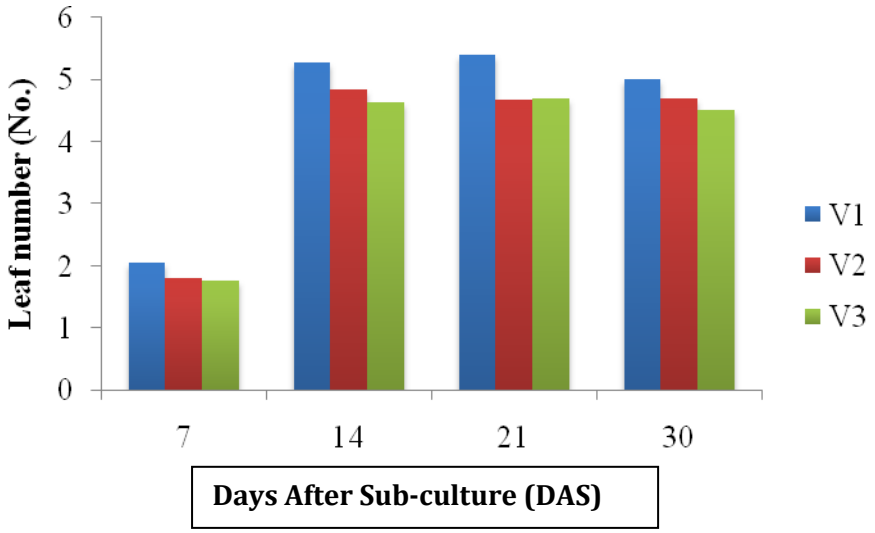


Figure 3. Effect of variety on the leaf number (no.) of potato plantlets at different days after sub-culture (DAS)

(LSD $_{(0.01)}$ = 0.09, 0.22, 0.21 and 0.20 at 7, 14, 21 and 30 DAS respectively)

V_1- Diamant Variety V_2- Cardinal Variety V_3- Asterix Variety

4.I.3.2 Effect of treatments on the leaf number

Among the six treatments, the maximum leaf number (2.92) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 7 DAS which was followed by MS standard dose (2.74 cm) While minimum leaf number (1.08) was found in 20 gmL^{-1} of β chemical treatment (T_6) which was statistically different from all other treatments at 7 DAS. At 14 days after sub-culture (DAS), the maximum leaf number (6.56) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments. On the other hand, minimum leaf number (3.28) was found in 20 gmL^{-1} of β chemical treatment (T_6) which was statistically different from all other treatments. The morphological appearance of

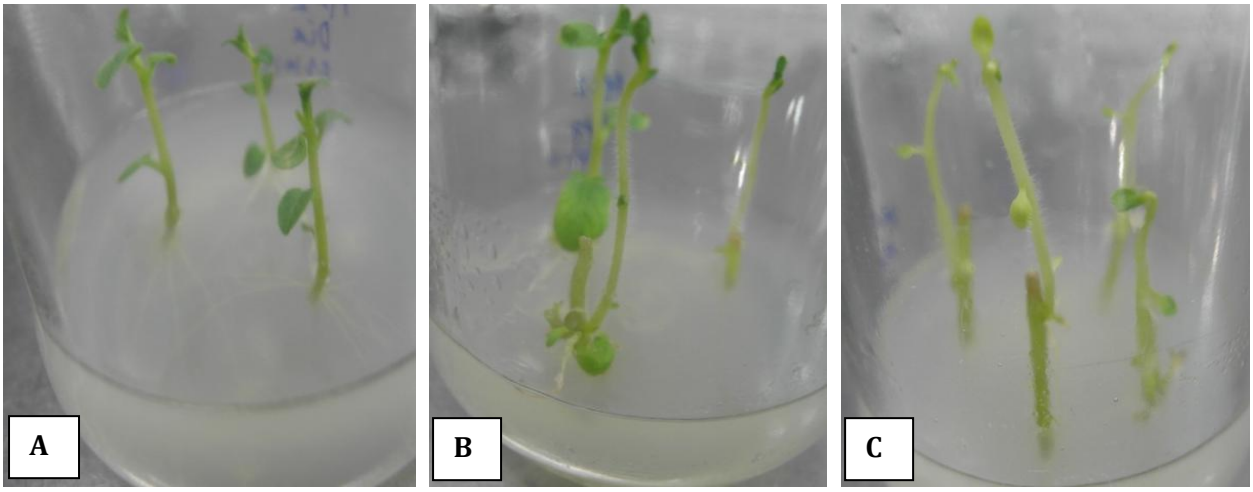
plantlets in the treatment (T_5) with 15 gmL^{-1} and treatment (T_6) with 20 gmL^{-1} of β chemical were turning to brownish and yellowish color.

The maximum leaf number (9.00) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the treatment of 15 gmL^{-1} and 20 gmL^{-1} of β chemical. Similar result also noticed at 30 days after sub-culture (DAS) (Table 6).

Table 6. Effect of treatment on the leaf number (no.) of potato plantlets at different days after sub-culture (DAS)

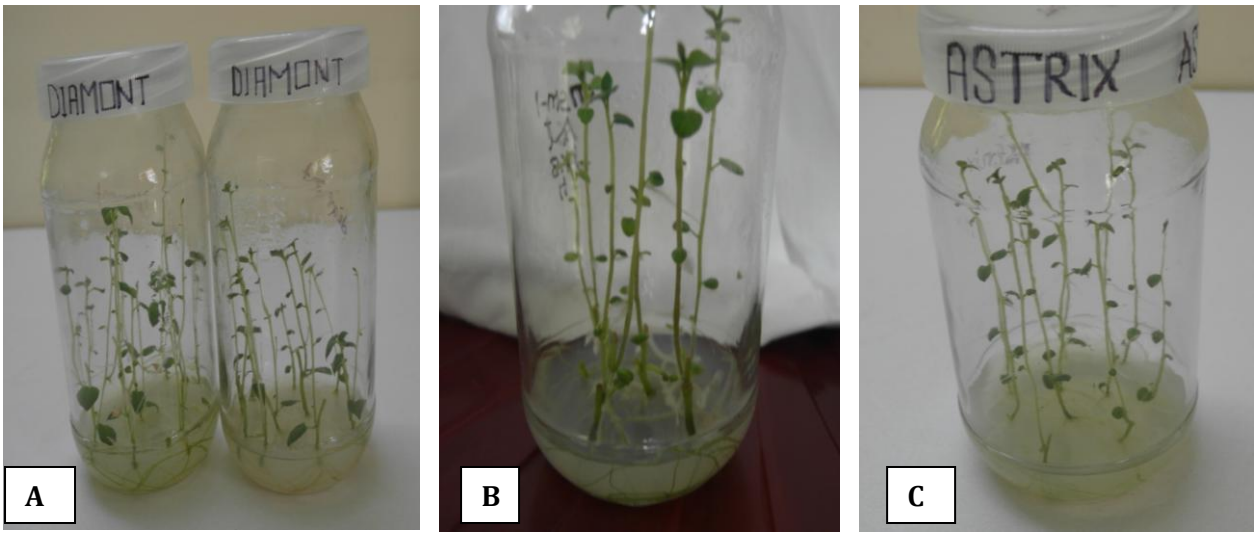
Treatment	Leaf number(no.) at different days after sub-culture (DAS)				
	7 DAS	**14 DAS**	**21 DAS**	**30 DAS**	**Morphological appearance at 14 DAS**
T_1	2.7 b	6.0b	6.48 c	6.3c	Greenish
T_2	1.74c	5.33c	8.00 b	8.4b	White
T_3	2.92a	6.56a	9.00 a	9.0a	White
T_4	1.52d	4.44d	4.22 d	4.5d	White
T_5	1.23e	3.90e	1.87 e	0.0e	Brownish
T_6	1.08f	3.28f	0.00 f	0.0e	Yellowish
LSD (0.01)	0.12	0.31	0.30	0.28	--
CV (%)	5.1	4.93	4.68	4.63	--

T_1= 16.50 gmL^{-1} of Ammonium nitrate
T_2= 1 gm of β chemical/litre
T_3= 5 gm of β chemical/litre
T_4=10 gm of β chemical/litre
T_5=15 gm of β chemical/litre
T_6=20 gm of β chemical/litre



A: Diamant variety **B: Cardinal variety** **C: Asterix variety**

Plate 13: Morphological performance of potato varieties at 14 days after Sub-culture (DAS)



A: Diamant variety **B: Cardinal variety** **C: Asterix Variety**

Plate 14: Morphological performance of potato varieties at 21 days after sub-culture (DAS)

4.I.3.3 Combined effect of variety and treatments on leaf number

Interaction effect of different varieties and different treatments in number of leaf of potato plantlet at different days after sub-culture (DAS) was presented in Table 7. The highest number of leaf (3.00) was found in combination of Diamant variety with 5 gmL^{-1} of β chemical (V_3T_6) which was statistically similar with MS standard dose and different from all other combinations at 7 DAS. The lowest number of leaf (1.02) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was

statistically similar to combination of Cardinal variety with 20 gmL^{-1} of β chemical (V_2T_6) & combination of Diamant variety with 20 gmL^{-1} of β chemical (V_1T_6) but different from all other combinations at 7 DAS.

The highest number of leaf (7.00) was found in combination of Diamant variety with 5 gmL^{-1} of β chemical (V_1T_2) which was statistically different from all other combinations at 14 DAS, While the lowest number of leaf (2.83) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically different from all other combinations at 14 Days after sub-culture.

The maximum leaf number (10.00) was found in combination of Diamant variety with 5 gmL^{-1} of β chemical treatment (V_1T_2) which was statistically different from all other combinations at 21 days after sub-culture (DAS). All plantlets were died at 21 (DAS) in the combination of varieties with 15 gmL^{-1} and 20 gmL^{-1} of β chemical.

Table 7. Combined effect of variety and treatment on the leaf number (no.) of potato plantlets at different days after sub-culture (DAS)…Cont.

Treatment	Leaf number (no.) at different days after sub-culture (DAS)				
	7 DAS	14 DAS	21 DAS	30 DAS	Morphological performance at 21 DAS
V_1T_1	2.97 a	6.33 bc	9.00 b	9.00 b	Greenish
V_1T_2	3.00 a	7.00 a	10.00 a	10.00 a	Greenish
V_1T_3	1.97 d	6.00 cd	6.33 e	6.00 e	Greenish
V_1T_4	1.90 d	4.67 ef	4.33 g	5.00 f	Greenish
V_1T_5	1.33 f	4.00 gh	2.73 i	0.00 h	Died
V_1T_6	1.17 fg	3.67 hi	0.00 l	0.00 h	Died
V_2T_1	2.67 bc	6.00 cd	8.00 c	8.27 c	Greenish
V_2T_2	2.93 a	6.67 ab	9.00 b	9.17 b	Greenish
V_2T_3	1.59 e	5.00 e	6.00 e	6.17 e	Greenish
V_2T4	1.33 f	4.33 fg	3.33 h	4.53 f	Greenish
V_2T_5	1.19 fg	3.73 hi	1.77 j	0.00 h	Died
V_2T_6	1.07 g	3.33 ij	0.00 l	0.00 h	Died
V_3T_1	2.59 c	5.67 d	7.00 d	8.00 c	Greenish
V_3T_2	2.83 ab	6.00 cd	8.00 c	8.00 c	Greenish
V_3T_3	1.67 e	5.00 e	7.10 d	7.00 d	Greenish

V_3T_4	1.33 f	4.33 fg	5.00 f	4.00 g	Greenish
V_3T_5	1.17 fg	3.97 gh	1.12 k	0.00 h	Died
V_3T_6	1.02 g	2.83 j	0.00 l	0.00 h	Died
LSD $_{(0.01)}$	0.21	0.54	0.51	0.49	--
CV (%)	5.1	4.93	4.68	4.63	--

V_1- Diamant Variety, V_2- Cardinal variety and V_3- Asterix Variety

T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre

T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre

T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

4.I.4.1 Effect of variety on node number

Significant variation was observed among the varieties in respect of the node number (no.) of potato plantlets at different days after sub-culture (DAS). It was presented in Fig 4. The maximum node number (4.33) was recorded in Diamant variety (V_1) which was statistically similar to all other varieties at 14 DAS. Similar result was also observed at 21 and 30 DAS respectively in Diamant variety (V_1). On the other hand, the minimum number (3.55) of node was recorded in Asterix variety (V_3) from which was statistically similar to Cardinal variety (V_2) at 14 days after sub-culture (DAS). Significant variation was also noticed at 21 & 30 DAS for the same traits.

