A Short Review and a Preliminary Regeneration Study of a Commercially Important Bangladeshi Potato Variety: Diamant

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Dedicated to

my parents and my teachers

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis title entitled **'A Short Review and a Preliminary Regeneration Study of a Commercially Important Bangladeshi Potato Variety: Diamant'** submitted by the undersigned has been carried out under supervision of Dr. Aparna Islam, Professor, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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(Samia Afroz)

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List of Abbreviations

The following abbreviations have been used throughout the text

BAP	6-Benzylaminopurine
EDTA	Ethylenediaminetetraacetic acid
GA_3	Gibberelic Acid
HCl	Hydrochloric Acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kn	Kinetin
MS	Murashige and Skoog (1962) medium
NaOH	Sodium Hydroxide
mg	milligram
g	gram
ml	millilitre
l	litre

Abstract

Potato is one of the most important solanaceous tuber crops occupying the fourth position worldwide among all the food crops. Despite not being native to our country, potato production is growing in last few decades. Potato is important to tackle nutritional security as it is a very good source of many essential vitamins, minerals and also, carbohydrate. But with the climate change, salt water intrusion to inland soil and loss of cultivable land, development of high yielding variety of potato with salt tolerance is imperative. Moreover, pathogen infestation is also a problem in production. For this reason, it is essential to increase cultivation and yield of potato in Bangladesh. Plant biotechnology is a good option for improvement. But before proceeding to that, an efficient and reliable protocol for *in vitro* regeneration is necessary. For this, an extensive study of the existing *in vitro* protocols was performed here. In light of that, later, nodal explants from a high yielding farmer popular variety, Diamant, was evaluated for regeneration capacity. In terms of shooting response (92.08%), highest shoot length (11.38cm), highest rooting response (98.02%), maximum root length (15.68cm) and survivability percentage after acclimatization in nature (96%), hormone free MS media gave the best response. With increase in concentrations of cytokinins, early shoot initiation occurred but shoots became stunted. However, low concentration of Kn was seen to be favourable in terms of multiple shooting. After acclimatization in nature, highest (96%) percentage of plantlets survived that were cultured in hormone free MS media.

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Chapter – 1: Introduction

CHAPTER – 1
INTRODUCTION

Potato (*Solanum tuberosum*) is a starchy, tuber of a plant that belongs to the Solanaceae family. It is the world's fourth largest food crop and it is the third most popular crop in Bangladesh after rice and wheat, respectively in respect to land under cultivation (Sarker and Mustafa, 2002). So, it is a vegetable crop of major economic importance. Potato tuber provides a critically important element to the diets of many people in Bangladesh as a source of vitamin C and amino acids that is not provided by rice, and is a source of cash income to farmers and labourers which complements other staple crops (World Potato Atlas, 2009). It has a greater scope and potential for food security, nutritional security and poverty alleviation in Bangladesh.

1.1 Food Security

Bangladesh is predominantly an agro-based country. This agricultural sector has a profound effect on major macroeconomic objectives, such as, food security, poverty alleviation, employment generation, etc. It is a densely populated country with an area of 14.48 million ha. According to a study, net cultivable land would decrease from 8.42 million ha in 2000 to 7.89 million ha in 2025 and population would increase from 127.22 million in 2000 to 168.96 million in 2025. The per capita net cultivable land would reduce from 0.066 ha in 2000 to 0.047 ha in 2025 (Uddin, 2010).

In addition to that, coastal population in Bangladesh will become more vulnerable to salinity intrusion in a changing climate. As climate changes, this situation is believed to worsen. According to a study by The World Bank, climate change will cause significant changes in river salinity in the southwest coastal region during the dry season (October to May) by 2050. This will likely lead to shortages of drinking and irrigation water and cause changes in aquatic ecosystems. Soil salinity will also significantly increase in many areas of Barisal, Chittagong and Khulna districts leading to a significant decrease in High Yielding Variety (HYV) crops (The World Bank, 2015). With the consequence of climate change, this saltwater intrusion is gradually extending towards inland water and soil. The

total amount of salinity affected land in Bangladesh was 83.3 million hectares in 1973, which had been increased up to 102 million hectares in 2000 and the amount has raised to 105.6 million hectares in 2009 and continuing to increase (Mahmuduzzaman et al., 2014). Therefore, new coping mechanisms need to be adopted to address this impending situation to feed the dense population within limited amount of land.

1.2 Characteristics of potato plant

Potato plants are herbaceous perennials that grow about 60 cm high depending on variety (Fig 1.2).

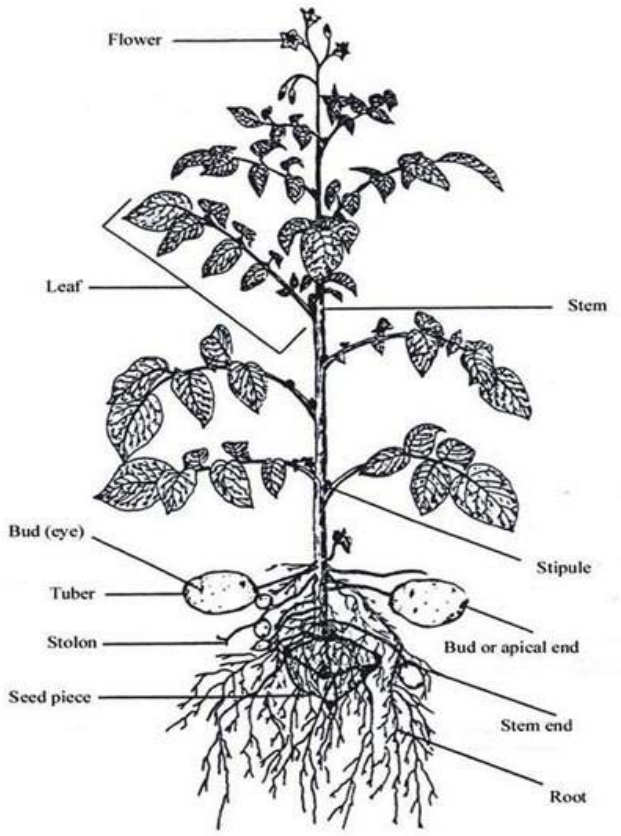

Figure 1.2: A typical potato plant

The plant is sparsely hairy. Leaves are spirally arranged; each leaf is 20–30 cm long and consists of a terminal leaflet and two to four pairs of leaflets. The stems extend underground into structures called stolons. The ends of the stolons may enlarge greatly to

