DEVELOPMENT OF STRESS TOLERANT POTATO (*SOLANUM TUBEROSUM* L.) THROUGH GENETIC TRANSFORMATION

Ph.D. THESIS

BY

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Dedicated To My Respected Parents

CERTIFICATE

This is to certify that the thesis entitled "**Development of stress tolerant potato (*Solanum tuberosum* L.) through genetic transformation"** submitted by Sanjida Rahman Mollika has been carried out under our supervision in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. This is further to certify that it is an original work and suitable for submission for the award of Ph.D. in Botany.

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– The Author

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ABSTRACT

Potato (*Solanum tuberosum* L.*)* is a herbaceous and tuberous plant belongs to the family Solanaceae. *It* is one of the most commonly cultivated vegetable crops in Bangladesh as well as all over the world. Potato is energy rich, nutritious and easy to grow on small pots and ready to cook without any expensive processing. Improvement of potato production faces several constraints; various biotic and abiotic stresses limit the production and quality of potato. Abiotic stresses include drought, salinity, extreme temperature, chemical toxicity and oxidative stress that are serious threats to agriculture including potato. The severity of such issue goes big when it comes as obstacle to ensure an optimum agricultural production for a country like Bangladesh. Even if a gene for drought or salt tolerance is found in a wild relative of potato, it is difficult to integrate the gene into the cultivated potato varieties due to the reproductive barriers. Moreover, in Bangladesh true potato breeding is hampered due to absence of required day length for potato flowering

The present investigation was carried out for the establishment of *in vitro* regeneration and *Agrobacterium*- mediated genetic transformation in potato (*Solanum tuberosum* L.). Four potato varieties cultivated in Bangladesh, namely, Asterix, Diamant, Granola and Lady Rosseta were used to develop a suitable *in vitro* regeneration and efficient genetic transformation protocol for potato using marker as well as salinity and drought tolerant genes. Nodal segments, microtuber discs, leaf and intermodal segment explants were used for direct and indirect regeneration of shoots. The best responses obtained when the explants of nodal segments and microtuber discs were cultured on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA. In case of indirect shoot regeneration leaf and internodal segment explants were cultured on MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3. Among the explants, the best response towards multiple shoot regeneration was obtained from nodal segments on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA. Hormone free MS medium was found to be most effective for healthy root induction from the *in vitro* raised excised shoots. Following root induction, *in vitro* regenerated plantlets were successfully transplanted and established to soil.

Transformation experiments were performed using *Agrobacterium* strain LBA4404/pBI121 harboring *GUS* and *nptII* and EHA105/pCAMBIA 1301-PDH45 harboring *PDH45* and *hptII* genes. *Agrobacterium tumefaciens* strain LBA4404/pBI121

showed maximum transformation efficiency in nodal segment with bacterial suspension having an optical density of 0.6 at 600 nm in Asterix. Moreover, 30 min incubation period followed by 72 hrs of co-cultivation was found the most effective for transformation as determined by transient GUS histochemical assay. Transformed shoots were selected using MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA, 0.5 mg/l GA_3, 300 mg/l carbenicillin and 200 mg/l kanamycin. In case of strain EHA105/pCAMBIA 1301-PDH45, maximum transformation efficiency was observed in nodal segment with bacterial suspension having an optical density 0.5 at 600 nm, 15 min incubation and 48 hrs co-cultivation period. In this case transformed shoots were selected using 20 mg/l hygromycin. Stable integration of *GUS*, *nptII*, *PDH45* and *hptII* genes were confirmed through PCR analysis using the genomic DNA isolated from transformed shoots.

Transgenic tuber generation (TG_2) and control plants were subjected to salt and drought stress. After 15 days of treatment, plant tissues were used to evaluate different physiological parameters i.e. chlorophyll, carotenoid content, proline content, H_2O_2 content and water loss, etc. Transgenic plants challenged to NaCl stress showed better performance towards growth, chlorophyll and carotenoid content than wild type (WT) control. Similarly, less amount of water loss was observed in transgenic lines. Transgenic lines (TG_1 and TG_2 generations of *PDH45*-potato) performed well under stress condition were maintained in the greenhouse. Molecular characterization and phenotypic analysis of TG_1 generation were performed. Amplification of 1200 bp band for *PDH45* gene and 750 bp band for *hptII* gene in transformed lines confirmed presence of *PDH45* and *hptII* gene.

1. INTRODUCTION

1. INTRODUCTION

Potato (*Solanum tuberosum* L.) a common solanaceous crop ranks third among edible crops and top among non-cereal crops in terms of consumption. It is the fifth-most significant food crop in the world after wheat, maize, rice, and sugar cane (Dangol *et al.* 2018). About 1.3 billion people consume it regularly, and because of its nutrient-dense tubers, it is gaining popularity almost everywhere in the world (Stokstad 2019). It is assumed that by 2050, there will be 9.7 billion people on the planet, and the potato may be a crucial candidate crop for preventing a global food crisis (Dangol *et al*. 2018). The term "potato" can be used to describe both the plant and the edible tuber. This plant produces starchy tubers that are primarily utilized as vegetables. It is one of the most widely grown crops both in Bangladesh and around the world. Potatoes are grown in Bangladesh as a rabi crop. It leads all vegetable crops grown in Bangladesh in terms of both productivity and acreage, and it alone accounts for nearly 53% of total vegetable production.