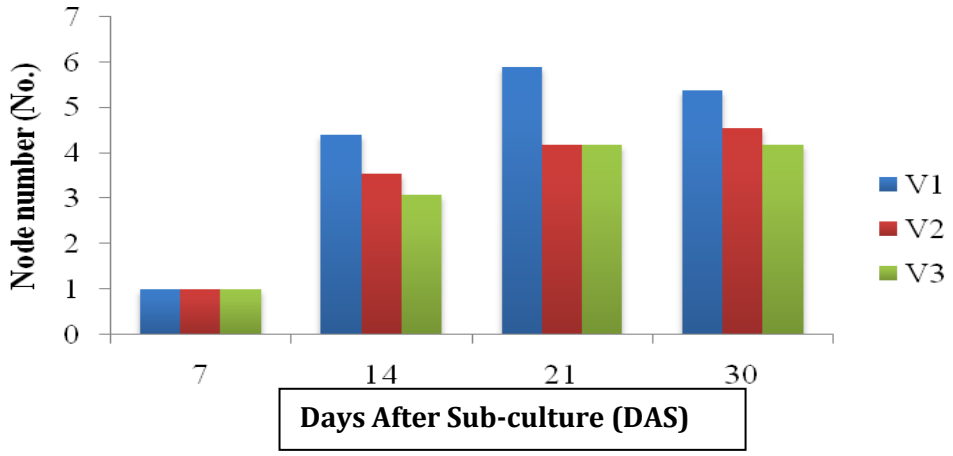


Figure 4. Effect of variety on the node number (no.) in potato plantlets at different days after sub-culture (DAS)

(LSD $_{(0.01)}$ = NS, 0.17, 0.23 and 0.21 at7, 14, 21 and 30 DAS, respectively)

V_1- Diamant Variety V_2- Cardinal Variety V_3- Asterix Variety

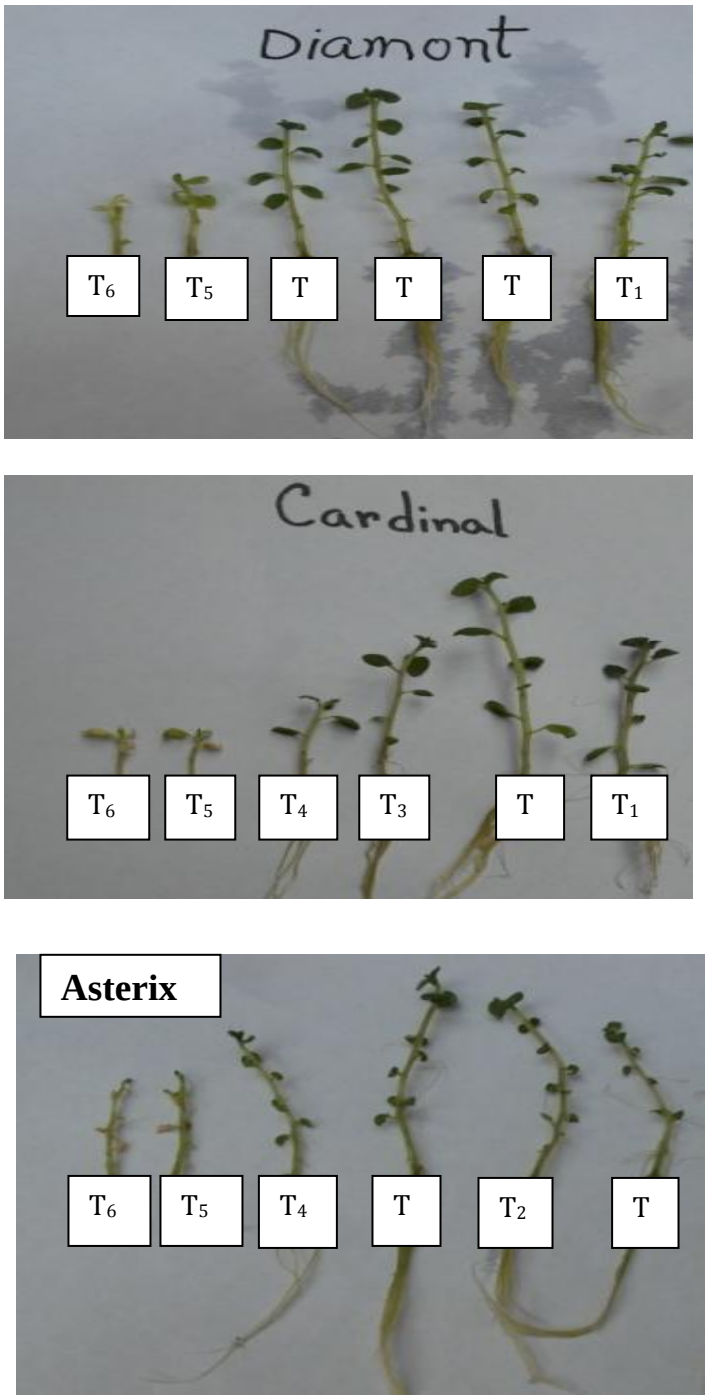
4.I.4.2 Effect of treatment on the node number

At 14 days after sub-culture (DAS), the maximum node number (5.69) was found in 1 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments. On the other hand, the minimum node number (1.47) was found in 20 gmL^{-1} of β chemical treatment (T_6) which was statistically different from all other treatments. The morphological appearance of plantlets in the treatment (T_5) with 15 gmL^{-1} and treatment (T_6) with 20 gmL^{-1} of β chemical were turning to brownish and yellowish color. The maximum node number (7.67) was found in 1 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the treatment of 15 gmL^{-1} and 20 gmL^{-1} of β chemical. Similar result also noticed at 30 days after sub-culture (DAS) (Table 8 and plate 15, Plate 16).

Table 8. Effect of treatments on the node number (no.) of potato plantlet at different days after sub-culture (DAS)

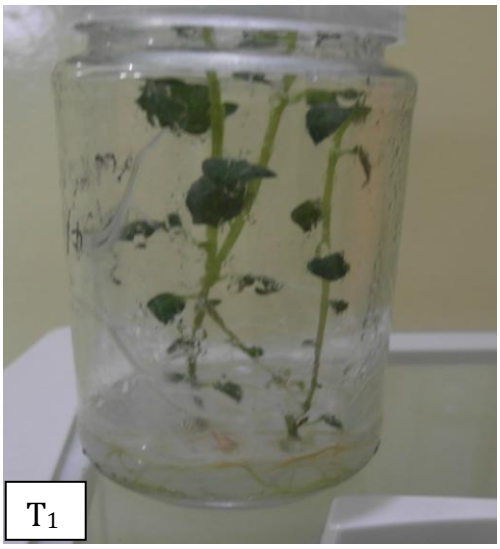
Treatment	Node number (no.) at different days after sub-culture (DAS)				
	7 DAS	**14 DAS**	**21 DAS**	**30 DAS**	**Morphological appearance at 21 DAS**
T_1	1.00	4.56 b	6.80 b	7.36 b	Alive
T_2	1.00	5.69 a	7.67 a	8.01 a	Alive
T_3	1.00	4.44 b	5.78 c	6.78 c	Alive
T_4	1.000	3.89 c	5.23 d	6.09 d	Alive
T_5	1.00	2.00 d	3.00 e	0.00 e	Died
T_6	1.00	1.47 e	0.00 f	0.00 e	Died
LSD $_{(0.01)}$	NS	0.24	0.32	0.30	--
CV (%)	0	5.2	5.33	4.9	--

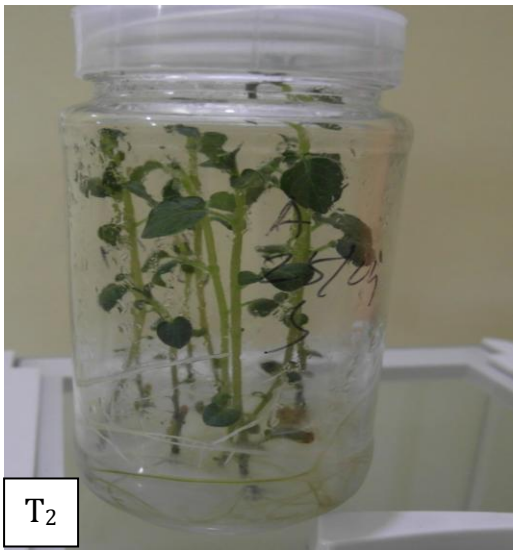
T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre
T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre
T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

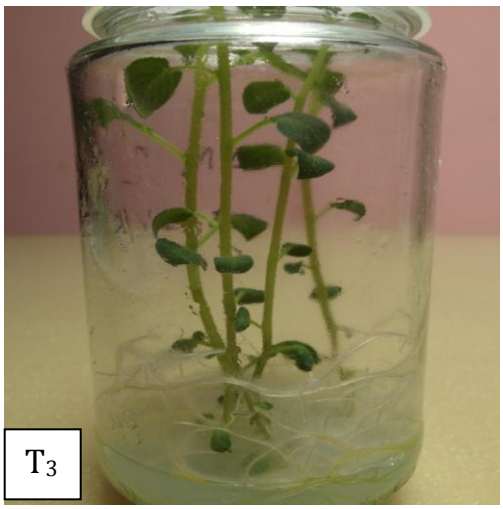


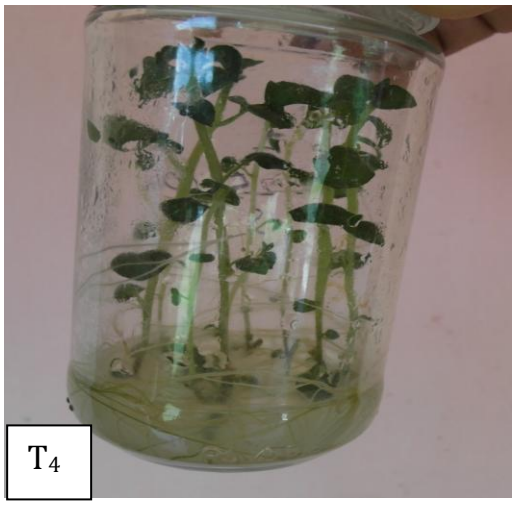
T_1= 16.50 gm/L of ammonium nitrate
T_2= 1gm of urea /litre
T_3= 5gm of urea /litre
T_4=10 gm of urea /litre
T_5=15gm of urea /litre
T_6=20gm of urea /lit

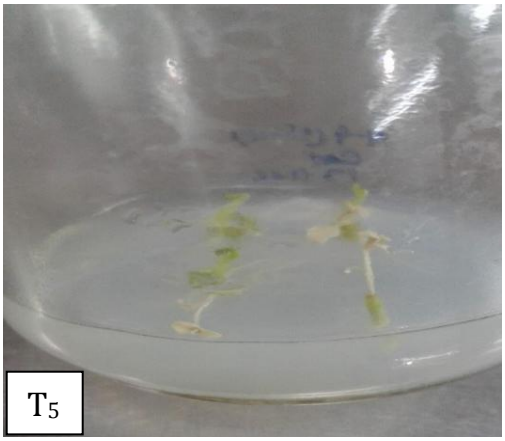
Plate 15: Effects of different treatments on varieties at 21 days after sub-culture (DAS)

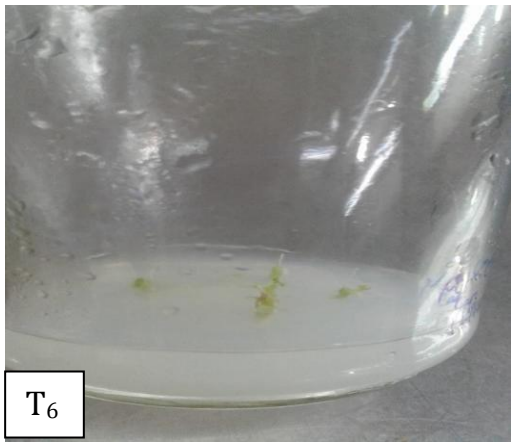












T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre
T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre
T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

Plate 16: Effects of different treatments on varieties at 30 days after sub-culture (DAS)

4.I.4.3 Combined effect of variety and treatment on node number

Interaction effect of different varieties and different concentrations of β chemical in number of node of potato plantlets at different days after sub-culture (DAS) was presented in Table 9. There was no significant variation found in node number among the different treatments at 7 DAS. The highest number of node (6.00) was found in combination of Diamant variety with 1 gmL^{-1} of β chemical (V_1T_2) which was statistically different from all other combinations at 14 DAS, While the lowest number of node (1.07) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically different from all other combinations at 14 Days after sub-culture(DAS). The morphological appearance of plantlets in the combination of varieties with 15 gmL^{-1} and 20 gmL^{-1} of β chemical was turning to brownish and yellowish. The maximum node number (9.33) was found in combination of Diamant variety with 1 gmL^{-1} of β chemical treatment (V_1T_2) which was statistically different from all other combinations at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the combination of varieties with 15 gmL^{-1} and 20 gmL^{-1} of β chemical treatment in (V_1T_5, V_1T_6, V_2T_5, V_2T_6, V_3T_5, V_3T_6) combinations. Similar result also noticed at 30 days after sub-culture (DAS).