form a few to more than 20 tubers, of variable shape and size, usually ranging in weight up to 300 grams (10 ounces) but occasionally to more than 1.5 kg (3.3 pounds) (Britannica, 2018). As the potato plants become mature and the tubers are fully formed, the leaves become gradually yellowish and then brownish, and finally the plants die. It is always better to harvest the crop after these signs are evident in the field (Banglapedia, 2014).

1.3 Suitable growing conditions

Potatoes are essentially a crop which thrive in cooler conditions (Kerr, 2012). It is fundamentally a winter crop with temperature being a limiting factor on production: tuber growth is sharply inhibited in temperatures below 10°C (50°F) and above 30°C (86°F), while optimum yields are obtained where mean daily temperatures are in the 18 to 20°C (64 to 68°F) range. This is why, potato is widely cultivated in all the districts of Bangladesh during winter when the temperature is around 15–21°C and varieties are harvested during February-March period.

The potato is a very accommodating and adaptable plant, and will produce well without ideal soil and growing conditions. The potato can be grown almost on any type of soil, except saline and alkaline soils. Naturally loose soils, which offer the least resistance to enlargement of the tubers, are preferred, and loamy and sandy loam soils that are rich in organic matter, with good drainage and aeration, are the most suitable. Soil with a pH range of 5.2-6.4 is considered ideal (FAO, 2008).

1.4 Potato cultivation technique in Bangladesh

Potato is widely cultivated in all the districts of Bangladesh. Total area under potato crop was estimated 11,74,978 acres (4,75,488 hectares) and an estimate of 94,74,098 metric tonnes of potatoes were produced in year 2015-2016 (Bangladesh Bureau of Statistics, 2017). Virtually all potatoes are planted manually. Row spacing is usually from 45 to 60 cm, with optimal depth of planting depending on local soil type and moisture. If initial planting is shallow (around 5cm deep), soil must be gradually ridged over the rows to cover the developing tubers and protect them from light and pests. Mulching is a common practice, utilizing locally available materials, such as, rice straw and water

hyacinth, to preserve soil moisture and control weeds. Harvesting is also performed manually, using spades or other simple tools (World Potato Atlas, 2009).

1.5 Pests and diseases

Potatoes are vulnerable to many pests and diseases. Cutworm, crickets, leafhoppers, potato tuberworm, aphids, flea beetles, root knot nematode, and golden nematode are the notorious pests and cause most of the damage. Various fungal, viral and bacterial attacks lead to serious constraints in potato production.

1.5.1 Potato pests

Cutworms (*Agrotis ipsilon*), aphids (*Macrosiphum euphorbiae*), potato tuberworms (*Phthorimaea operculella*), leaf hoppers (*Empoasca fabae*) are the major insect pests of potato in Bangladesh. Among these insect pests, cutworm attacks seedling. Aphids produce no significant impact on potato yield unless they are especially numerous. Aphids pierce veins, stems and growing tips while, potato tuberworm feeds on potato leaves, stems, petioles, and more importantly potato tubers in the field and in storage, leaf hopper causes the curling up of leaves. Some other minor insects include leaf miner (*Liriomyza huidobrensis*), field cricket (*Gryllotalpa pennsylvanicus*), yellow mites (*Polyphagotarsonemus latus*) and many more (Islam, 2015).

1.5.2 Potato diseases

Late Blight is the most serious and widespread of all potato diseases. The causal organism is *Phytophthora infestans*, a parasitic fungus. The first signs of the disease are brownish to black lesions on any portions of the plant tops, principally on the leaves (Banglapedia, 2014). This downy mildew fungus was responsible for the Irish Potato Famine, also known as the Great Hunger, in Ireland in 1845. The infestation ruined up to one-half of the potato crop that year, and about three-quarters of the crop over the next seven years. Since the farmers of Ireland had relied heavily on the potato, as a source of food, the infestation had a catastrophic impact on Ireland and its population. Before it ended in 1852, the Potato Famine resulted in the death of roughly one million Irish from starvation and related causes, with at least another million forced to leave their homeland as refugees (Irish Potato Famine, 2017).

Early Blight is another serious fungal infection caused by *Alternaria solani*. Brown to black spots with concentric rings develop on leaves during this disease. These spots appear in numerous numbers and get scattered all over the leaves, occurring before tuber initiation but continuing to develop until the death of the affected plant (Agropedia, 2010). Both Late Blight and Early Blight are found in Bangladesh.

Among the bacterial diseases, Brown Rot is an alarming disease at present and previous time in context of Bangladesh. The causative agent of this disease is *Ralstonia solanacearum*. The major areas of Bangladesh have faced many hampers due to this disease. The potato growers and businessmen of Bangladesh are facing much problems on this disease especially in case of export (Chakraborty and Roy, 2016). This disease causes the leaves to wilt, shrivel, and finally, leads to the death of the plant (Banglapedia, 2014).

The Black Scurf disease, although not usually very serious, may cause occasionally considerable damage. It is a fungal disease of potatoes and is caused by *Rhizoctonia solani*. It is usually found as irregular, black, scab-like marks on the skin of the tubers. They are easily scraped off with a fingernail and this releases a distinctive 'fungal' smell. (http://www.downgardenservices.org.uk/potato_black_scurf.htm, date: 21. 05. 18).