The plant is often an erect perennial herb that is 50 to 90 cm tall, strong, glabrous, and covered in short, simple, glandular hair. The height of potato plants, which are herbaceous perennials, reaches about 60 cm (24 inch). Following flowering, fruiting, and tuber production, leaves begin to die. They produce white, pink, red, blue, or purple blooms with yellow stamens (Winch 2006). The majority of potato plants are cross-pollinated, however, self-fertilization also occurs to a significant extent. Potato plants yield little green fruits that resemble green cherry tomatoes after flowering. Fruit should not be consumed since they contain the poisonous alkaloid solanine. The seeds, also known as "true potato seed," "TPS," or "botanical seed," are used to grow all new potato cultivars.

The potato was domesticated between 7,000 and 10,000 years ago in the Andes (Spooner *et al*. 2005). Worldwide, there are around 5,000 different kinds of potato. There are more than 3,000 species, mostly found in Peru, Bolivia, Ecuador, Chile, and Colombia.. There are 200 or more species and subspecies which are found as the wild. *Solanum tuberosum*, a tetraploid with 48 chromosomes, is the most common species planted globally, and current variants of this species are mostly grown everywhere (Jacobs *et al*. 2011).

In countries with various climatological zones, including temperate, subtropical, and tropical locations, potato is grown as a major crop under various agro ecological zones. This crop is currently farmed in around 130 different nations. Shape, size, color, texture, and nutritional value of varieties vary widely. Asian countries have seen the fastest growth in potato production. In the second half of the 16th century, the Spanish brought potatoes from the Americas to Europe. Today, the potato has spread around the world and has become a staple item, making up a large portion of the global food supply.

The two main subspecies of *Solanum tuberosum*-andigena, or Andean, and *tuberosum*, or Chilean were distinguished by Spooner and Raker in 2002. *S. andigenum* Juz. and Bukasov and *S. tuberosum* L., as well as two cultivar groupings within *S. tuberosum*, have been used to refer to the Andean and Chilean tetraploid farmed potatoes. The Chilean potato, a native of the Chiloé Archipelago, is adapted to the long day circumstances common in the higher latitude region of southern Chile, unlike the Andean potato, which is adapted to the short-day conditions common in mountainous equatorial and tropical regions where it originated (Rodríguez 2007). While 100 wild species and about 10 cultivars of *Solanum* species have been identified, *Solanum tuberosum* is the only botanical species that accounts for the majority of potato variations grown worldwide (Burlingame *et al.* 2009).

Two categories of potato varieties can be found in Bangladesh: modern or high yielding variants and native or regional potato varieties. In Bangladesh, modern potato types were first introduced in the early 1960s. Native potato types are those that have been around for a while but have lost their uniqueness and have degenerated because of their low yield potential and other agricultural limitations. However, some of them are still grown and widely used in various local names in various parts of the country (Siddique 1995). Bangladesh Agricultural Research Institute has released 73 high yielding potato cultivars (BARI). Currently, the most popular potato types are Diamant, Cardinal, Asterix Granola, and Lady Rosseta.

Total area of the world under potato crop is 1,73,40,986 ha and total potato production is 37,04,36,581 tons with average yield rate of 19.5 tons per ha (BBS 2020). In Bangladesh, there are believed to be 4,68,375 ha of total potato cultivation, yielding a total of 96,55,082 metric tons of potatoes. 20,614 metric tons of potatoes are produced on average per hectare (FAOSTAT 2020).

In 2018, 368.2 million tons of potatoes were produced globally across 193.02 lakh hectares of harvested potato land (FAOSTAT 2020). More than twice as many potatoes are produced in China (18.7% of global production), compared to India, the next-largest producer.

Potato production in 2019

Country	Production (millions of tones)
China	98.3
India	48.5
Russia	22.5
Ukraine	22.5
United States	20.6
World	368.2

Source: FAOSTAT 2020

In Bangladesh, potato cultivation covered 4.99 lakh hectares in 2018, with a yield of 20.44 tons/ha and production totaling 102.1 lakh tons (FAOSTAT 2020; BBS 2020).

Potato is grown almost in all districts of Bangladesh. Rangpur, Bogura, Dinajpur, Thakurgaon, Gaibandha, Joypurhat, Munshigonj, Chandpur, Sherpur, and Kishoregonj are major potato-growing regions. Potato harvesting season runs from mid-January to mid-March. Seed tubers are sown between mid-September and mid-November.

Potatoes are still under scrutiny for their potential applications in managing health and wellbeing, including the avoidance of the onset of chronic diseases, in addition to their usage as a staple meal and source of energy. In comparison to many other vegetables, potatoes have a better overall nutrient-to-price ratio, and they are a significant staple food around the world. Potato is a crucial crop in the developing countries because it can generate significant yields on small plots of land and be marketed as a cash crop to support the incomes of small farmers (Ramani and Mouille 2019). Compared to other possible food crops, it can produce more carbohydrates, proteins, minerals, and vitamins per unit of land and take less time to grow (Zaheer and Akhtar 2016). Vitamin C, potassium, and dietary fiber are just a few of the essential elements that potatoes bring to the table (McGill *et al*. 2013). Nowadays, potatoes make up about half of all consumed root crops, making roots and tubers the third-largest source of carbohydrates in the world (International Potato Center 2018).