Treatment	Node number (no.) at different days after sub-culture (DAS)				
	7 DAS	14 DAS	21 DAS	30 DAS	Morphological performance at 21 DAS
V_1T_1	1.00	5.00 cd	8.00 b	8.17 b	Greenish
V_1T_2	1.00	6.00 a	9.33 a	8.87 a	Greenish
V_1T_3	1.0	5.33 bc	7.33 cd	7.93 b	Greenish
V_1T_4	1.00	5.00 cd	7.67 bc	7.33 c	Greenish
V_1T_5	1.00	3.00 h	3.00 k	0.00 g	Yellowish, Brownish
V_1T_6	1.00	2.00 i	0.00 l	0.00 g	Brownish, Yellowish
V_2T_1	1.00	4.67 de	6.00 g	7.33 c	Greenish
V_2T_2	1.00	5.67 ab	6.67 ef	8.00 b	Greenish
V_2T_3	1.00	4.33 ef	5.00 h	6.00 e	Greenish
V_2T_4	1.00	3.67 g	4.37 i	5.93 e	Greenish
V_2T_5	1.00	1.67 ij	3.00 k	0.00 g	Yellowish, Brownish
V_2T_6	1.00	1.33 jk	0.00 l	0.00 g	Brownish, Yellowish
V_3T_1	1.00	4.00 fg	6.40 fg	6.57 d	Greenish
V_3T_2	1.00	5.40 bc	7.00 de	7.17 c	Greenish
V_3T_3	1.00	3.67 g	5.00 h	6.40 de	Greenish

V_3T_4	1.00	3.00 h	3.67 j	5.00 f	Greenish
V_3T_5	1.00	1.33 jk	3.00 k	0.00 g	Yellowish, Brownish
V_3T_6	1.00	1.07 k	0.00 l	0.00 g	Brownish, Yellowish
LSD $_{(0.01)}$	NS	0.42	0.56	0.51	--
CV (%)	0	5.2	5.33	4.9	--

Table 9. Combined effect of variety and treatment on the node number (no.) of potato plantlets at different days after sub-culture (DAS)…Cont.

V_1- Diamant Variety, V_2- Cardinal variety and V_3- Asterix Variety

T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre

T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre

T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

4.I.5.1 Effect of variety on root length

Significant variation was observed among the varieties in respect of the root length (cm) of potato plantlets at different days after sub-culture (DAS). It was presented in Fig 5. The maximum length of root (4.17) was recorded in Diamant variety (V_1) which was statistically different from all other varieties at 14 DAS. Similar result was also observed at 21 and 30 DAS respectively in Diamant variety (V_1). Meanwhile, the minimum length of root (3.11cm) was recorded in Asteix variety (V_3) which was statistically different from Cardinal variety (V_3) at 14 days after sub-culture (DAS). Significant variation was also noticed at 21 & 30 DAS for the same traits.

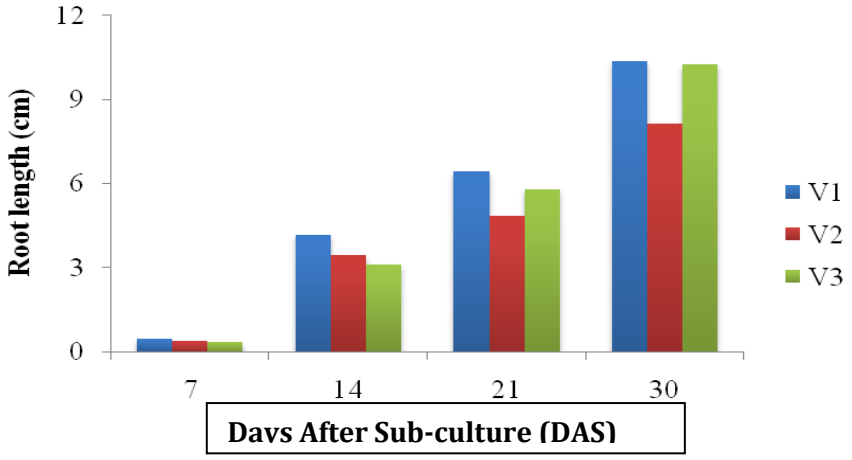


Figure 5. Effect of variety on the root length (cm) of potato plantlet at different days after sub-culture (DAS)

(LSD $_{(0.01)}$ = 0.03, 0.13, 0.23 and 0.50 at 7, 14, 21 and 30 DAS respectively)

4.I.5.2 Effect of treatment on the root length (cm)

At 14 days after sub-culture (DAS), The maximum root length (6.11 cm) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments. On the other hand, minimum root length (0.96 cm) was found in 20 gmL^{-1} of β chemical treatment (T_6) which was statistically different from all other treatments (Table 10). The maximum root length (8.80 cm) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the treatment of 15 gmL^{-1} and 20 gmL^{-1} of β chemical. Similar result also noticed at 30 days after sub-culture (DAS).

Table 10. Effect of treatment on the root length (cm) of potato plantlets at different days after sub-culture (DAS)

Treatment	Root length (cm) at different days after sub-culture (DAS)		
	14 DAS	21 DAS	30 DAS
T_1	5.2 b	7.68 b	13.0b
T_2	4.11c	6.07 c	11.33c
T_3	6.11a	8.80 a	16.33a
T_4	3.11d	6.16 c	10.97c
T_5	1.94e	5.40 d	0.00 d
T_6	0.00f	0.00 e	0.00 e
LSD (0.01)	0.19	0.32	0.71
CV (%)	4.14	4.39	5.76

T_1= 16.50 gm/L of ammonium nitrate T_2= 1 gm of β chemical/litre
T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre
T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

4.I.5.3 Combined effect of variety and treatment on root length

Interaction effect of different varieties and different treatments in length of root (cm) of potato plantlet at different days after sub-culture (DAS) was presented in Table 11. The highest length of root (7.00 cm) was found in combination of Diamant variety with 1 gmL^{-1} of β chemical (V_1T_2) which was statistically different from all other combinations at 14 DAS, While the lowest length of root (0.97 cm) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically similar to combination of Cardinal variety with 20 gmL^{-1} of β chemical (V_2T_6 ,0.90 cm) & combination of Diamant variety with 20 gmL^{-1} of β chemical (V_1T_6 , 1.00 cm) but different from all other combinations at 14 Days after

sub-culture (DAS). The maximum root length (10.10 cm) was found in combination of Diamant variety with 1 gmL^{-1} of β chemical treatment (V_1T_2) which was statistically different from all other combinations at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the combination of varieties (Diamant, Cardinal & Asterix) with 15 gmL^{-1} and 20 gmL^{-1} of β chemical treatment in (V_1T_6, V_2T_6, V_3T_6) combinations. Similar result also noticed at 30 days after sub-culture (DAS), It may be due to toxic effect of β chemical in MS media concentration (Table 11).

Table 11. Combined effect of variety and treatments on the root number (no.) of potato plantlets at different days after sub-culture (DAS)

Treatment	Root length (cm) at different days after sub-culture (DAS)		
	14 DAS	**21 DAS**	**30 DAS**
V_1T_1	7.00 a	9.30 b	14.00 cd
V_1T_2	6.00 b	10.10 a	18.00 a
V_1T_3	4.00 g	7.00 de	12.00 ef
V_1T_4	3.67 h	6.40 f	11.00 fg
V_1T_5	3.33 i	5.70 g	7.30 hi
V_1T_6	1.00 l	0.00 j	0.00 k
V_2T_1	5.00 d	7.20 d	12.00 ef
V_2T_2	6.00 b	8.20 c	15.00 bc
V_2T_3	4.00 g	4.90 h	10.00 g
V_2T_4	3.33 i	4.79 h	7.90 h
V_2T_5	1.50 k	3.90 i	3.90 j
V_2T_6	0.90 l	0.00 j	0.00 k
V_3T_1	4.67 e	6.53 ef	13.00 de
V_3T_2	5.33 c	8.10 c	16.00 b
V_3T_3	4.33 f	6.30 f	12.00 ef
V_3T_4	2.33 j	7.30 d	14.00 cd
V_3T_5	1.00 l	6.60 ef	6.60 i
V_3T_6	0.97 l	0.00 j	0.00 k
LSD $_{(0.01)}$	0.33	0.55	1.23
CV (%)	4.14	4.39	5.76

V_1- Diamant Variety, V_2- Cardinal variety and V_3- Asterix Variety
T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre
T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre
T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

4.I.6.1 Effect of variety on root number

Significant variation was observed among the varieties in respect of the root number of potato plantlets at different days after sub-culture (DAS). It was presented in Fig 6. The maximum root number (6.71) was recorded in Diamant variety (V_1) which was statistically different from all other varieties at 14 DAS. Similar result (8.25, 8.28) was also observed at 21 and 30 DAS respectively in Diamant variety (V_1). On the other hand,the minimum root number (4.11) was recorded in Asterix variety (V_3) which was statistically significant from Cardinal variety (V_2) at 14 days after sub-culture(DAS). Significant variation was also noticed at 21 & 30 DAS for the same traits (Fig 6 and Plate 17).