1.6 Uses of potato

Potatoes are a very popular food and particularly used as a vegetable in our country. However, they constitute the staple nourishment in numerous countries on the world. Potatoes are a healthy source of vitamin B6 and are also good source of potassium, copper, vitamin C, manganese, phosphorus, niacin, dietary fibre, and pantothenic acid. Potatoes also contain a variety of phytonutrients that have antioxidant activity. Among these important health-promoting compounds are carotenoids, flavonoids, and caffeic acid, as well as unique tuber storage proteins, such as, patatin, which exhibit activity against free radicals (The World’s Healthiest Foods, 2018).

They are utilized as fodder for livestock. Potato starch is used in the food industry as a thickener and binder for soups and sauces, in the textile industry as an adhesive, and for

the manufacturing of papers and boards. In addition to these, potatoes are also used in the production of alcoholic beverages.

1.7 Potato varieties

There are several hundred varieties of potatoes grown worldwide. These contrast in appearance, structure, size and colour, time of development, etc. It is not necessary that all the varieties must grow well in one area. Potato varieties that are cultivated in Bangladesh are classified as either locals or high yielding varieties. There are about 27 local varieties which include commercial and indigenous ones. However, many high yielding varieties have been brought to Bangladesh and tried experimentally under local conditions before being recommended for general cultivation. Many of these varieties have been introduced in the Bangladeshi market in last few years. (Banglapedia, 2014).

1.7.1 Diamant variety of Bangladesh

The potato variety that has been used in this study is the Diamant variety. Diamant is both high-yielding and farmer popular cultivar. It is originally a Holland variety but modified by Bangladesh Agriculture Research Institute (BARI) and was released in the year 1993. This variety is virus tolerant (Digital Herbarium of Crop Plants, 2017). The tuber is pale yellow in colour. It has smooth skin having an oval or oblong shape and is cultivated throughout Bangladesh during the month of November. Yield ranges from 18-24 metric tonnes per hectare (Banglapedia, 2014).

According to a study by Haque et al. (2012), Diamant is the highly adopted potato variety in Bangladesh. On an average, 48% of all the land, subjected to potato cultivation, is covered by Diamant variety and farmers are more enthusiastic in cultivating this cultivar due to its higher demand and yield compared to other varieties (Haque et al., 2012).

1.8 Objectives

Under the current scenario of Bangladesh to meet the demand of potato seedlings by producing virus free plantlets, the present study was conducted to achieve following objectives:

1. A short review on *in vitro* regeneration protocol of potatoes in Bangladesh
2. Determination of best shooting medium of Diamant variety
3. Determination of best rooting medium of the same variety
4. Acclimatization of the regenerated plantlets.

Chapter 2: Materials and Methods

CHAPTER – 2
MATERIALS AND METHODS

This study was carried out at the Plant Biotechnology & Biosafety Laboratory of BRAC University. The materials and methods used in this study are described below.

2.1 Materials

2.1.1 Plant material

In this study, potatoes of Diamant variety were supplied by ACI Agribusiness, ACI Limited. The potatoes were, then, allowed for sprouting in the laboratory of BRAC University.

2.1.2 Sterilizing agents

Trix® was used in the initial washing of the potatoes. Besides, 70% ethanol and mercury chloride ($HgCl_2$) were used in the major sterilization step.

2.1.3 Components of stock solutions for plant regeneration media

To prepare the appropriate stock solutions of plant regeneration media, following components were needed in particular amounts (Table 2.1 and Table 2.2):

Table 2.1: Components of macro and micro nutrient stock solutions:

Macro components	Amount (mg/l) (10X strength)	Micro Components	Amount (mg/l) (100X strength)
KNO_3	1900	KI	0.83
NH_4NO_3	1650	H_3BO_3	6.2
$MgSO_4.2H_2O$	370	$MnSO_4.4H_2O$	22.3
$CaCl_2.2H_2O$	440	$ZnSO_4.7H_2O$	8.6
KH_2PO_4	170	$Na_2MoO_4.2H_2O$	0.25
		$CuSO_4.5H_2O$	0.025
		$CoCl_2.6H_2O$	0.025

Table 2.2: Components of Iron EDTA and Organic stock solutions:

Iron EDTA Components	Amount (mg/l) (100X strength)	Organic Components	Amount (mg/l) (100X strength)
$FeSO_4.7H_2O$	27.8	Nicotinic Acid	0.5
$Na_2EDTA.2H_2O$	37.3	Pyridoxin HCl	0.5
		Thiamine HCl	0.1
		Glycine	2.0

2.2 Methodology

2.2.1 Database search for review

The search engine that was used for the review was Google Search. The main keywords were *in vitro* regeneration, potato, Diamant, Bangladesh, etc. The journals that were accessed to retrieve articles were Banglajol, Bangladesh Association for Plant Tissue Culture & Biotechnology, ResearchGate, etc.

2.2.2 Preparation of Mercury Chloride (HgCl2) stock solution

To prepare 0.1% of $HgCl_2$ solution, 1g of $HgCl_2$ powder was measured and was dissolved in 1000ml of distilled water.

2.2.3 Media preparation

In this study, MS medium (Murashige and Skoog, 1962), supplemented with ten combinations of BAP and Kn, were used for shoot induction. Half strength MS medium was used for rhizogenesis.

2.2.3.1 Preparation of stock solutions required for MS medium

For the preparation of MS medium, many constituents are needed in respective quantities. To make the work easier, stock solutions of macro-nutrients, micro-nutrients, organics and Na-Fe-EDTA were prepared before media preparation.

The four different stock solutions were made by dissolving their respective constituents (as mentioned in Table 2.1 and Table 2.2) one-by-one in distilled water. These stocks were then mixed together with other components like phytoormones, as needed, to prepare MS medium or ½ MS medium.

2.2.3.2 Preparation of plant growth regulatory hormones

In this study, cytokinins BAP and Kn were used in the regeneration media. Both of the hormones dissolve readily in NaOH. So, 10 mg of each hormone was first dissolved with a few drops of NaOH. Then, solutions were volume with 100 ml of distilled water. The hormone stocks were, later, added to MS media in appropriate quantities to prepare shooting media.