Potato is widely utilized in industry to produce biofuels, alcohol, starch, animal feed, and processed food goods (Liang and McDonald 2014). After soybean, potato has the highest protein content, and "patatin" is the main storage protein (Liedl *et al*. 1987). Important dietary sources of starch, protein, vitamins, and antioxidants can be found in potato tubers (Burlingame *et al*. 2009). 100 g of potatoes are thought to contain 322 (KJ) of calories, 17 g of carbohydrate, 2 g of protein, 0.09 g of fat, and 2.2 g of fiber. A medium-sized 150 g (5.3 oz) potato with the skin contains 1.3 mg iron (6% of DV), 48 mg magnesium (5% of DV), 620 mg potassium (18% of DV), 0.2 mg vitamin B6, and trace levels of thiamin,

riboflavin, folate, niacin, phosphorus, and zinc. Vitamin C is present in trace amounts as well. Depending on the makeup of the flesh, potatoes should be regarded as a vegetable with a high antioxidant capacity. Chlorogenic acid is the main phenolic component found in potatoes. The amount of flavonoids in the flesh of white-fleshed potatoes is up to 30 g per 100 g FW, while the amount in red- and purple-fleshed potatoes is about twice as high. Epicatechin and catechin are the main flavonoids (Brown 2005). Like sweet potatoes, potatoes have the greatest Harvest Index (HI), and their worth is around 60% (Mazurczyk *et al*. 2009). High HI crops are crucial for the development of sustainable civilizations in the future since HI reflects the amount of each crop that is now available.

The production and quality of potatoes are constrained by a number of biotic and abiotic stresses. Its cultivation and output suffer significant harm from biological pressures such bacteria, viruses, fungi, and insects. One of the most significant causes of its poor yield is disease. Fungal, bacterial, and viral diseases that influence the development of potato crops are brought on by farmers' intensification and inexperience, which results in significant yearly economic losses. Typically, monoculture and haphazard crop rotation are the main causes of soil-borne diseases. Lack of disease-resistant clones and inadequate germplasm may cause some illnesses to spread. There have been 57 different potato diseases reported in Bangladesh so far (Hossain *et al*. 2008). The most significant diseases among them are late blight, stem rot, Sclerotium rot, wilt, common scab, potato leaf roll, and mosaic (Ahmed *et al*. 2000). Drought, salt, extremely high temperatures, chemical toxicity, and oxidative stress are only a few of the abiotic factors that pose major risks to agriculture (Wang *et al.* 2003). Climate change, industrialization, and urbanization are just a few of the reasons that have put too much pressure on the world's food supplies and agricultural areas (Badami and Ramankutty 2015). In a world that is becoming more populated, there will be a proportionate rise in the demand for food

sources. Numerous biotic and abiotic stresses have an impact on crop production globally which contribute decreased food production. Additionally, it is essential to undertake research with objectives in nutrition, health, and agriculture. It is not unexpected that increasing emphasis is being placed on agriculture and food in the creation of new, sustainable crops to address stress-related issues. Utilizing the existing germplasm and technical breakthroughs, a number of crops with improved nutritional profiles have been produced through public-private partnerships (Ricroch and Henard-Damave 2016).

Abiotic factors that are experienced by cultivated potatoes include salinity, drought, cold, and heat extremes. Stresses from the environment are to blame for the decline in agricultural growth. The transgenic strategy is one way to reduce abiotic stress (Shimazaki *et al*. 2016). Global crop productivity is severely hampered by salinity. It is a significant abiotic stress that lowers plant productivity (Gupta and Huang 2014). Around the world, nearly 800 million ha of land, more than 6% of the total land area is susceptible to salinity (Munns 2005). Nearly 20% of the world's agricultural land and roughly 50% of all irrigated fields are affected by salinity (Zhu 2001). 40% of the world's food and feed comes from about 20% of the irrigated arable land (Kondrak *et al*. 2012). However, salt damage affects 50% of irrigated land (Geilfus *et al*. 2010). Within 25 years, about 30% of arable land will be gone, and by 2050, about 50% of arable land will be destroyed (Wang *et al*. 2003). Currently, agriculture uses 70% of the fresh water in the world (Kondrak *et al*. 2012). Worldwide, crop yields and the extent of arable land are both significantly impacted by land salinization and the deterioration of water resources, which have an adverse impact on irrigated agriculture in arid and semiarid regions (Munns and Gilliham 2015). Approximately 600 million people today live in low-elevation coastal zones as a result of growing salinization (Payo *et al.* 2017).

At the meeting of the Ganges, Brahmaputra, and Meghna river systems is Bangladesh, a low-lying, flat delta. Twenty per cent of the nation is made up of coastal regions. Numerous environmental difficulties and issues are impeding Bangladesh's coastal livelihood development. One of their most significant problems is salinity. Climate change and sea level rise both contribute to salinity, which eventually has an impact on crop output (Hossain *et al.* 2015). Salinity stress affects more than 400 million hectares of land (Martinez *et al.* 2012). Due to long periods of drought, climate change could result in increased soil surface salinity (Yeo *et al*. 2017) in Bangladesh.

A rise in arable land salinization by the middle of the twenty-first century appears to cause a loss of 50% of the arable land (Sekmen *et al*. 2007). Being a country bordering the sea, Bangladesh is particularly affected negatively by saltwater intrusion. Salinity is mostly a problem for the land and water in coastal regions. As a result of climate change, it increasingly spreads to inland water and soil. Bangladesh's total salinity-affected land area was 83.3 million hectares in 1973; it climbed to 102 million hectares in 2000; it increased to 105.6 million hectares in 2009; and it is still rising. In the past 35 years, salinity had increased by about 26% in this nation (Mahmuduzzaman *et al.* 2014).