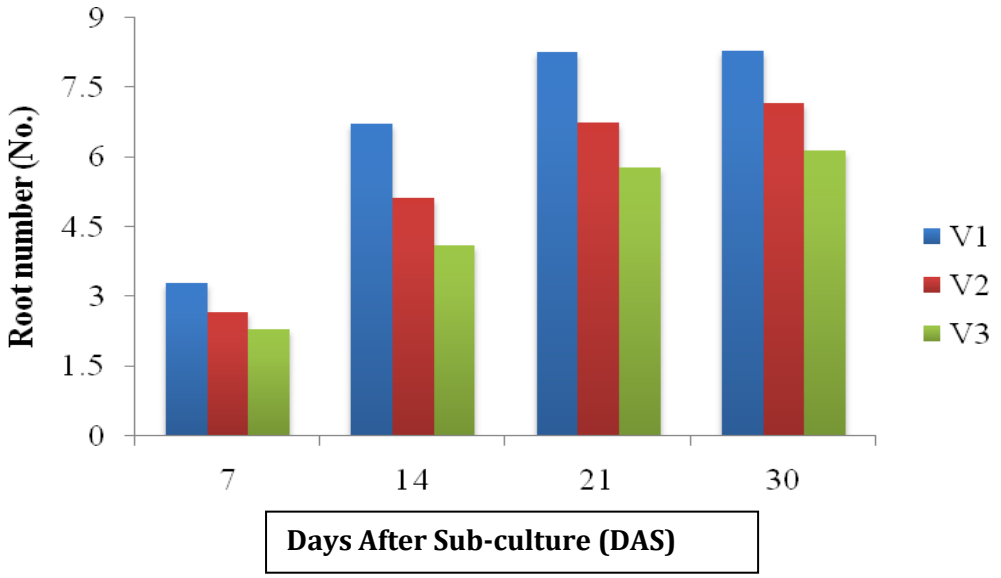
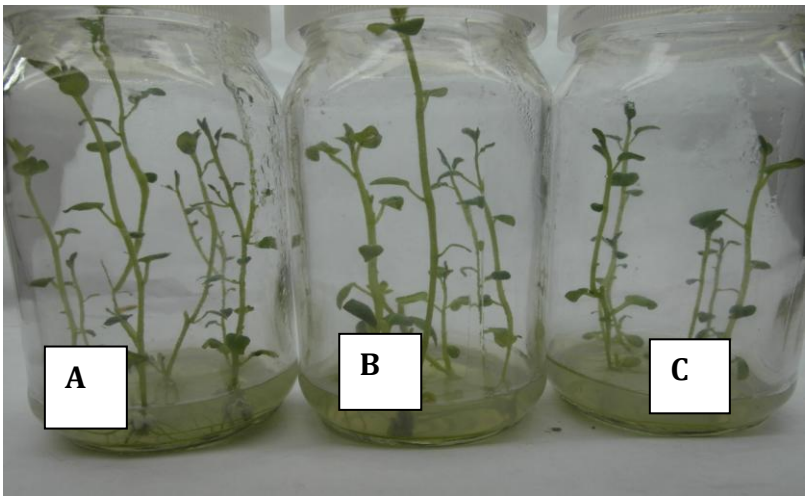


Figure 6. Effect of variety on the root number (no.) of potato plantlet at different days after sub-culture (DAS)

(LSD $_{(0.01)}$ = 0.11, 0.18, 0.29 and 0.40 at 7, 14, 21 and 30 DAS respectively)

V_1- Diamant Variety V_2- Cardinal Variety V_3- Asterix Variety



A: Diamant variety B: Cardinal variety C: Asterix variety

Plate 17: Phenotypic appearance of potato varieties at 30 days after sub-culture (DAS)

4.I.6.2 Effect of treatments on the root number

At 14 days after sub-culture (DAS), the maximum root number (8.00) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments. On the other hand, minimum root number (2.89) was found in 20 gmL^{-1} of β chemical treatment (T_6) which was statistically different from all other treatments (Table 12). The maximum root number (10.67) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the treatment of 15 gmL^{-1} and 20 gmL^{-1} of β chemical. Similar result also noticed at 30 days after sub-culture (DAS), It may be due to toxic effect of β chemical.

Table 12. Effect of treatments on the root number (no.) of potato plantlets at different days after sub-culture (DAS)

Treatment	Root number (no.) at different days after sub-culture (DAS)		
	14 DAS	21 DAS	30 DAS
T_1	6.33 b	9.33 b	9.67 b
T_2	8.00 a	10.67 a	10.67 a
T_3	5.49 c	8.33 c	8.78 c
T_4	5.11 d	7.02 d	7.78 d
T_5	4.09 e	6.17 e	6.28 e
T_6	2.89 f	0.00 f	0.00 f
$LSD_{(0.01)}$	0.26	0.41	0.56
CV (%)	3.81	4.6	6.09

T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre
T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre
T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

4.I.6.3 Combined effect of variety and treatment on root number

Interaction effect of different varieties and different treatments in root number of potato plantlets at different days after sub-culture (DAS) was presented in Table 13. The lowest number of root (0.00) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically similar to combination of Cardinal variety with 20 gmL^{-1} of β chemical (V_2T_6) & combination of Diamant variety with 20 gmL^{-1} of β chemical(V_1T_6) but different from all other combinations at 7 DAS (Table 13).The highest number of root (9.00) was found in combination of Diamant variety with 5 gmL^{-1} of β chemical (V_1T_2) which was statistically different from all other combinations at 14 DAS, While the lowest number of root (2.00) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically different from all other combinations at 14 Days after sub-culture (DAS). The morphological appearance of roots in the combination of varieties with 15 gm/L and 20 gm/L of β chemical was turning to white color. The maximum root number (13.00) was found in combination of Diamant variety with 5 gmL^{-1} of β chemical treatment (V_1T_2) which was statistically different from all other combinations at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the combination of varieties (Diamant, Cardinal &Asterix) with 15 gmL^{-1} and 20 gmL^{-1} of β chemical treatment in (V_1T_5 , V_1T_6, V_2T_5, V_2T_6, V_3T_5, V_3T_6) combinations.

Table 13. Combined effect of variety and treatment on the root number of potato plantlet at different days after sub-culture (DAS)

Treatment	Root number(no.) at different days after sub-culture (DAS)		
	14 DAS	21 DAS	30 DAS
V_1T_1	8.00 b	11.00 b	11.00 b
V_1T_2	9.00 a	13.00 a	13.00 a
V_1T_3	7.00 c	10.00 c	10.00 c
V_1T_4	7.00 c	8.00 e	9.00 d
V_1T_5	5.60 d	7.50 ef	6.67 fg
V_1T_6	3.67 gh	0.00 j	0.00 i
V_2T_1	5.67 d	9.00 d	9.00 d
V_2T_2	8.00 b	10.00 c	10.00 c
V_2T_3	5.13 e	8.00 e	9.00 d
V_2T_4	5.00 e	7.13 f	8.00 e
V_2T_5	4.00 fg	6.33 gh	7.00 fg
V_2T_6	3.00 ij	0.00 j	0.00 i
V_3T_1	5.33 de	8.00 e	9.00 d
V_3T_2	7.00 c	9.00 d	9.00 d
V_3T_3	4.33 f	7.00 fg	7.33 ef
V_3T_4	3.33 hi	5.93 h	6.33 g
V_3T_5	2.67 j	4.67 i	5.17 h
V_3T_6	2.00 k	0.00 j	0.00 i
LSD $_{(0.01)}$	0.45	0.71	0.97
CV (%)	3.81	4.6	6.09

V_1- Diamant Variety, V_2- Cardinal variety and V_3- Asterix Variety

T_1= 16.50 gmL^{-1} of Ammonium nitrate T_2= 1 gm of β chemical/litre

T_3= 5 gm of β chemical/litre T_4=10 gm of β chemical/litre

T_5=15 gm of β chemical/litre T_6=20 gm of β chemical/litre

4.II.1 Sub-Experiment II: Comparative performance of different MS media for *in vitro* regeneration of potato

4.II.1.1 Effect of potato variety on shoot length

The maximum lengths of shoot 7.04 cm, 8.63 cm and 9.80 cm were recorded in Diamant variety (V_1) at 14, 21, 30 DAS respectively which were statistically different from all other varieties. Meanwhile, the minimum lengths of shoot 6.65 cm, 8.04 cm and 9.5cm were recorded in Asterix (V_3) which was statistically different from others varieties at 14, 21 & 30 DAS for the same trait (Fig 7 and Plate 18).

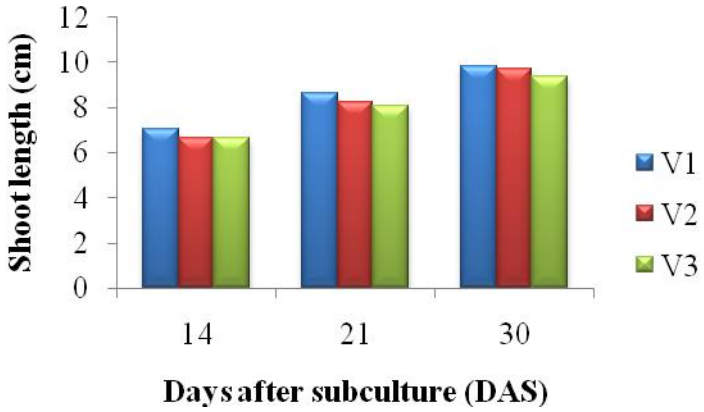


Figure 7. Effect of variety on the shoot length of potato plantlet at different days after sub-culture

(LSD $_{(0.01)}$ = 0.14, 0.13 and 0.13 at 14, 21 and 30 DAS, respectively),
V_1– Diamant, V_2 – Cardinal, V_3 – Asterix

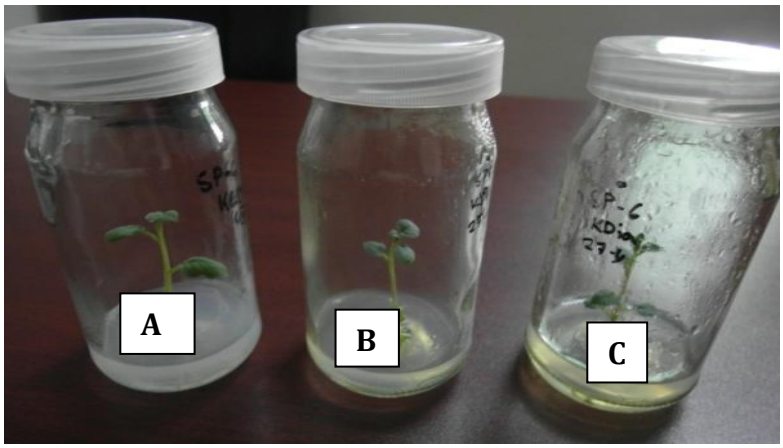


Plate 18: Varietal performance at 14 Days After Sub-Culture (DAS)

A. Diamant **B**. Cardinal **C**. Asterix

4.II.1.2 Effect of different treatment formulations on shoot length

Among the four treatments (Formulation), the maximum shoot length (7.33, 8.60 and 9.93 cm) was found in 5 gmL^{-1} of β chemical (T_4) treatment which was statistically non significant to ready MS powder (T_1) at 14, 21 and 30 DAS respectively. While minimum shoot length (6.02, 7.98, 9.28 cm) was found in 1 gmL^{-1} of β chemical (T_3) which was statistically different from all other treatments at 14, 21 and 30 DAS respectively (Table 14 & Plate 19).

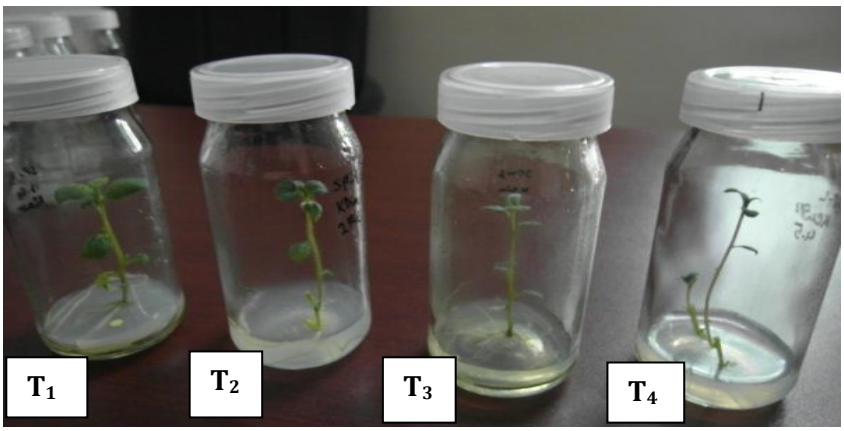


Plate 19. Effect of different formulations on shoot length of potato at 14 DAS

T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate T_3=1 gm of β chemical/Litre T_4= 5 gm of β chemical/Litre

4.II.1.3 Combined effect of variety and different formulation on shoot length

The combined effect of variety and treatment (different formulations) is presented in Table 15. The highest length of shoot (7.43 cm, 9.00cm & 10.10 cm) were found in Diamant variety with 5 gmL^{-1} of β chemical treatment (V_1T_4) which was statistically similar in the same with ready MS standard dose (V_1T_1) but different from all other combinations at 14, 21 & 30 DAS respectively. On the other hand, the lowest lengths of shoot (5.07 cm , 7.77 cm & 8.73 cm) were found in Asterix (V_3) with 5 gmL^{-1} of β chemical (V_3T_4) which having statistically dissimilarity from all other combinations at 14, 21 and 30 DAS respectively. Qadri et al. (2015) reported that Growth, yield and quality of potato are greatly affected by its nutritional management. Foliar application of urea reduces nitrogen losses and increases plant nitrogen use efficiency. Foliar applications of nitrogenous fertilizer (urea) were given 30 days after sowing with one week interval in five split doses. Results indicated that T_3 (DAP 120+ Urea 8 kg/ acre) remained better regarding productivity and quality of potato.