2.2.3.3 Preparation of regeneration media

The MS medium was used throughout the study for regeneration of plantlets. The medium was prepared according to following calculations (Table 2.3):

Table 2.3: Components and their quantities required to prepare 1L basal MS medium

Components	Amount for 1L MS medium	Amount for 1 L ½MS medium
Macronutrients (10x)	100 ml	50 ml
Micronutrients (100x)	10 ml	5 ml
Organic (100x)	10 ml	5 ml
Na-Fe-EDTA (100x)	10 ml	5 ml
Sucrose	30 g	30 g
Myo-inositol	0.1 g	0.1 g
Agar	8.0 g	8.0 g

All the components were carefully measured and taken into a conical flask. If hormone supplements were needed, the solutions were added too upon requirement. Then, the final volume was made to 1000 ml by adding distilled water. The pH of the media was adjusted to 5.8 by either NaOH or HCl. Then, agar was added in 0.8% (w/v) ratio and the

media was heated in a microwave oven. Finally, the media was split into several test tubes and were sealed properly with two layers of aluminium foil papers and sterilized by autoclaving at 15psi pressure at 121°C for 20 minutes (Model: CL – 32L, ALP Co., Ltd). The media was cooled and stored at 25±2°C.

2.2.3.4 Preparation of rooting media

The *in vitro* shoots which did not develop roots were transferred to rooting media. For rooting, ½ strength MS media without hormone was used.

2.2.4 Sterilization of the sprouts

The sprouts were washed in running tap water with few drops of Trix® for an hour. Then, they were taken under laminar hood and washed with distilled water. After that, the sprouts were rinsed thoroughly with 70% ethanol for 30 seconds and washed with distilled water twice. Finally, they were rinsed thoroughly with 0.1% $HgCl_2$ (w/v) solution for 3 minutes and washed with distilled water thrice to remove any traces of impurities.

2.2.5 Excision and inoculation of sprouts

After the sterilization procedure, the ends of some of the sprouts turned brownish. So, those parts were excised off with the help of sterilized scalpel and forceps and then, they were placed into sterile test tubes containing regeneration media. The inoculated test tubes were incubated at around 22 ± 2°C with a photoperiod of 16/8 hrs light/dark cycle at 2000-3000 lux light intensity with cool white fluorescent lights. The relative humidity was 60-65%. The test tubes were checked daily to note the response and the development of contamination.

2.2.6 Collection and inoculation of sprouts

After the sprouts regenerated into well developed shoots, nodal segments were collected as explants and were placed into sterile test tubes containing regeneration media with the help of sterilized scalpel and forceps and the inoculated test tubes were kept in the same culture conditions.

2.2.7 Subculture of explants

The cultures were subcultured on fresh media regularly after 4 weeks depending on regeneration response. Regular monitoring was also done to check any morphological change.

2.2.8 Root induction

The well developed shoots which did not grow proper roots were transferred to rooting media to initiate proper rooting.

2.2.9 Acclimatization of *in vitro* plantlets

The shoots that had developed proper and sufficient roots in the rooting media were then transferred to soil for acclimatization in nature. The plantlets were taken out of test tubes and washed carefully under running tap water to remove any trace of media. The water flow had to be sufficiently low and roots were gently pressed to avoid breakage. Once the roots were cleaned, plantlets were transferred to little pots containing autoclaved soil mixed with organic fertilizer at a ratio of 4:1 respectively. They were watered just enough to moisten the soil. The pots were covered with perforated clear plastic bags, and water was sprayed on the inside of the bag. After 2 weeks, the plastic bags were daily removed and replaced, with the duration of removal gradually increasing until they were no longer needed.

Chapter 3: Results

CHAPTER – 3
RESULTS

3.1 Plant biotechnological endeavours on potato varieties in Bangladesh: a review

Potato (*Solanum tuberosum* L.) is a major vegetable that plays a vital role in global food and nutritional security and alleviation of poverty, especially in Bangladesh. Although, the soil and climatic conditions of Bangladesh are favourable for potato cultivation, every year production is hampered due to use of low quality seeds (Shaheb et al., 2014). This makes potato the subject of constant interest and numerous studies have been conducted in our country to ameliorate the issue including those classified as plant biotechnology. In the rapidly developing field of plant biotechnology, the use of plant *in vitro* culture techniques enable us to excise from plants and culture their cells, tissues, organs or even the whole plants under controlled, axenic laboratory conditions (Vinterhalter et al., 2008).

In vitro regeneration allows potato plantlets to be multiplied an unlimited number of times, by cutting them into desired explants and cultivating the cuttings. The plantlets can either be induced to produce small tubers directly within containers or transplanted to the field, where they grow and produce potato tubers naturally (Potato and Biotechnology, 2008). *In vitro* tuberization not only enables maintenance and propagation of disease-free planting materials in the laboratory for the potato breeders and farmers but also produce planting materials which are convenient to handle in transport, storage, and field planting (Zakaria et al., 2014).

In Bangladesh, the Ministry of Agriculture has shown awareness to expand the production of quality potato seeds, their preservation and distribution. Many agencies like Bangladesh Agricultural Research Institute (BARI), Bangladesh Agricultural Development Corporation (BADC), Department of Agriculture Extension (DAE), Seed Certification Agency (SCA) etc. are working together to improve the potato sector of our country by producing breeder seeds of potato through tissue culture technology in a bid to reduce import dependence, collecting local and foreign germplasm to innovate high-yielding, salt and drought-tolerant varieties and so on. At the same time, the government

is also encouraging private sector to strengthen stable seed potato production and supply system etc. (Shaheb et al., 2014).