About 29,000 km^2 (20% of Bangladesh's total land area) is made up of the country's coastal region. Out of that, 53% had salinities between medium and high levels (Haque 2006). Although different potato cultivars react differently to salinity stress, potatoes are generally thought to be a crop that is somewhat sensitive to salt (Martinez *et al*. 1996 and Ochat *et al*. 1999). Soil salinity hinders the development of tubers and plants. Salinity reduced the amount of fresh and dry tubers per plant and accelerated the aging, yellowing, and desiccation of leaves (Akhtar *et al.* 2015 and Jaarsma *et al.* 2013). Therefore, limiting the spread of salinization and improving the salt tolerance of high-yielding crops are currently the major global challenges. Global climate change, in the form of excessive

heat and drought, poses a significant threat to sustainable food production by influencing plant performance and crop yield. Increased evapotranspiration and drought severity, soil salinity, and dangers from insects and diseases are all caused by further temperature rise and ongoing greenhouse gas emissions. Plant scientists have a significant challenge in meeting the world's growing food demand, which necessitates an urgent increase in yield of important food crops by twofold. As the fourth main food crop, the production of the potato is crucial for ensuring food security for a growing population (Dahal *et al*. 2019).

In many parts of the world, drought severely restrict plant growth and terrestrial ecosystem productivity (Chaves *et al.* 2003). Nearly every regions experiences drought, which has afflicted more people globally in the past 40 years than any other natural hazard (Obidiegwu *et al.* 2015). The world's land surface is estimated to be over 35% arid or semi-arid, with precipitation levels too low for the majority of agricultural uses (Wood 2006). When a drought strikes, agriculture is both the first and most severely impacted sector, taking up to 80% of all direct impacts and having a variety of implications on agricultural production, food security, and rural lives (Haque 2006). In case of drought, the cytoplasm becomes dehydrated and loses some of its enzymatic activity. In this instance, pH shift and ions accumulation are both present (Sinha 2004). Potatoes are vulnerable to water stress, and soil water is a significant limiting factor for potato productivity and quality (McKersie and Leshem 1994; Costa *et al.* 1997).

The cultivation of potatoes is still difficult in regions with variable rainfall or insufficient water supplies (Thiele *et al*. 2010). Potato vegetative growth, including shoot length, leaf size, and leaf quantity, is impacted by drought (Kiziloglu *et al*. 2006*).* Yearly variations in the length and severity of the drought stress period are possible (Simelton *et al*. 2012). Water reduction after tuber start seriously impairs physiological plant processes and reduces tuber production (Costa *et al.* 1997). This yield decrease is the consequence of a

significant reduction in tuber number (Deblonde *et al.* 1999 and Eiasu *et al.* 2007), but also a consequence of a reduction in tuber size (Schafleitner *et al.* 2007).

It is well recognized that abiotic stress factors like drought, extremes in temperature, and salinity have an impact on the occurrence and spread of diseases, insects, and weeds (Scherm and Coakley 2003; Peters *et al.* 2014). Through modifications to plant physiology and defense mechanisms, these stress conditions also have a direct impact on plant-pest interactions (Scherm and Coakley 2003). Abiotic stress circumstances, such as drought, can favor competitive interactions between weeds and crops because many weeds use water more effectively than crops (Patterson 1995 and Valerio *et al.* 2013).

It has been estimated that drought and global warming will cause a significant drop in potato crop by 2055 (Holden *et al.* 2003). In another study, it has been estimated that biotic and abiotic pressures brought on by climate change will cause a drop in global potato production of 18-32% from 2040-2069 (Hijmans 2003). According to previous reports, *Solanum tuberosum* L. is quite sensitive to drought stress, and its leaves are more sensitive due to the plant's decreased ability to absorb water from the soil (Weisz *et al.* 1994). Because potatoes have a shallow root system (rooting zone between 50 and 80 cm deep), their tuber yield and quality can dramatically decline under drought, high salinity, and excessive heat conditions (Bouaziz *et al*. 2013).

Drought hinders photosynthesis and metabolic processes, which results in a drop in transpiration rate and nutrient uptake as well as a reduction in the fresh weight of biomass in potato leaves. It also affects the number of leaves, the size of the leaves, and the length of the shoots (Evers *et al.* 2010 and Li *et al*. 2015). When the temperature exceeds 25°C, the growth of the tuber stops; however, when the temperature reaches 39°C, the growth of the stem and leaf stops (Krauss *et al*. 1984). Stress has the most negative effects on tuberization, bulking, and tuber production due to lower rates of carbon absorption and

lower assimilate partitioning to tubers (Obidiegwu *et al.* 2015). Furthermore, under 5.9 mS/m electric conductivity, potato tuber production in field conditions was cut in half (Kotuby-Amacher *et al*. 2000).

Therefore, it is essential to find out the best production techniques and create new potato cultivars that are best suited for the anticipated climatic change in order to increase potato yield. To fulfill the growing population's worldwide food need, increased potato output is crucial. By increasing the potato plant's exposure to these stressful conditions, climate change will probably worsen tuber production losses. Therefore, it is vital to produce heat, drought, pest, and pathogen resistant crop cultivars that are adequately developed for the changing environment in order to respond to the emerging agricultural difficulties. To address the harmful effects of climate change and stress, it is crucial to implement genetic and management treatments in an environmentally friendly way. There are not many studies that identify the features that persisted in modern potato cultivars that could serve as a foundation for increasing the potato's resistance to abiotic stress. Although cultivars that are regarded moderately salt tolerant have been developed via significant breeding and selection of features for abiotic stress resistance. Wild potatoes growing in the Andes are relatively stress tolerant (Arvin *et al.* 2008). However, there is a significant information vacuum about the underlying processes of potato plant resistance to biotic and abiotic stress as well as the capacity to foretell future results. Finding ways to increase tuber yield in the face of stress from rising CO_2, high temperatures and drought, as well as shifting patterns of pest and pathogen infestations, is difficult for plant scientists (Dahal *et al*. 2019).