4.II.2.1 Effect of variety on number of leaf

Significant variation was observed among the varieties in respect of leaf number in potato plantlets at different days after sub-culture (Fig 8). The maximum leaf number (6.3) was recorded in Diamant variety (V_1) which was statistically different to all other varieties at 14 DAS. Similar results were also observed at 21 and 30 DAS respectively in Diamant variety (V_1). On the other hand, the minimum leaf number (5.7) was recorded in Asterix (V_3) which was statistically different from Cardinal variety (V_2) at 14 days after sub-culture (DAS). Significant variation was also noticed at 21 and 30 DAS for the same trait.

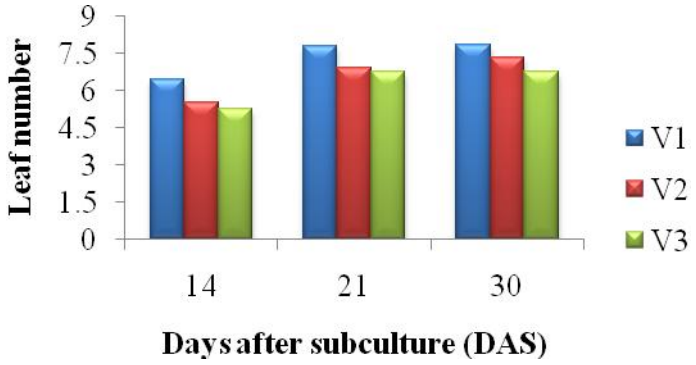


Figure 8. Effect of variety on the leaf number of potato plantlet at different days after sub-culture

($LSD_{(0.01)}$ = 0.29, 0.30 and 0.28 at 14, 21 and 30 DAS respectively)

V_1- Diamont ,V_2-Cardinal, V_3- Asterix

4.II.2.2 Effect of treatment on the leaf number

The maximum leaf number (6.77, 9.11, 9.30) were found in 5 gmL^{-1} of β chemical (T_4) which was statistically non significant with ready MS powder (T_1) but significant for all other treatments at 14, 21& 30 DAS respectively (Table 2). While minimum leaf number 4.44, 4.22 and 4.51) were found in 5 gmL^{-1} (T_3) which was statistically different from all other treatments at 14, 21 and 30 DAS respectively (Table 14).

4.II.2.3 Combined effect of variety and treatment on number of leaf

The highest numbers of leaf 8.20, 10.50 and 10.40 at 14, 21 and 30 DAS respectively were observed in Diamant variety with 5 gmL^{-1} of β chemical (V_1T_4) showing statistically non-similarity with all other combinations. On the other hand, the lowest numbers of leaf 4.33, 5.00 and 4.00 at 14, 21 and 30 DAS were noted in Asterix variety with 5 gmL^{-1} of β chemical (V_3T_4) which was statistically different from all other combinations (Table 15).

4.II.3.1 Effect of variety on root length (cm)

There was a significant variation among the varieties in respect of root length (cm) of potato plantlets at different DAS (Fig 9). The maximum lengths of root (5.17, 9.00 and 14.35 cm) were recorded in Diamant (V_1) which was statistically different from all other varieties at 14, 21 and 30 DAS respectively. On the other hand, the minimum lengths of root (4.07, 6.43 and 12.94 cm) were recorded in Asterix (V_3) showing statistically difference from others varieties at 14, 21 and 30 DAS for the same trait.

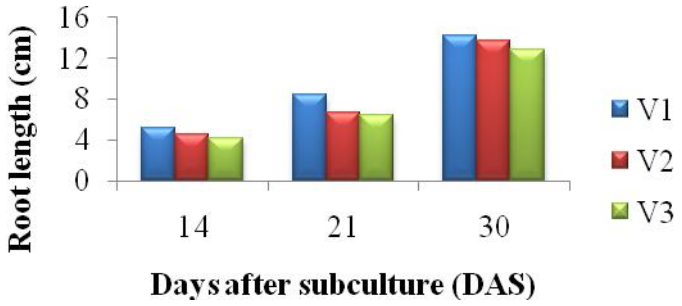


Figure 9. Effect of variety on the root length of potato plantlets at different days after sub-culture

(LSD $_{(0.01)}$ = 0.20, 0.39 and 1.19 at 14, 21 and 30 DAS respectively),V_1- Diamont ,V_2- Cardinal, V_3- Asterix

4.II.3.2 Effect of treatment on the root length (cm)

Treatment effect on root length was presented in Table 14. At 14 days after sub-culture (DAS), the maximum root length (6.11cm) was found in liquid MS standard dose (T_2) which was statistically different from all other treatments. While minimum root length (3.11cm) was found in 5 gmL^{-1} of β chemical (T_4) which was statistically different from all other treatments at 14 DAS. The maximum root lengths (8.72 cm and 14.00 cm) were found in ready MS powder (T_1) which was statistically different from all other treatments at 21 and 30 DAS respectively. In contrast, minimum root lengths (5.70 cm and 12.47 cm) were found in 5 gmL^{-1} of β chemical treatment (T_4) which was statistically different from all other treatments at 21 & 30 DAS respectively. It is well established that, vegetative growth of plant (Shoot length, more leaf etc.) increase due to application of β chemical or nitrogenous fertilizer. It also slidely reduce the root growth.

Probabily due to that, the treatment 5 gmL^{-1} of β chemical showed minimum root length in the regenerated plantlet.

4.II.3.3 Combined effect of variety and treatment on root length (cm)

The highest lengths of root (7.00, 10.40, 15.00 cm) were found in Diamant with ready MS standard dose (V_1T_1) which was statistically similar in Diamant with 5 gmL^{-1} of β chemical (V_1T_4) but different from all other combinations at 14, 21 and 30 DAS respectively. On the other hand, the lowest lengths of root (2.33, 5.90 and 12.40 cm.) were found in Asterix with 5 gmL^{-1} of β chemical (V_3T_4) having statistical difference from all other combinations at 14,21 and 30 DAS respectively (Table 15 & Plate 20).

Table 14: Effect of treatments on shoot length, number of leaf and root length at 14, 21 and 30 DAS

Treatment	Shoot length (cm)			Number of leaf			Root length (cm)		
	14 DAS	21 DAS	30 DAS	14 DAS	21DAS	30 DAS	14 DAS	21 DAS	30 DAS
T_1	7.32 a	8.52 a	9.79 a	6.42 b	8.82 a	9.06a	5.22 b	8.72 a	14.00ab
T_2	6.46 b	8.13 b	9.60 b	5.33 c	6.48 b	6.39b	6.11 a	8.21 b	12.89bc
T_3	6.02 c	7.98 c	9.28 c	4.44 b	4.22 c	4.51c	4.11 c	6.07 c	15.00 a
T_4	7.33 a	8.60 a	9.93 a	6.77 a	9.11 a	9.30a	3.11 d	5.70 c	12.47 c
LSD (0.01)	0.16	0.15	0.15	0.33	0.35	0.32	0.23	0.45	1.37
CV (%)	1.77	1.39	1.20	4.38	3.71	3.30	3.81	4.74	7.66

T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate (Liquid MS standard dose)

T_3=1 gm of β chemical/Litre T_4= 5 gm of β chemical/Litre

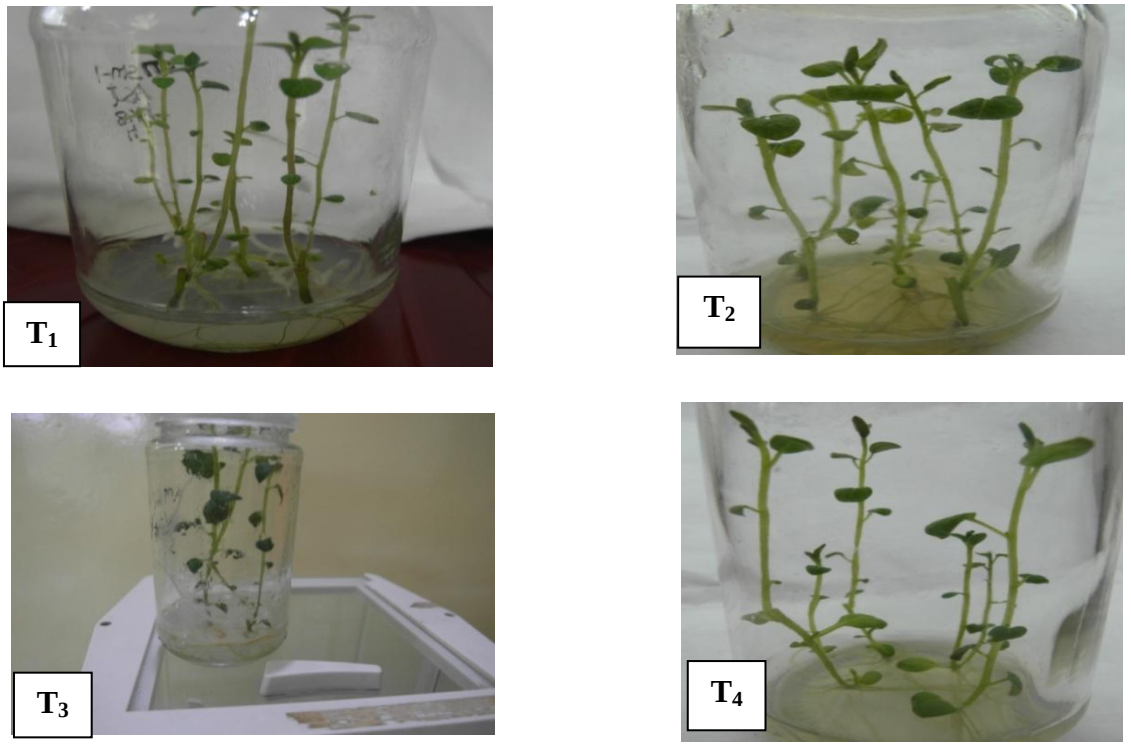


Plate 20: Effects of four (4) treatments at 30 DAS in potato varieties

T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate (Liquid MS standard dose)

T_3=1 gm of β chemical/Litre T_4= 5 gm of β chemical/Litre

Table 15: Combined effect of variety and treatment on shoot length, leaf number &root length

Treatment	Shoot length (cm)			Number of leaf			Root length (cm)		
	14 DAS	21 DAS	30 DAS	14 DAS	21 DAS	30 DAS	14 DAS	21 DAS	30 DAS
V_1T_1	7.20 a	8.77 a	9.83 a	4.67 ef	4.33 f	5.00 g	7.00 b	10.40 a	15.00 ab
V_1T_2	6.70 b	8.40 bc	9.60 cf	7.00 b	10.00 a	10.00b	7.00 a	10.10 a	13.67 ac
V_1T_3	6.37 c	8.37 bc	9.70 be	6.00 cd	6.33 d	6.00 f	4.00 fg	7.00 cd	16.00 a
V_1T_4	7.43 a	9.00 a	10.10 a	8.20 a	10.50 a	10.40 a	3.67 gh	6.40 ce	12.00 c
V_2T_1	6.37 c	8.50 b	9.90 ab	6.43 bc	9.27 b	9.50 bc	5.00 cd	8.60 b	14.00 ac
V_2T_2	7.33 a	8.20 c	9.80b-d	6.27 c	9.00 b	9.17 c	6.00 b	8.20 b	13.00 bc
V_2T_3	5.80 d	8.50 b	9.70 be	5.00 e	6.00 d	6.17 f	4.00 fg	4.90 f	15.00 ab
V_2T_4	7.20 a	7.80 d	9.50 ef	4.33 f	3.33 g	4.53 gh	3.33 h	4.79 f	13.00 bc
V_3T_1	6.30 c	8.30 bc	9.80 bd	5.67 d	7.57 c	8.00 d	4.67 de	7.17 c	13.00 bc
V_3T_2	7.20 a	7.80 d	9.57 df	6.00 cd	7.47 c	8.00 d	5.33 c	6.33 de	12.00 c
V_3T_3	5.90 d	8.30 bc	9.40 f	5.00 e	7.10 c	7.00 e	4.33 ef	6.30 de	14.00 ac
V_3T_4	5.07 e	7.77 d	8.73 g	4.33 f	5.00 e	4.00 h	2.33 i	5.90 e	12.40 c
LSD $_{(0.01)}$	0.27	0.26	0.26	0.57	0.61	0.55	0.40	0.78	2.38
CV (%)	1.77	1.39	1.20	4.38	3.71	3.30	3.81	4.74	7.66

T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate (Liquid MS standard dose)

T_3=1 gm of β chemical/Litre & T_4= 5 gm of β chemical/Litre

4.II.4.1 Effect of variety on shoot number

There was no significant variation among the varieties in respect of shoot number of potato plantlets at 7, 14, 21, 30 different days after sub-culture (DAS) respectively. It was presented in Fig 10.