As far as data could be retrieved, tissue culture of potato was initiated in our country by S. C. Debnath (1991) who compared the growth of the *in vitro* potato cultures in different nutrient media where MS medium was found to be better responsive. Afterwards, in another prefatory study by Hossain (1994), the relation to different nodes and the different position of nodes into culture media was determined and concluded that second node was the best performer for most of the characteristics, such as, days to new shooting and rooting, plant height and growth rate, etc. However, in the following years, much attention was shed onto the *in vitro* regeneration of sweet potatoes until the early 2000s when interest got diverted into local and commercial potatoes.

Most of the data presented in this review part comes from research articles published in leading journals of our country specialised for basic or applied plant research which is summarised below (Table 3.1):

Table 3.1: Summary of some of the major tissue culture work conducted on potatoes in Bangladesh.

Article titles	Authors (Year)	Varieties	Explants	Findings
Regeneration and *Agrobacterium*-mediated Genetic Transformation of Two Indigenous Potato Varieties of Bangladesh	R. H. Sarker and Barkat Murtaja Mustafa (2002)	Lal Pakri and Jam Alu	Nodal segments	- Lal Pakri showed better response in terms of higher number of shoots, nodes and shoot length - Best shooting medium was semi-solid MS medium + 1.0 mg/l BAP + 0.1 mg/l GA_3

Article titles	Authors (Year)	Varieties	Explants	Findings
				- Best rooting medium was ½ MS + 0.1 mg/l IAA
In vitro Microtuberisation in Potato Obtained from Diverse Sources	M. J. Hossain (2005)	17 varieties including Cardinal, Diamant, Heera, Lal Pakri, etc.	Meristem	- Microtuberisation potential, is a variety-dependent character, influenced by environmental factor - In presence of light, tuber initiation appears earlier than in dark and produces higher number of microtubers
In vitro propagation of Elite Indigenous Potato of Bangladesh (Indurkani)	Sardar Nasir Uddin (2006)	Indurkani	Nodal segments	- Combined effect of Kn and BA is better for shooting than Kn alone - Combination of 2.0 mg/l Kn and 3.0 mg/l BA gave the best shooting response - For rooting, MS + 0.5 mg/l IBA gave the best result

Article titles	Authors (Year)	Varieties	Explants	Findings
Effect of Nitrogen and Potassium on *in vitro* Tuberization of Potato	Zakaria et al. (2007)	Diamant	Nodal segments	- Microtuberization was delayed with increasing rate of nitrogen - As the nitrogen concentration increased, number of microtubers per plantlet reduced but average weight of microtubers increased
Agrobacterium-mediated Genetic Transformation for Local Cultivars of Potato Using Marker Genes	Rita Sarah Borna, M. I. Hoque and R. H. Sarker (2010)	Diamant, Cardinal and Granola	Nodal and intermodal segments	- Diamant showed better shooting response in MS supplemented with 4.0 mg/l BAP +1.0 mg/l IAA - Diamant showed more spontaneous *in vitro* tuberization than other varieties
In Vitro Meristem Culture and Regeneration of Three Potato Varieties of Bangladesh	Farhana Rumzum Bhuiyan (2013)	Esprit, Lady Rosseta and Meridian	Meristem	- For Esprit and Meridian, MS medium supplemented with 1.0 mg / l BAP+1.0 mg / l GA_3 was proved best for

Article titles	Authors (Year)	Varieties	Explants	Findings
				shooting - For Rosseta, MS medium fortified with 1.0 mg/l BAP+0.5 mg/l GA3 was the best - For rooting, MS medium supplemented with 0.5 mg/l IAA showed the best result
Micropropagation of Environmental Stress Tolerant Local Potato (*Solanum tuberosum* L.) Varieties of Bangladesh	Fazlima Parveen, Mahmuda Khatun and Aparna Islam (2014)	Zaubilati, Shadaguti and Challisha	Leaf, shoot apex, nodal and intermodal segments	- Nodal segment was the best responsive explant - Hormone free MS medium was found to be best for nodal explants of both Zaubilati and Challisha varieties - For rooting, MS medium supplemented with 0.5 mg/l IBA gave the best result
In vitro plant regeneration of potato (*Solanum*	Sayeed Shahriyar and Soleh Akram	Cardinal	Shoot tips and nodal segments	- Shoot tips showed better response as explants

Article titles	Authors (Year)	Varieties	Explants	Findings
tuberosum L.) at the rate of different hormonal concentration	(2015)			- MS medium with no hormonal supplementation was shown to be the best for both shooting and rooting

3.2 Protocol establishment for potato: an *in vitro* study

In light of the previous studies, this part was carried out with an aim to study *in vitro* regeneration of commercially important potato variety, Diamant. From sprouting to shooting and rooting, finally transplantation was done.

3.2.1 Comparison of sprout development through natural process and GA3 treatment

Twenty potatoes were allowed for both natural process of sprouting and treatment with 50 mg/l GA_3 solution. GA_3 is believed to influence the dormancy of potato tubers by reducing the dormancy period which can be deduced from the following data (Table 3.2):

Table 3.2: A comparison between natural and GA_3 treated sprouts in terms of different parameters

Parameters	Natural	GA_3 treated
Length (cm)	2.0 ± 0.46	3.25 ± 1.72
No. of sprouts per potato	4.5 ± 2.80	17.5 ± 2.92
Morphology	Thick	Thin
Days to sprout initiation	28 ± 3.81	8 ± 2.79

Although sprouting response was better in hormone treated sprouts, they showed more

tissue damage after the sterilization treatment. Therefore, naturally developed sprouts were used in further study (Figure 3.1).