Cross-breeding has previously been carried out often to add pest and disease resistances from the gene pool of wild species to the gene pool of domesticated potato species (Helgeson *et al.* 1998). *Solanum tuberosum* (2n = 4x = 48) is an example of a polyploid

crop that is heterozygous. This crop's heterozygosity and allopolyploidy make breeding difficult. Multiple alleles segregate at a specific location to introduce genetic variation by crossing two heterozygous parents. The unique gene mix within a selected variety will be destroyed if backcrossing techniques are utilized to enhance features. Therefore, it becomes almost impossible to introduce new traits while maintaining all advantageous attributes of a potato variety after it has become established. Instead of using real seeds, farmers use seed tubers, which produce genetically similar plants.

As previously indicated, the potato crop is subject to a variety of biotic and abiotic challenges, including drought, salinity, cold, heat, weeds, insects, viruses, fungi, and nematodes, which are the main causes restricting its growth. Water scarcity will be a danger in the ensuing decades as a result of global warming (Simelton *et al*. 2012). For the purpose of breeding innovative potato cultivars using both conventional and biotechnological techniques, it is essential to have access to a variety of genetic resources, including both farmed types and wild species (Heinonen *et al*. 2016).

Transferring a gene from a domesticated cultivar to a wild relative of the crop is a significant issue with conventional breeding because of reproductive barriers and linkage drag (Rahman *et al*. 2014). Limited success has been achieved in potato breeding efforts to improve specific traits utilizing wild species (Carputo and Barone 2005). It is challenging for researchers and breeders to properly modify the genome using traditional breeding strategies because the cultivated potato (*Solanum tuberosum*) has a tetraploid (2n = 4x = 48) genome with four copies (alleles) of genes (Consortium 2011). Using conventional breeding, gene stacking in polyploid crops is a challenging, tedious, and time-consuming process (Weeks 2017). Due to the lack of salt tolerance genes in the existing potato germplasms, traditional breeding methods have limited success in producing potatoes that can withstand salt. Additionally, even if a gene for salt tolerance

is discovered in a wild relative of the potato, the reproductive hurdles make it challenging to incorporate the gene into the cultivated potato varieties. Furthermore, the lack of the necessary day length for potato flowering in Bangladesh hinders real potato breeding.

Utilizing plant biotechnology, one can get over the constraints of conventional breeding. At the turn of the 20th century, plant biotechnology entered its current era. It relates to the capacity for *in vitro* plant cell and tissue growth, plant regeneration and cloning, and molecular breeding techniques like molecular marker-assisted selection (MAS), genetic modification (GM), and more recently genome editing. In clonally propagated plants, genetic engineering, which is generally defined as a technique to alter an organism's genome using biotechnology, has the potential to overcome the drawbacks of conventional breeding because it can avoid issues with inbreeding depression, protracted breeding cycles, and sexual incompatibility (Ko *et al*. 2018). Due to its adaptability to plant transformation and tissue culture, the cultivated potato (*Solanum tuberosum* L.), the fourth most significant food crop in the world after rice, maize, and wheat, needs special attention for genetic engineering (Halterman *et al*. 2016).

With the development of genetic engineering techniques, potato has undergone genetic alteration. The researchers were able to insert the genes with economic significance using this technology. There have been numerous research for abiotic (drought, cold, heat, salt) tolerances and improvements in nutrient quality. Additionally, it is believed that because potatoes are autotetraploid, improving abiotic stress tolerance without changing cultivar features is challenging. Using transgenic techniques, various crop species have been given increased abiotic stress tolerance. The transgenic strategy has been shown to be an appealing and effective way to increase crop stress tolerance since it involves the direct introduction of genes through genetic engineering (Shimazaki *et al*. 2016).

Based on prior investigations of DNA transfer from *Agrobacterium tumefaciens* into plants during crown gall formation, the identification and sequencing of plant genomes became a standard practice (Altman 2019). Microorganisms found in soil called *Agrobacterium* have been used for their capacity to introduce DNA into plant genomes. This method has transformed agriculture by allowing for the identification and testing of gene function as well as the introduction of superior trait genes into crops without the introduction of undesirable genes that occur during breeding programs. The stability of the inserted genes is a significant factor in DNA transfer into plants (McCue *et al*. 2019). The mainstay of plant genome engineering has been the bacterium *Agrobacterium tumefaciens*. A sequence of interest known as Transfer-DNA (T-DNA) was able to be inserted into the nuclear genome of plants through the customized substitution of natural tumor-inducing (Ti) plasmid components. In clonally propagated crops like potatoes, plant transformation is a complicated process that involves transgene delivery, random genome integration of T-DNA, selection and plant regeneration from callus (Halterman *et al*. 2016).

The first genetically modified potato was New Leaf TM, a Colorado potato beetle-resistant variety that Monsanto released for sale in 1995 (Halterman *et al*. 2016). Twenty years later, the United States Department of Agriculture (USDA) approved the first generation of Innate potatoes, which were made to reduce bruising and browning using RNA interference, and the J.R. Simplot Corporation launched them in 2014 (Waltz 2015). Direct genetic transformation at CIP has been successful in creating new potato cultivars resistant to late blight (Ghislain *et al.* 2019).

Historically, improved potato cultivars have been developed by conventional breeding. Potato production and quality attributes are being improved through the insertion, expression, or silencing of commercially significant genes, without affecting the best

allele combinations in the current varieties (Diretto *et al.* 2007; Clasen *et al.* 2016; Andersson *et al.* 2018; McCue *et al.* 2018).