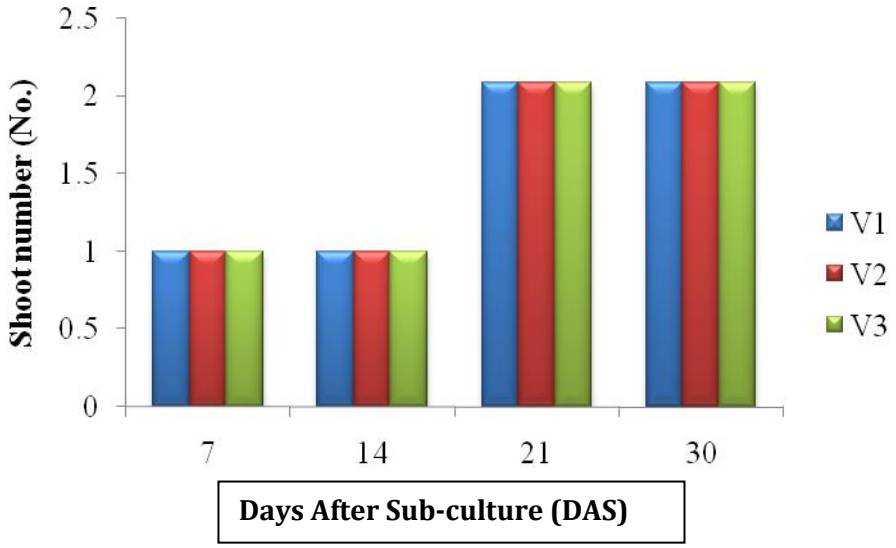


Figure 10. Effect of variety on the shoot number (no.) of potato plantlets at different days after initiation (DAS)

(LSD $_{(0.01)}$ = NS, NS, NS and NS at 7, 14, 21 and 30 DAS respectively)

V_1 - Diamant V_2 - Cardinal V_3 – Asterix

4.II.4.2 Effect of treatment on the shoot number

There was no significant variation among the treatments in respect of shoot number at 14 DAS. The maximum shoot number (2.33 & 2.33) were found in ready MS powder standard dose (T_1) which was statistically non significant with 16.50 gmL^{-1} of ammonium nitrate (Liquid MS standard dose) but different from all other treatments at 21 & 30 DAS. While minimum shoot length (1.67 & 1.67) were found in 5 gmL^{-1} of β chemical (T_4) which was statistically different from all other treatments at 21 & 30 DAS respectively (Table 16).

Asma et al. (2001) also recorded the highest number of shoots (4) was obtained when 2.0 mg/L BAP was applied properly.

Table 16. Effect of treatment on the shoot number (no.) of potato plantlets at different days after Sub-culture (DAS)

Treatment	Shoot number (no.) at different days after Sub-culture (DAS)		
	14DAS	21 DAS	30 DAS
T_1	1.00	2.33 a	2.33 a
T_2	1.00	2.33 a	2.33 a
T_3	1.00	2.00 ab	2.00 ab
T_4	1.00	1.67 b	1.67 b
LSD $_{(0.01)}$	NS	0.66	0.66
CV (%)	0.00	24.00	24.00

T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate T_3=1 gm of β chemical/Litre T_4= 5 gm of β chemical/Litre

4.II.5.1 Effect of variety on number of node

Significant variation was observed among the varieties in respect of number of node of potato plantlets at different days after sub-culture (DAS). It was presented in Fig 11. The highest node number (1, 5.71. 7.92, 8.52.) was recorded in Diamant variety (V_1) which was statistically significant to all other varieties at 7, 14, 21 and 30 DAS respectively. On the other hand, the lowest leaf number (1, 4.39, 5.79, 6.56) was recorded in Asterix variety (V_3) which was statistically different from Cardinal variety (V_2) at 7, 14, 21 and 30 days after sub-culture (DAS).

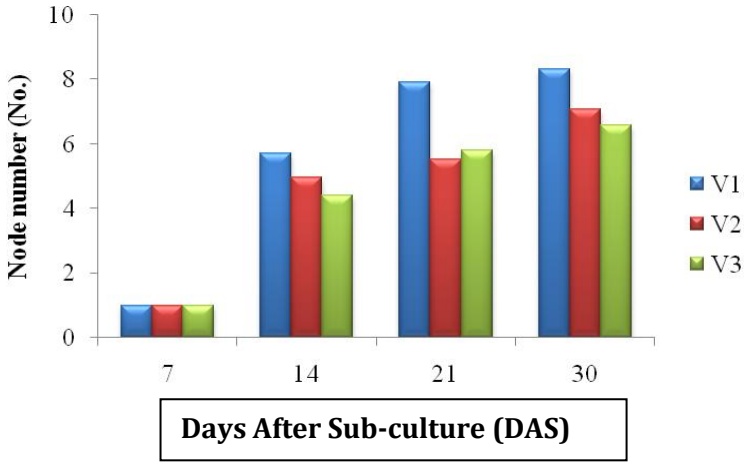


Figure 11. Effect of variety on the node number (no.) of potato plantlet at different days after initiation (DAS)

(LSD $_{(0.01)}$ = NS, 0.22, 0.28 and 0.28 at 7, 14, 21 and 30 DAS respectively)

V_1- Diamont V_2- Cardinal V_3- AsteriX

4.II.5.2 Effect of treatment on number of node

The maximum node number (6.06 ,7.51 , 8.39) were found in ready MS powder treatment (T_1) which was statistically different from all other treatments at 14, 21& 30 DAS respectively. While minimum node number (3.89, 5.23, 6.09) were found in 5 gm of β chemical/Litre (T_4) which was statistically different from all other treatments at 14, 21& 30 DAS respectively (Table 17 and Plate 21).

Table 17. Effect of treatment on the node number (no.) of potato plantlets at different days after sub-culture (DAS)

Treatment	Node number (no.) at different days after sub-culture (DAS)			
	7 DAS	14 DAS	21 DAS	30 DAS
T_1	1.00	6.06 a	7.51 a	8.39 a
T_2	1.00	5.69 b	7.13 b	8.01 b
T_3	1.00	4.44 c	5.78 c	6.78 c
T_4	1.00	3.89 d	5.23 d	6.09 d
LSD $_{(0.01)}$	NS	0.25	0.32	0.32
CV (%)	0.00	3.86	3.77	3.29

T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate (Liquid MS standard dose)

T_3=1 gm of β chemical/Litre T_4= 5 gm of β chemical/Litre

4.II.5.3 Combined effect of variety and treatment on number of node

Interaction effect of different varieties and different MS media in number of node of potato plantlet at different days after sub-culture (DAS) was presented in Table 21. The highest number of node (6.50, 8.50 , 9.10) were found in combination of Diamant variety with ready MS standard dose (V_1T_1) which was statistically similar with combination of Diamant variety with 1gm/L of β chemical treatment (V_1T_3) combination but different from all other combinations at 14, 21 & 30 DAS respectively. On the other hand, the lowest number of node (3.00, 3.67& 5.00) was found in combination of Asterix variety with 5 gmL^{-1} of β chemical treatment (V_3T_4) which was statistically different from all other combinations at 14,21& 30 DAS respectively (Table 21).

Table 18. Combined effect of variety and treatment on the node number of potato plantlet at different days after sub-culture (DAS)

Treatment	Node number (no.) at different days after sub- culture (DAS)		
	14 DAS	**21 DAS**	**30 DAS**
V_1T_1	6.50 a	8.50 a	9.10 a
V_1T_2	5.33 de	7.33 cd	7.93 cd
V_1T_3	6.00 bc	8.17ab	8.87 ab
V_1T_4	5.00 e	7.67 bc	7.33 ef
V_2T_1	6.17 ab	6.53 ef	8.33 bc
V_2T_2	5.67 cd	6.23 f	8.00 cd
V_2T_3	4.33 f	5.00 g	6.00 g
V_2T_4	3.67 g	4.37 h	5.93 g
V_3T_1	5.50 d	7.50 cd	7.73 de
V_3T_2	5.40 de	7.00 de	7.17 f
V_3T_3	3.67 g	5.00 g	6.40 g
V_3T_4	3.00 h	3.67 i	5.00 h
LSD $_{(0.01)}$	0.44	0.55	0.55
CV (%)	3.86	3.77	3.29

V_1- Diamont V_2- Cardinal V_3- Asterix

T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate (Liquid MS standard dose)

T_3=1 gm of β chemical/Litre and T_4= 5 gm of β chemical/Litre

4.II.6.1 Effect of variety on number of root

There was a significant variation among the varieties in respect of root number (no.) of potato plantlets at different days after sub-culture (DAS). It was presented in Fig 12. The maximum number of root (4.82, 7.83, 11.07, 12.00) was recorded in Diamant variety (V_1) which was statistically different from all other varieties at 7, 14, 21 and 30 DAS respectively. On the other hand, the minimum number of root (3.46, 5.44, 7.83, 8.00) was recorded in Asterix variety (V_3) which was statistically different from others variety at 7, 14, 21 and 30 days after sub-culture (DAS).

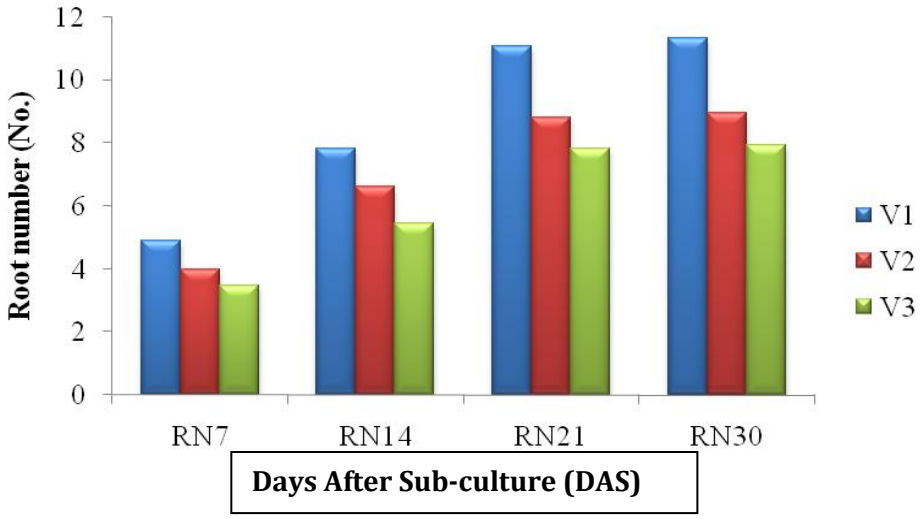


Figure 12. Effect of variety on the root number of potato plantlet at different days after initiation

(LSD $_{(0.01)}$ = 0.17, 0.28, 0.35 and 0.52 at 7, 14, 21 and 30 DAS respectively)