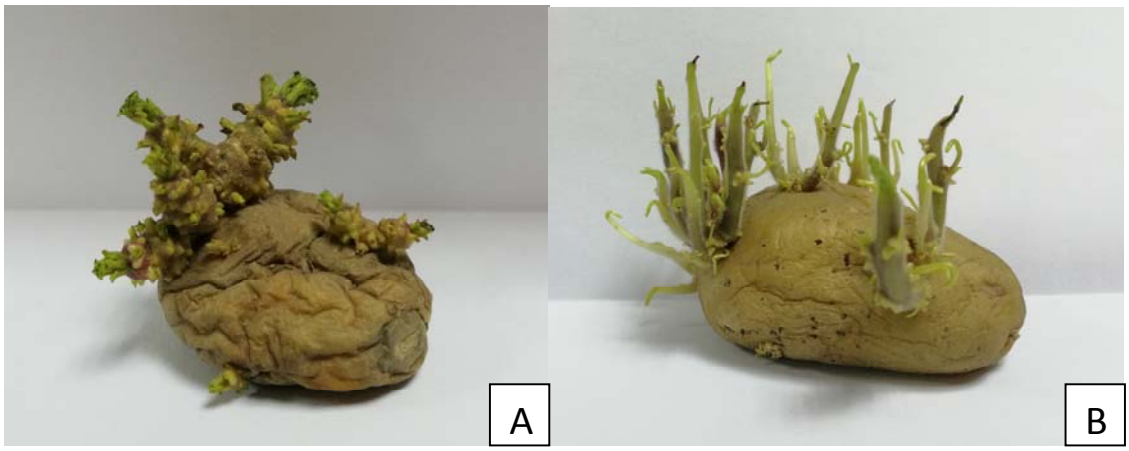

Figure 3.1: Potatoes showing **A.** natural sprouts and **B.** GA_3 treated sprouts

3.2.2 Effect of different concentrations of BAP and Kn on shoot formation

Nodal segments of Diamant cultivar were cultured on ten different hormonal treatments and data were recorded accordingly which is presented in Table 3.3.

In terms of overall shooting response, explants were seen to be better responsive in MS medium with no hormonal supplement (Figure 2B). The maximum response (92.08%) was recorded in basal MS medium followed by 3.0 mg/l BAP and 1.0 mg/l Kn (90.40%) and 3.0 mg/l BAP and 0.5 mg/l Kn (90.0%) respectively. However, the least response (65.0%) was recorded in 1.0 mg/l BAP and 1.0 mg/l Kn. This difference was believed to be due to seasonal variation since inoculation in each hormonal treatment was done at different months of the year.

All media compositions gave shoots via direct organogenesis. The explants elongated in size before shoot proliferation. However, few of them started adventitious rooting before shooting. The cytokinins significantly had an effect on time requirement for shoot initiation. With the increase in hormonal concentration, day requirement for shoot initiation gradually started to decrease. Maximum days (7.78) were recorded for basal MS, whereas, the least (3.26) was recorded for 3.0 mg/l BAP and 1.0 mg/l Kn. A slight

variation was observed for a combination of 1.0 mg/l BAP and 0.5 mg/l Kn and for 2.0 mg/l BAP singly. These two treatments were expected to have early shoot proliferation but delayed a little instead.

Different concentrations of cytokinins were also tested to find out their effects on multiple shoot induction from nodal explants. The plantlets, in average, had similar number of shoots from all the media compositions. However, the maximum (4.08) number of shoots was obtained from MS medium supplemented with 1.0 mg/l BAP. From table 3.3, it can be seen that concentrations of Kn had an effect on number of shoots. Combinations with 0.5 mg/l of Kn gave slightly more number of shoots than 1.0 mg/l Kn.

Significant variation was observed in this study regarding length of shoot. Maximum (11.38cm) length was achieved by basal MS medium. However, as the concentration of cytokinins increased, the plantlets started to exhibit poorer length and there wasn't any remarkable difference in shoot length in rest of the hormonal treatments.

3.2.3 Effect of cytokinins on *in vitro* tuberization

Effect of different levels of cytokinins on *in vitro* tuberization was also observed in this study. Despite having cytokinins or not in media, all the shoots developed *in vitro* microtubers except the shoots that were cultured on MS media having combinations of 3.0 mg/l BAP. Nevertheless, MS supplemented with 1.0 mg/l BAP and 0.5 mg/l Kn gave the highest (72.2%) number of microtubers followed by MS fortified with only 1.0 mg/l BAP (50%). The lowest (10%) number of microtubers was recorded in media that had a combination of 1.0 mg/l BAP and 1.0 mg/l Kn. It was also observed that Kn reduced the average number of microtuber per shoot (Table 3.4).

3.2.4 Analysis of rhizogenesis of the regenerated shoots

Shoots regenerated at basal MS without any hormone spontaneously produced concomitant roots. The roots were developed enough to directly go for acclimatization in nature. However, shoots that were cultured on 1.0 mg/l BAP and under combination of 1.0 mg/l BAP and 0.5 mg/l Kn, either produced very few roots or no roots at all. Hence,

adequate root induction was necessary for these regenerated shoots. These shoots were collected and placed in rooting medium which was ½ MS. This is because nutrient deficits act as powerful stimulants for rhizogenesis. The shoots from other hormonal treatments were fragile and short enough to undergo root induction.

Rhizogenesis was observed for a span of 30 days. Highest (98.02%) rooting response and highest (15.68cm) average root length were recorded for basal MS (Figure 2C). On the other hand, shoots cultured on 1.0 mg/l BAP and 0.5 mg/l Kn produced highest (8.80) number of roots although day requirement for root initiation was similar for all the shoots despite the treatment (Table 3.5).

3.2.5 Analysis of transplantation and acclimatization in nature

Plantlets that produced well developed roots were transferred to soil for acclimatization (Figure 2D). Most of the plantlets, that made it through the acclimatization phase, survived more than 100 days. Plantlets cultured in MS0 gave the highest (96%) percentage of survivability followed by the plantlets cultured in 1.0 mg/l BAP (91.7%). However, a drastic fall was observed in survivability for plantlets that were cultured in combination of 1.0 mg/l BAP and 0.5 mg/l Kn (Table 3.6).

After 100 days of acclimatization in nature, the leaves of the plantlets started to wither and eventually turned yellowish. As the plantlets died, they were uprooted to check if the respective plantlets produced any minitubers (Figure 2F).