It has been observed that a large number of stress-specific genes, including functional genes, transcriptional regulators and transcriptional activators, play a significant role in enhancing stress tolerance in plants (Tuteja 2010; Amuda and Balasubramani 2011; Dang 2011; Amin 2011). Transcriptional activators, especially helicases, have been found to be particularly important in reducing stress. "Helicases" have been identified as important and efficient participants in reducing multiple abiotic stresses among the variety of genes used for abiotic stress tolerance (Pascuan *et al.* 2016). Helicases, commonly referred to as molecular motors, are involved in a variety of cellular functions that provide plants an innate resistance to abiotic stressors. Helicases unwind the duplex nucleic acids and are essential for many biological activities, such as transcription, recombination, repair, and replication (Tuteja and Tuteja 2004; Tuteja *et al.* 2012). The DEAD-box helicase *PDH45* (pea DNA helicase 45*)* actively promotes salinity stress tolerance, while the exact mechanism is unknown (Nath *et al.* 2016).

The cellular ratio of K+ to Na+ is significantly impacted by salt stress due to excessive Na+ buildup, which may cause cell death (Tracy *et al.* 2008). The capacity of the transgenic plants that overexpress *PDH45* to sustain cell viability in the face of salt stress suggests that *PDH45* regulates the vitality of root cells. Salinity, dehydration, wounding, and low temperature all increase *PDH45* expression, indicating that it plays a widespread role in abiotic stress response (Sanan-Mishra *et al.* 2005). This was supported by the increased abiotic stress tolerance that *PDH45* overexpression in numerous crops showed (Amin *et al*. 2011; Sahoo *et al*. 2012; Manjulatha *et al.* 2014; Augustine *et al.* 2015; Nath *et al.* 2015). It also contains RNA unwinding activity that is ATP-dependent. PDH45 shares 86% of its amino acid sequence with the tobacco translation initiation factor eIF-

4A and 58% of it with the eIF-4A found in the plants *Arabidopsis thaliana*, maize, rice, and wheat (Pham *et al.* 2000). Previous studies demonstrated that over-expression of salt-inducible *PDH45* in tobacco and rice (IR64 and PB1) results in the tolerance of salinity stress without reducing output (Gill *et al.* 2013; Sahoo *et al.* 2012; Sanan-Mishra *et al.* 2005).

Present investigation was conducted with the aim of developing stress tolerant local potato varieties, namely, BARI Alu-25 (Asterix), BARI Alu-7 (Diamant), BARI Alu-13 (Granola) and BARI Alu- 28 (Lady Rosseta) in Bangladesh through the introduction of *PDH45* gene using *Agrobacterium*-mediated genetic transformation. Prior to that, experiments were carried out to establish a suitable and reproducible *in vitro* regeneration system for potato to facilitate genetic transformation. Therefore, the overall objectives of the present investigation were designed as below:

1. Establishment of an efficient and reproducible regeneration system for Asterix, Diamant, Granola and Lady Rosseta varieties of potato.
2. Optimization of various factors influencing *Agrobacterium*-mediated genetic transformation.
3. Development of salinity and drought tolerant potato varieties by over expressing *PDH45* gene through *Agrobacterium*-mediated genetic transformation.
4. Characterization of putative transgenic plants through molecular techniques.
5. Comparative analysis for stress tolerance using different morphological and biochemical parameters.

2. MATERIALS

2. MATERIALS

2.1 Plant materials

The following four varieties of potato (*Solanum tuberosum* L.) were used in the present investigation:

(i) Asterix (BARI Alu-25)

(ii) Diamant (BARI Alu-7)

(iii) Granola (BARI Alu-13)

(iv) Lady Rosseta (BARI Alu-28)

2.1.1 Source

Tubers of the above mentioned varieties were collected from Tuber Crop Research Centre of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. These potato varieties were maintained in the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka. Fig. 1(a-d) represents the tubers of potato varieties used in the present investigation.

2.1.2 Description of the potato varieties

The important characteristics of these varieties are presented below:

Asterix (BARI Alu-25)

Origin: Developed by Bangladesh Agriculture Research Institute (BARI).

Year of release: 2005.

Main characteristics: Tuber oval shaped, medium to large in size, skin smooth and light red in colour, colour of flesh pale yellow, sprout color reddish violet and slight hairy. Plant strong and rapid growing, stem tall and stout, leaf slight large and deep green, seed dormancy 70-75 days in normal temperature, crop duration 90-95 days.

Planting season and time: Rabi and November.

Harvesting time: February.

Yield: 25-30 t/ha.

Diamant (BARI Alu-7)

Origin: The origin of Diamant is in the Netherlands and developed by Bangladesh Agriculture Research Institute (BARI).

Year of release: 1993.

Main characteristics: Tuber white, oval, medium to large size, skin smooth, light yellow, shallow eye, at first sprout color reddish violet and slight hairy. Plant strong and rapid growing, number of stems is lower but tall and stout, leaf slight large and deep green, seed dormancy 75-80 days in normal temperature, crop duration 90-95 days.

Planting season and time: Rabi and November.

Harvesting time: February.

Yield: 25-30 t/ha.

Granola (BARI Alu-13)

Origin: The origin of Granola is in the Netherlands and developed by Bangladesh Agriculture Research Institute (BARI).

Year of release: 1994.

Main characteristics: Tubers are oval in shape, medium size, skin rough and light bronze yellow and color of flesh pale yellow and eye slight shallow. Sprout color bronze violet, slight hairy. Plant slight bushy, number of stems more and green, at first plant grown slowly but later grow rapidly. Seed dormancy 75-85 days in normal temperature, crop duration 85-90 days. Because of more dormancy, potato can be stored 4-5 month in ambient temperature.

Planting season and time: Rabi and November.

Harvesting time: February.

Yield: 25-30 t/ha.

Resistance/tolerance: Tolerant to virus and drought stress.