V_1- Diamont V_2- Cardinal V_3- Asterix

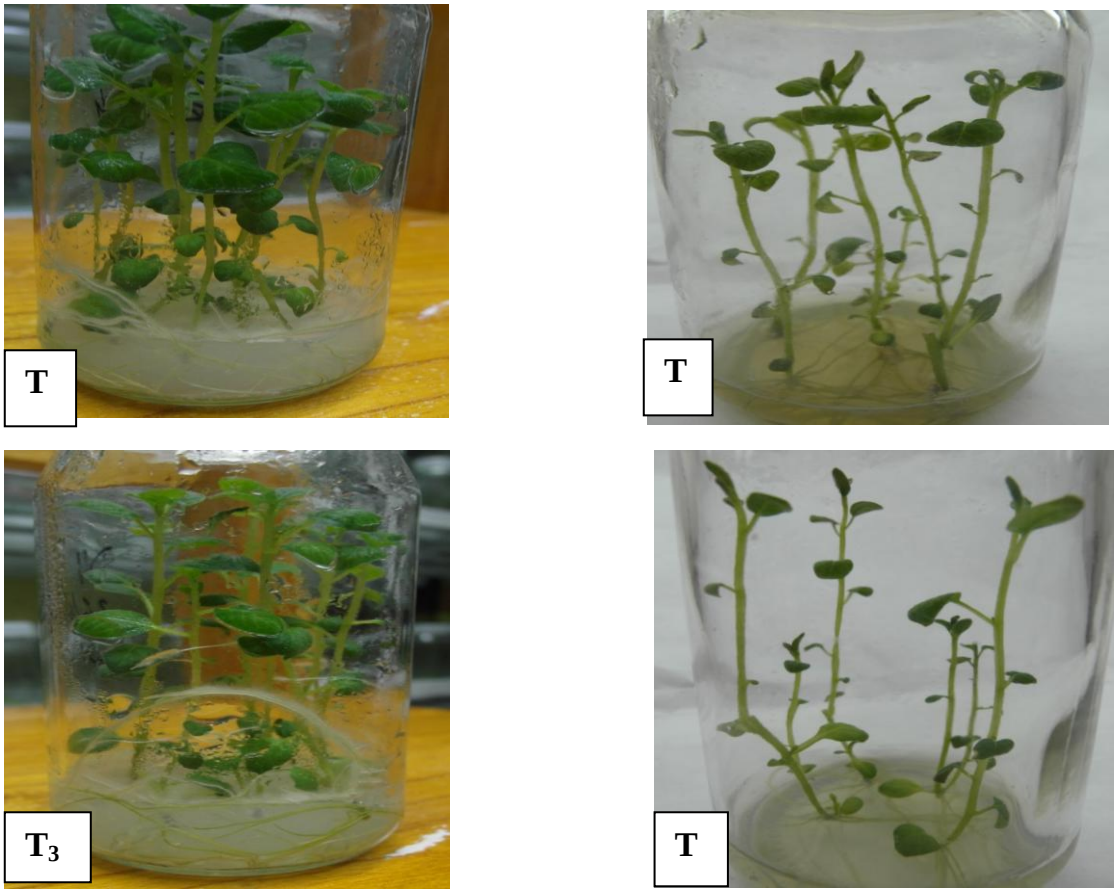
4.II.6.2 Effect of treatment on the root number

Among the four treatments, the maximum root number (4.50) was found in ready MS powder standard dose (T_1) which was statistically different from all other treatments at 7 DAS. While minimum root number (3.44) was found in with 5 gmL^{-1} of β chemical treatment (T_4) which was statistically different from all other treatments at 7 DAS (Table 19). At 14 days after sub-culture (DAS), the maximum root number (8.16) was found in ready MS powder standard dose (T_1) which was statistically different from all other treatments. While minimum root number (5.11) was found in with 5 gmL^{-1} of β chemical treatment (T_4) which was statistically different from all other treatments at 14DAS. The maximum root number (10.93 & 10.76) were found in ready MS powder standard dose (T_1) which was statistically similar with 1 gmL^{-1} of β chemical treatment . While minimum root number (7.02 & 7.78) were found with 5 gmL^{-1} of β chemical treatment (T_4) which was statistically different from all other treatments at 21 & 30 DAS respectively (Table 19 and Plate 21).

Table 19. Effect of treatment on the root number (no.) of potato plantlet at different days after sub-culture (DAS)

Treatment	Root number (no.) at different days after sub-culture (DAS)		
	14 DAS	21 DAS	30 DAS
T_1	8.16 a	10.93 a	10.76 a
T_2	7.78 b	8.33 b	8.78 b
T_3	5.49 c	10.67 a	10.30 a
T_4	5.11 d	7.02 c	7.78 c
LSD $_{(0.01)}$	0.32	0.40	0.60
CV (%)	3.70	3.31	4.80

T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate T_3=1 gm of β chemical/Litre and T_4= 5 gm of β chemical/Litre



T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate T_3=1 gm of β chemical/Litre and T_4= 5 gm of β chemical/Litre

Plate 21: Effects of Four (4) treatments at 30 days after sub-culture (DAS) of varieties

4.II.6.3 Combined effect of variety and treatment on root number

The highest number of root (9.00,13.27,13.67) were found in combination of Diamant variety with ready MS standard dose (V_1T_1) which was statistically similar with combination of Diamant variety with 1 gmL^{-1} of β chemical treatment (V_1T_3) combination but different from all other combinations at 14, 21 & 30 DAS respectively. On the other hand, the lowest number of root (3.33, 5.93, 6.33) was found in combination of Asterix variety with with 5 gmL^{-1} of β chemical treatment (V_3T_4) which was statistically different from all other combinations at 14, 21& 30 DAS respectively (Table 20).

Table 20. Combined effect of variety and treatment on the root number of potato plantlet at different days after sub-culture (DAS)

Treatment	Root number (no.) at different days after sub-culture (DAS)		
	14 DAS	**21 DAS**	**30 DAS**
V_1T_1	9.00 a	13.27 a	13.67
V_1T_2	7.00 b	10.00 a	10.00 a
V_1T_3	8.00 c	13.00 bc	12.67 b
V_1T_4	7.00 c	8.00 e	9.00 bc
V_2T_1	8.37 b	10.13 b	9.60 b
V_2T_2	8.00 b	10.00 bc	9.23 b
V_2T_3	5.13 d	8.00 e	9.00 bc
V_2T_4	5.00 d	7.13 f	8.00 cd
V_3T_1	7.10 c	9.40 cd	9.00 bc
V_3T_2	7.00 c	9.00 d	9.00 bc
V_3T_3	4.33 e	7.00 f	7.33 de
V_3T_4	3.33 f	5.93 g	6.33 e
LSD $_{(0.01)}$	0.56	0.70	1.03
CV (%)	3.70	3.31	4.80

V_1- Diamont V_2- Cardinal V_3- Asterix

T_1= Ready made MS powder (Duchefa), T_2= 16.50 gmL^{-1} of Ammonium nitrate (Liquid MS standard dose)

T_3=1 gm of β chemical/Litre and T_4= 5 gm of β chemical/Litre

CHAPTER V

SUMMARY AND CONCLUSION

5.1 SUMMARY

The experiment was conducted at the Biotechnology Laboratory and Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh during the period of January, 2015 to June, 2016 to identify a new chemical for the substitute of explosive chemical Ammonium nitrate (NH_4NO_3) in MS media composition for *in vitro* regeneration of potato (*Solanum tuberosum* L.) The key findings were given below.

5.1.1 Sub-Experiment I: *In vitro* plantlets regeneration of potato varieties using β chemical as a substitute of NH_4NO_3

The treatment 5 gmL^{-1} and 1 gmL^{-1} of β chemical showed better result in respect of different parameters under investigation. On the contrary, 15 gmL^{-1} and 20 gmL^{-1} of β chemical showed toxic effect on plantlets and due to that, all the explants died within 21 DAS. It can be concluded that, different modifications of stock solution-A by β chemical has significant effect on plantlet regeneration and its development. The amount of β chemical in stock solution-A has tremendous role on *in vitro* regeneration of potato.

Significant variation was observed among the varieties in respect of the shoot length of potato plantlets at different days after sub-culture (DAS). The maximum length of shoot (2.93 cm, 3.61cm, 5.22 cm, 5.37 cm) were recorded in Diamant variety (V_1) which was statistically different from all other varieties at 7, 14, 21, 30 DAS respectively. On the other hand, the minimum length of shoot (2.59cm, 3.09 cm, 4.87 cm, 4.82 cm) were recorded in Asterix variety (V_3) which was statistically different from all other varieties at 7, 14, 21, 30 DAS respectively.

The maximum shoot length (7.52cm) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the treatment of 15 gmL^{-1} of β chemical and 20 gmL^{-1} of β chemical. Similar result also noticed at 30 days after sub-culture (DAS), It may be due to toxic effect of β chemical.

The maximum leaf number (2.92) was recorded in Diamant variety (V_1) which was statistically significant to all other varieties at 7 DAS. Similar result (6.56, 9.00, 9.06) was also observed at 14, 21, 30 DAS respectively in Diamant variety (V_1). On the other hand,the minimum leaf number (1.08, 3.28, 0.00, 0.00) was recorded in Asterix variety (V_3) which was statistically different from Cardinal variety (V_2) at 7, 14, 21 and 30 days after sub-culture (DAS) respectively.

The maximum leaf number (10.00) was found in combination of Diamant variety with 5 gmL^{-1} of β chemical treatment (V_1T_2) which was statistically different from all other combinations at 21 days after sub-culture (DAS). All plantlets were died at 21 (DAS) in the combination of varieties with 15 gmL^{-1} of β chemical and 20 gmL^{-1} of β chemical.

At 14 days after sub-culture (DAS), the maximum node number (5.69) was found in 1 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments. On the other hand, the minimum node number (1.47) was found in 20 gmL^{-1} of β chemical treatment (T_6) which was statistically different from all other treatments. The morphological appearance of plantlets in the treatment (T_5) with 15 gmL^{-1} of β chemical and treatment (T_6) with 20 gmL^{-1} of β chemical were turning to brownish and yellowish color.

The maximum node number (7.67) was found in 1 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the treatment of 15 gmL^{-1} of β chemical and 20 gmL^{-1} of β chemical. Similar result also noticed at 30 days after sub-culture (DAS).

The highest number of node (6.00) was found in combination of Diamant variety with 1 gmL^{-1} of β chemical (V_1T_2) which was statistically different from all other combinations at 14 DAS, While the lowest number of node (1.07) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically different from all other combinations at 14 Days after sub-culture (DAS). The morphological appearance of plantlets in the combination of varieties with 15 & 20 gmL^{-1} of β chemical was turning to brownish and yellowish.

The maximum node number (9.33) was found in combination of Diamant variety with 1 gmL^{-1} of β chemical treatment (V_1T_2) which was statistically different from all other combinations at 21 days after sub-culture (DAS).
All plantlets were died at 21 days after sub-culture (DAS) in the combination of varieties with 15 & 20 gmL^{-1} of β chemical treatment in (V_1T_5 , V_1T_6 , V_2T_5 ,

V_2T_6, V_3T_5, V_3T_6) combinations. Similar result also noticed at 30 days after sub-culture (DAS).

The maximum root length (8.80 cm) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the treatment of 15 & 20 gmL^{-1} of β chemical. Similar result also noticed at 30 days after sub-culture (DAS).

The highest length of root (7.00 cm) was found in combination of Diamant variety with 5 gmL^{-1} of β chemical (V_1T_2) which was statistically different from all other combinations at 14 DAS, While the lowest length of root (0.97 cm) was found in combination of Asterix variety with 20 gmL^{-1} of β chemical (V_3T_6) which was statistically similar to combination of Cardinal variety with 20 gmL^{-1} of β chemical (V_2T_6 ,0.90 cm) & combination of Diamant variety with 20 gmL^{-1} of β chemical (V_1T_6 , 1.00 cm) but different from all other combinations at 14 Days after sub-culture (DAS).

The maximum root length (10.10 cm) was found in combination of Diamant variety with 5 gmL^{-1} of β chemical treatment (V_1T_2) which was statistically different from all other combinations at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the combination of varieties (Diamant, Cardinal & Asterix) with 15 & 20 gmL^{-1} of β chemical treatment (V_1T_6, V_2T_6, V_3T_6) combinations. Similar result also noticed at 30 days after sub-culture (DAS), It may be due to toxic effect of β chemical in MS media concentration.