Table 3.3: Effect of different combinations of hormonal supplementations on the *in vitro* shoot regeneration from nodal explants of Diamant potato variety

Hormones		Type of response	Average days to shoot initiation ± SD	Average number of shoots ± SD	Average shoot length (cm) ± SD	Shooting response (%)
BAP (mg/l)	Kn (mg/l)					
-	-	Direct	7.78 ± 3.39	2.35 ± 1.88	11.38 ± 1.12	92.08
1.0	-	Direct	6.96 ± 2.63	4.08 ± 2.10	5.24 ± 0.76	89.3
1.0	0.5	Direct	7.26 ± 1.09	3.40 ± 1.92	5.83 ± 0.95	83.3
1.0	1.0	Direct	5.29 ± 1.58	2.30 ± 0.94	4.20 ± 1.04	65.0
2.0	-	Direct	5.90 ± 1.91	3.30 ± 1.31	2.80 ± 0.82	76.9
2.0	0.5	Direct	5.36 ± 1.67	3.82 ± 1.60	2.18 ± 0.80	78.6
2.0	1.0	Direct	4.50 ± 1.56	3.00 ± 0.94	2.60 ± 0.35	85.7
3.0	-	Direct	4.20 ± 1.98	2.41 ± 1.23	2.27 ± 0.67	78.5
3.0	0.5	Direct	3.50 ± 1.51	3.12 ± 0.98	2.96 ± 0.49	90.0
3.0	1.0	Direct	3.26 ± 1.72	2.89 ± 0.54	2.80 ± 0.83	90.4

Table 3.4: Effect of hormonal treatments on microtuber formation in respective media compositions

Hormones		No. of shoots inoculated	Shoots developing microtubers (%)	Average no. of microtuber per shoot ± SD
BAP (mg/l)	Kn (mg/l)			
-	-	100	15	1.60 ± 0.74
1.0	-	100	50	1.86 ± 0.86
1.0	0.5	100	72	0.26 ± 0.38
1.0	1.0	50	10	0.76 ± 0.82
2.0	-	50	16	1.56 ± 0.48
2.0	0.5	50	24	0.78 ± 0.57
2.0	1.0	50	14	1.19 ± 0.69

Table 3.5: Results of *in vitro* rhizogenesis for shoots cultured in three different shooting media

Shooting media	**Rooting media**	**Average days to root initiation ± SD**	**Average no. of roots ± SD**	**Average root length (cm) ± SD**	**Rooting response (%)**
Basal MS	Basal MS	5.29 ± 2.91	7.27 ± 0.61	15.68 ± 1.13	98.02
1.0 mg/l BAP	½ MS	5.09 ± 1.56	8.30 ± 1.20	8.43 ± 1.59	96
1.0 mg/l BAP + 0.5 mg/l Kn	½ MS	5.95 ± 1.45	8.80 ± 2.43	5.64 ± 2.14	93.75

*Data was collected after 30 days of root induction

Table 3.6: Effect of different hormonal treatments on acclimatization in nature

Shooting media	**Rooting media**	**Plantlets moved to soil**	**Surviving plantlets**	**Survivability rate (%)**	**Mature height (cm) after 100 days ± SD**
Basal MS	Basal MS	25	24	96.0	18.1 ± 3.56
1.0 mg/l BAP	½ MS	24	22	91.7	17.7 ± 2.76
1.0 mg/l BAP + 0.5 mg/l Kn	½ MS	21	7	33.3	17.2 ± 3.45