Quality of the product: Exportable quality.

Lady Rosseta (BARI Alu-28)

Origin: The origin of Lady Rosseta is in the Netherlands and developed by Bangladesh Agriculture Research Institute (BARI).

Year of release: 2008.

Main characteristics: Potato red round shape, medium size, skin smooth, skin and flesh yellowish white, Seed dormancy 75-80 days in normal temperature crop duration 85-90 days. Tubers oblong, reddish colour, smooth skin. Lady Rosetta shows late sprouting. Sprouts are long, purple colour, with small hairs, many sprouts per eye. Tuber becomes saggy after sprouting. Plant stem purple in colour, tall, hairy, leaves green, large.

Planting season and time: Rabi, November.

Harvesting time: February.

Yield: 25-30 t/ha.

Resistance/tolerance: None.

Quality of the product: This variety is suitable for food processing.

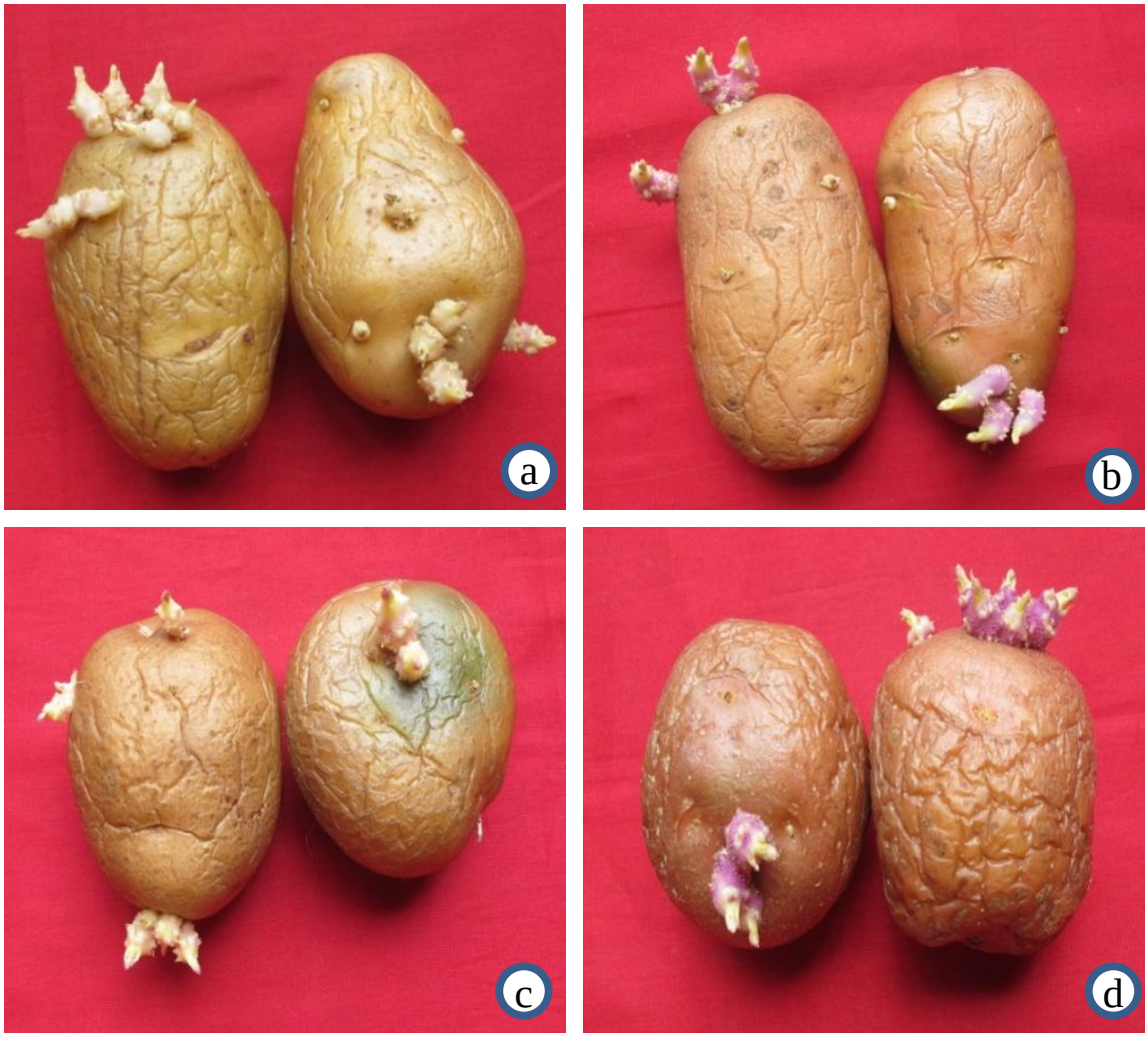

Fig. 1: (a-d): Sprouted tubers of four varieties of potato (*Solanum tuberosum* L.). (a) Asterix (BARI Alu-25); (b) Diamant (BARI Alu-7); (c) Granola (BARI Alu-13) and (d) Lady Rosseta (BARI Alu-28).

2.1.3 Explants for *in vitro* regeneration

Four different types of explants, namely, nodal segments, microtuber discs, internodal segments and leaf were used in the present investigation. Various types of explants have been presented in Fig. 2(a-d). Explants were collected from 15-20 days old *in vitro* grown shoot raised from sprout.