At 14 days after sub-culture (DAS), the maximum root number (8.00) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments. On the other hand, minimum root number (2.89) was found in 20 gmL^{-1} of β chemical treatment (T_6) which was statistically different from all other treatments.

The maximum root number (10.67) was found in 5 gmL^{-1} of β chemical treatment (T_2) which was statistically different from all other treatments at 21 days after sub-culture (DAS). All plantlets were died at 21 days after sub-culture (DAS) in the treatment of 15 & 20 gmL^{-1} of β chemical. Similar result also noticed at 30 days after sub-culture (DAS), It may be due to toxic effect of β chemical.

5.1.2 Sub-Experiment II: Comparative performance of different MS media for *in vitro* regeneration of potato

The regenerated plantlets reached optimum growth level at 21 DAS. The key finding of present experiment proved that 5 gmL^{-1} of β chemical showed the best performance on *in vitro* regeneration of potato in three varieties. Some parameters showed non significant difference among the treatment ready MS powder, standard dose of ammonium nitrate (NH_4NO_3) and 5 gmL^{-1} of β chemical. The variety and different formulations interaction showed that 5 gmL^{-1} of β chemical in Diamant variety produced highest shoot length and leaf number. It indicates that urea has positive effect on growth of potato plantlets. The objective of present study was to find out any alternate chemical which could be used instead of NH_4NO_3 in plant tissue culture medium. Hence, it is concluded that urea can be used as a substitute of NH_4NO_3 for the preparation of tissue culture medium.

The maximum length of shoot (5.03cm, 7.04 cm, 8.63 cm, 10.06 cm) was recorded in Diamant variety (V_1) which was statistically different from all other varieties at 7, 14, 21 & 30 DAS respectively. On the other hand, the minimum length of shoot (4.53 cm, 6.65 cm, 8.04 cm & 9.5cm) were recorded in Asterix variety (V_3) which were statistically different from others variety at 7, 14, 21 & 30 days after sub-culture (DAS) for the same traits.

Among the four treatments, the maximum shoot length (7.33, 8.60 and 9.93 cm) was found in ready made MS powder standard dose (T_1) which was statistically non significant to 5 gmL^{-1} of β chemical (T_3) treatment at 14, 21 and 30 DAS respectively. While minimum shoot length (6.02, 7.98, 9.28 cm) was found in 5 gmL^{-1} of β chemical (T_4) which was statistically different from all other treatments at 7, 14, 21. 30 DAS respectively.

The maximum leaf number was recorded in Diamant variety (V_1) which was statistically significant to all other varieties at 7 DAS. Similar result was also observed at 14, 21, 30 DAS respectively in Diamant variety (V_1). On the other hand,the minimum leaf number was recorded in Asterix variety (V_3) which was statistically different from Cardinal variety (V_2) at 7 days after sub-culture (DAS). Significant variation was also noticed at 14, 21 & 30 DAS for the same traits.

Among the four treatments, the maximum leaf number (2.71) was found in ready MS powder treatment (T_1) which was statistically different from all other treatments at 7 DAS. While minimum leaf number (1.56) was found in 5 gmL^{-1}

of β chemical (T_4) which was statistically different from all other treatments at 7 DAS.

The maximum leaf number (6.77, 9.11, 9.30) were found in ready MS powder treatment (T_1) which was statistically non significant (6.42, 8.82, 9.06) with 16.50 gmL^{-1} of ammonium nitrate but significant from all other treatments at 14, 21& 30 DAS respectively.

While minimum leaf number (4.44, 4.22, 4.51) were found in 5 gmL^{-1} of β chemical (T_4) which was statistically different from all other treatments at 14, 21& 30 DAS respectively.
The highest node number (1, 5.71. 7.92, 8.52.) was recorded in Diamant variety (V_1) which was statistically significant to all other varieties at 7, 14, 21 and 30 DAS respectively.

On the other hand, the lowest leaf number (1, 4.39, 5.79, 6.56) was recorded in Asterix variety (V_3) which was statistically different from Cardinal variety (V_2) at 7, 14, 21 and 30 days after sub-culture (DAS).

The maximum node number (6.06 ,7.51 , 8.39) were found in ready MS powder treatment (T_1) which was statistically different from all other treatments at 14, 21& 30 DAS respectively. While minimum node number (3.89, 5.23, 6.09) were found in 5 gmL^{-1} of β chemical (T_4) which was statistically different from all other treatments at 14, 21& 30 DAS respectively.
The maximum length of root (0.65cm, 5.17 cm, 8.48 cm & 12.35 cm) was recorded in Diamant variety (V_1) which was statistically different from all other varieties at 7, 14, 21 and 30 DAS respectively. On the other hand, the minimum length of root (0.54cm, 4.17 cm, 6.43 cm & 11.24 cm) was recorded in Asterix variety (V_3) which was statistically different from others variety at 7, 14, 21 & 30 DAS for the same traits.

At 14 days after sub-culture (DAS), the maximum root length (6.11cm) was found in liquid MS standard dose (T_2) which was statistically different from all other treatments. While minimum root length (3.11cm) was found in 5 gmL^{-1} of β chemical (T_4) which was statistically different from all other treatments at 14 DAS.

The maximum root length (8.72 cm & 14.00 cm) were found in ready MS powder standard dose (T_1) which was statistically different from all other treatments. At 21 and 30 DAS respectively. While minimum root length (5.70 cm & 12.47 cm) were found in 5 gmL^{-1} of β chemical (T_4) which was statistically different from all other treatments at 21 & 30 DAS respectively.

5.2.1 CONCLUSION

In vitro regeneration capacity, shoot and root morphology of three potato varieties were investigated in two experiments. The key findings revealed that, 1 gmL^{-1} or 5 gmL^{-1} of β chemical showed the best performance on *in vitro* regeneration of potato in three varieties. Higher dose 15 & 20 gmL^{-1} of β chemical showed toxic effect on requirements. Although, in some cases there was no significant difference among the standard dose of Ammonium nitrate (NH_4NO_3) and 1 & 5 gmL^{-1} of β chemical. β chemical can be used as a substitute of NH_4NO_3 for the preparation of tissue culture media. Formulation of β chemical concentration in stock solution-A is our new invention. Hence, it is patentable discovery of our experiment. The regenerated plantlets reach optimum growth condition at 21 days after sub-culture (DAS). Our present finding proved that, β chemical can be used as a substitute of NH_4NO_3 for the preparation of tissue culture media. Among the three varieties, the Diamant variety showed best performance in most of the morphological traits under *in vitro* condition.

5.2.2 Merits or advantages of newly identified β chemical for tissue culture

1. It is very cheap. MS powder is expensive, 220 gm MS powder's price is 8000/- but our new chemical have to pay only 5000/- only.
2. It is aviable in all over the country.
3. It is familiar to all levels of user.
4. It is needed in a very small amout compared to ammonium nitrate.
5. The regeneration performance using this chemical is better than the ready made MS powder and also by use of ammonium nitrate.
6. It has no harmful effect on environment.
7. It is user frienly to all.

CHAPTER VI
RECOMMENDATIONS

The present investigation revealed that Diamant variety showed better performance under *in vitro* condition. Further research may be carried out on the following mentioned points:

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

2. Population density per culture vial can be practiced with different number of explants.

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

CHAPTER VII
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CHAPTER VIII

APPENDICES

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

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

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

Appendix II. Mean square value of the data on the shoot length of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.689**	1.304**	0.919**	1.400**
Media (B)	5	4.905**	12.454**	98.963**	142.565**
Variety (A) x Media (B)	10	0.043^{NS}	0.114**	0.094**	0.174**
Error	36	0.022	0.022	0.036	0.017

**Significant at 1% level of significance

$^{NS=}$ Non significant

Appendix III. Mean square value of the data on the leaf number of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.452**	1.943**	3.000**	1.147**
Media (B)	5	5.439**	14.356**	112.574**	143.799**
Variety (A) x Media (B)	10	0.034**	0.174**	1.610**	0.859**
Error	36	0.009	0.059	0.053	0.048

**Significant at 1% level of significance

^{NS} = Non significant

Appendix IV. Mean square value of the data on the node number of potato as influenced by different variety and tratment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.000^{NS}	7.925**	17.625**	6.771**
Media (B)	5	0.000^{NS}	23.672**	71.324**	123.195**
Variety (A) x Media (B)	10	0.000^{NS}	0.275**	2.241**	0.916**
Error	36	0.000	0.036	0.064	0.053

**Significant at 1% level of significance

^{NS} =Non significant

Appendix V. Mean square value of the data on the shoot number of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.000^{NS}	0.079**	0.112**	0.012^{NS}
Media (B)	5	0.000^{NS}	2.131**	3.976**	9.395**
Variety (A) x Media (B)	10	0.000^{NS}	0.040**	0.054**	0.009**
Error	36	0.000	0.002	0.003	0.005

**Significant at 1% level of significance

$^{NS=}$ Non significant

Appendix VI. Mean square value of the data on the root length of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.082**	5.262**	11.494**	28.882**
Media (B)	5	0.947**	34.502**	83.611**	301.274**
Variety (A) x Media (B)	10	0.013**	0.877**	2.502**	4.540**
Error	36	0.001	0.022	0.062	0.306

**Significant at 1% level of significance

$^{NS=}$ Non significant

Appendix VII. Mean square value of the data on the root number of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	4.347**	30.883**	28.169**	20.597**
Media (B)	5	43.153**	28.273**	126.582**	132.508**
Variety (A) x Media (B)	10	0.525**	0.552**	1.629**	2.031**
Error	36	0.015	0.041	0.101	0.192

**Significant at 1% level of significance

^{NS} = Non significant

Appendix VIII. Mean square value of the data on the shoot length of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.795**	0.527**	1.081**	0.634**
Media (B)	3	3.304**	3.839**	0.812**	0.682**
Variety (A) x Media (B)	6	0.029**	0.029**	0.032**	0.088**
Error	24	0.014	0.014	0.013	0.013

**Significant at 1% level of significance

^{NS} = Non significant

Appendix IX. Mean square value of the data on the leaf number of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.325**	4.931**	3.651**	3.637**
Media (B)	3	2.434**	10.090**	46.997**	47.068**
Variety (A) x Media (B)	6	0.912**	0.683**	3.602**	1.804**
Error	24	0.035	0.063	0.071	0.058

**Significant at 1% level of significance

$^{NS=}$ Non significant

Appendix X. Mean square value of the data on the node number of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.000^{NS}	5.234**	20.525**	9.576**
Media (B)	3	0.000^{NS}	9.391**	10.560**	10.289**
Variety (A) x Media (B)	6	0.000^{NS}	0.346**	1.451**	0.421**
Error	24	0.000	0.037	0.059	0.058

**Significant at 1% level of significance

$^{NS=}$ Non significant

Appendix XI. Mean square value of the data on the shoot number of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.000^{NS}	0.000^{NS}	0.000^{NS}	0.000**
Media (B)	3	0.000^{NS}	0.000^{NS}	0.917**	0.917**
Variety (A) x Media (B)	6	0.000^{NS}	0.000^{NS}	0.000**	0.000**
Error	24	0.000	0.000	0.250	0.250

**Significant at 1% level of significance

$^{NS=}$ Non significant

Appendix XII. Mean square value of the data on the root length of potato as influenced by different variety and media

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	0.037**	3.028**	15.341**	5.434**
Media (B)	3	0.173**	15.361**	20.634**	11.729**
Variety (A) x Media (B)	6	0.003**	0.694**	2.878**	1.146**
Error	24	0.002	0.031	0.116	1.084

**Significant at 1% level of significance

^{NS} Non significant

Appendix XIII. Mean square value of the data on the root number of potato as influenced by different variety and treatment

Source of variation	df	Mean square value of			
		7 DAS	14 DAS	21 DAS	30 DAS
Variety (A)	2	6.521**	17.161**	32.968**	36.799**
Media (B)	3	2.859**	21.761**	31.930**	16.999**
Variety (A) x Media (B)	6	0.650**	0.940**	0.972**	2.024**
Error	24	0.022	0.060	0.094	0.204

**Significant at 1% level of significance

$^{NS=}$ Non significant