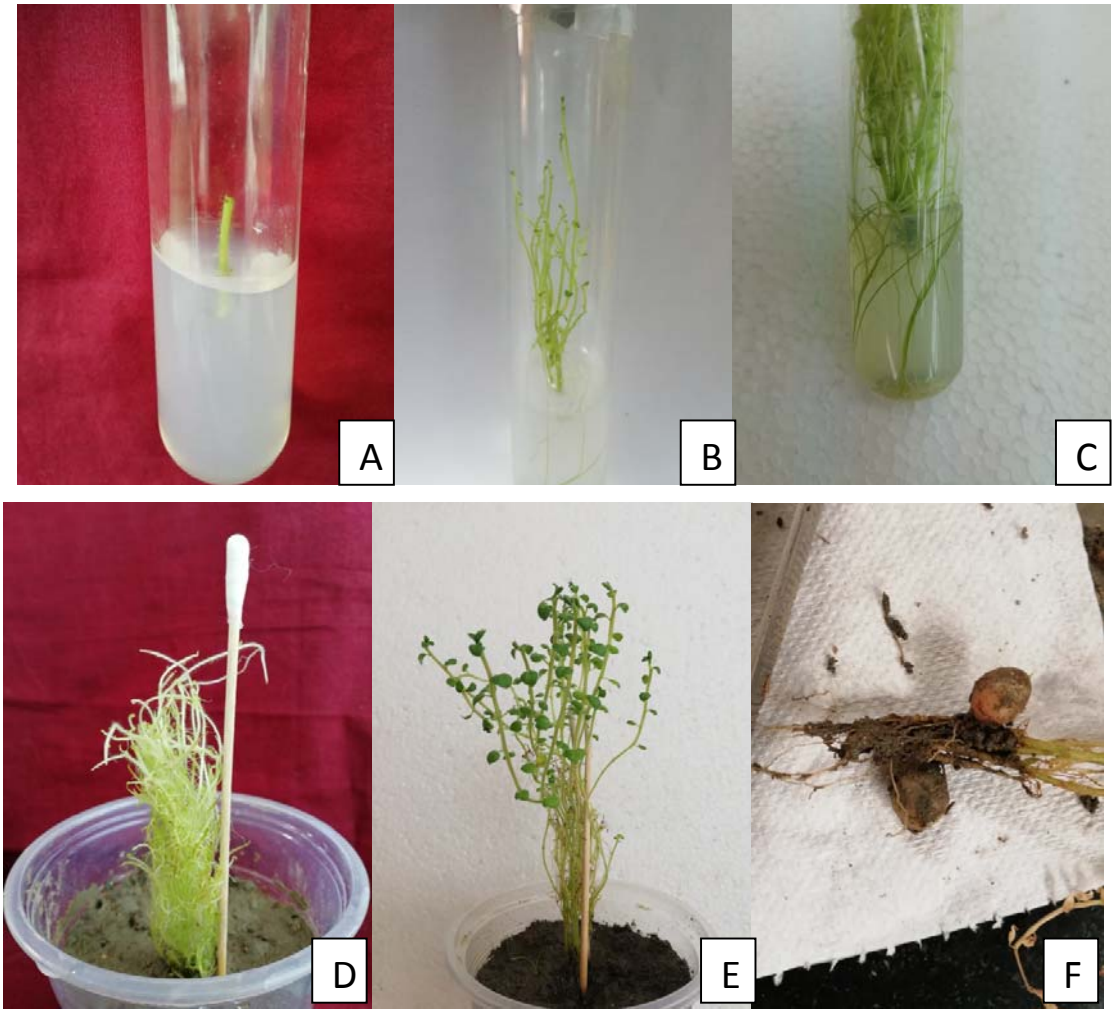

Figure 2: The *in vitro* regeneration of a potato. **A.** Nodal explant inoculated in basal MS media. **B.** Shoot formation from nodal explant in basal MS after 28days. **C.** Shoot with proper roots after 60 days in basal medium. **D.** Plantlet after being transferred to soil for acclimatization in nature. **E.** Plantlet after 28 days of acclimatization. **F.** Minitubers collected after about 120 days of acclimatization.

Chapter – 4: Discussion

CHAPTER – 4
DISCUSSION

Bangladesh is an agricultural country. Agriculture has a considerable contribution to the Gross Domestic Product (GDP) of the country (Chowdhury and Chowdhury, 2015) and potato crop is one of the most important ones. In the present study of establishing an *in vitro* regeneration protocol for potato, Diamant genotype was considered. This genotype is high-yielding, fairly resistant to pests and diseases and hence, farmer popular and commercially available. Hormone free basal media and different concentrations of cytokinins, viz. BAP and Kn were tested to find out the best media composition for micropropagation of potato. In terms of maximum shooting response, highest shoot length, highest rooting response, maximum root length and survivability percentage after acclimatization in nature, hormone free MS media gave the best response. Studies by Shahriyar et al. (2015) and Parveen, Khatun and Islam (2014) also indicated that hormone free basal media was the best for shooting regeneration by nodal explants of potato. Sanavy and Moeini (2003) also reported the same result but with different explants.

According to various studies by Liljana et al. (2012), Parveen et al. (2014) and Shahriyar et al. (2015), nodal segments responded the best as explants than any other tissue. Zakaria et al. (2007) and Sarker and Mustafa (2002) also worked with only nodal segments as explants. Hence, this present study was also conducted using nodal explants.

Although basal media delayed in shoot initiation by few days, it was outweighed by other parameters, such as, maximum shooting and rooting response, highest shoot and root length. It was also deduced that as the concentrations of BAP and Kn increased, earlier shoot initiations were observed. This was because cytokinins promote cell division in plant tissue culture. However, an exception was discerned with 1.0 mg/l BAP and 0.5 mg/l Kn. This media combination was supposed to initiate shooting earlier than 1.0 mg/l BAP singly as per the pattern. This anomaly can be rationalized by seasonal variation as inoculations in these two media were done at different months.

Increase in cytokinin concentrations also lead to stunted growth of the plantlets as stated by this study. This had a similar finding to the study by Sanavy and Moeini (2003). Their study demonstrated that application of BAP decreased plantlet length whereas, treatment without BAP produced taller plantlets.

Kn, in particular, was decided to be used in lower concentrations. According to Uddin (2010), lower concentrations of Kn were found suitable for potato micropropagation. In this present study, it was seen that 1.0 mg/l Kn inhibited shoot length and multiple shooting than 0.5 mg/l Kn which related with the study by Uddin (2010).

For *in vitro* microtuberization, 1.0 mg/l BAP and 0.5 mg/l Kn gave the highest number of microtubers followed by 1.0 mg/l BAP. In a study by Momena et al. (2014), Diamant variety performed better than other varieties and maximum number of microtubers was recorded by 1.0 mg/l BAP and 4.0 mg/l Kn. Although BAP concentration corresponded with the finding of the present study, concentration of Kn varied a lot. However, if *in vitro* microtuberization of potato is the goal of any study, then it is recommended to use 1.0 mg/l BAP in the media composition for achieving highest number of microtubers. This is because excess external use of hormones may interrupt the balance of endogenous level of growth regulator.

For rooting, hormone free MS media gave the maximum rooting response and the highest average root length. This finding was no exception to investigations by Shahriyar et al. (2015) and Sanavy and Moeini (2003). However, studies by Sarker and Mustafa (2002) and Parveen et al. (2014) showed half strength MS with little addition of auxins, 0.1 mg/l IAA and 0.5 mg/l IBA respectively, gave the best result.

After acclimatization in nature, it was observed that percentage of plantlets that grew in hormone free basal media survived the most for a period of more than 100 days. However, percentage survivability drastically fell for 1.0 mg/l BAP and 0.5 mg/l Kn. In investigations by Parveen et al. (2014) and Uddin (2010), 50% and 80% of the plantlets survived respectively.

In the light of current study, nodal segments gave better regeneration response in hormone free MS medium than any hormonal combination. Hence, regenerative tissue culture protocol can be established with only basal medium. This will likely reduce the cost of the entire process that would, otherwise, be costly if hormones were used. The present study was conducted in a small scale. In future, repetition of this protocol and also microtuberization needs to be studied. Finally, suitability of these potato plantlets for potato production in field needs to be studied.

Chapter – 5: References

CHAPTER – 5
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