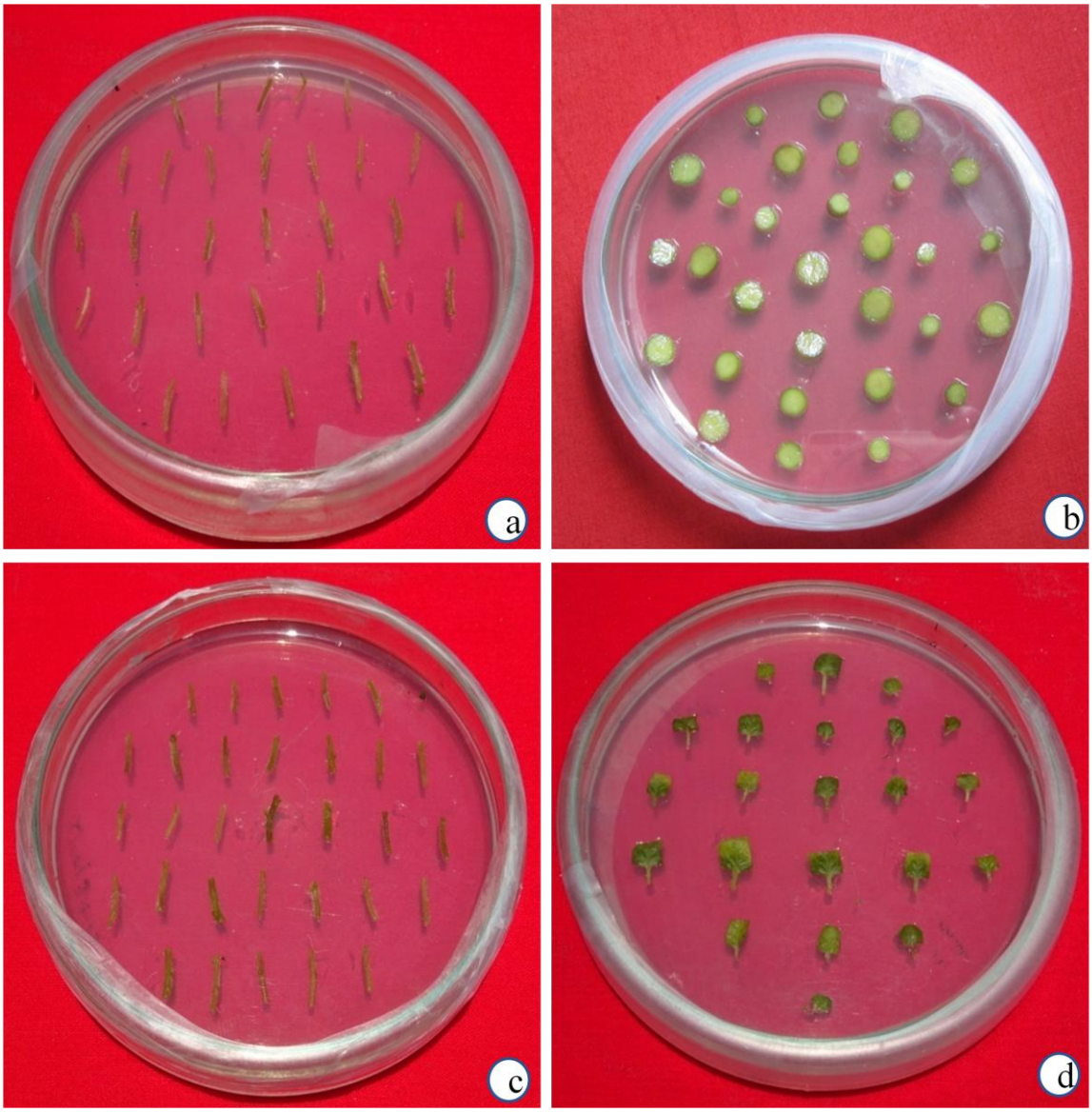

Fig. 2 (a-d): Four type of explants from Asterix variety of potato. (a) nodal segments; (b) microtuber discs; (c) internodal segments and (d) leaf.

2.2 *Agrobacterium* strain and vector plasmids

Two different genetically engineered constructs were used in transformation experiments. These are-

1. pBI121GUS-NPTII
2. pCAMBIA1301-PDH45

Agrobacterium strain namely LBA4404 and EHA105 were used for the transformation experiments.

2.2.1 Constract I (pBI121GUS-NPTII)

Agrobacterium tumefaciens strain LBA4404 contains plasmid pBI121 (binary vector). This binary vector has two genes within the right border (RB) and left border (LB) region of the gene construct (Fig. 3a):

(i) The *udi*A gene (Jefferson *et al*. 1987) encoding GUS (β-glucuronidase), driven by CaMV 35S promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation. The *nptII* gene (Herrea-Estrella *et al*. 1983) encoding neomycin phosphotransferase II conferring kanamycin resistance, driven by NOS promoter and NOS terminator.

The reporter gene *GUS* can be used to assess the efficiency of transformation. In this strain, the transformed tissue as well as transgenic plant can be selected by using kanamycin.

2.2.2 Construct II (pCAMBIA1301-PDH45)

Genetically engineered *Agrobacterium tumefaciens* strain EHA105 containing plasmid pCAMBIA1301was used for transformation experiments. This construct was kindly provided by Dr. Narendra Tuteja, of International Center of Genetic Engineering and

Biotechnology (ICGEB), New Delhi, India. This vector contains following genes within the right border (RB) and left border (LB) region are shown in (Fig. 3b).

(i) The *hpt* gene (Waldron *et al*. 1985; van den Elzen *et al*., 1985) encoding hygromycin phosphotransferase conferring hygromycin resistance, driven by CaMV 35S promoter and terminator.

(ii) It also contains *PDH45* gene (pea DNA helicase 45) conferring salinity and drought tolerance driven by CaMV 35S promoter and NOS terminator.

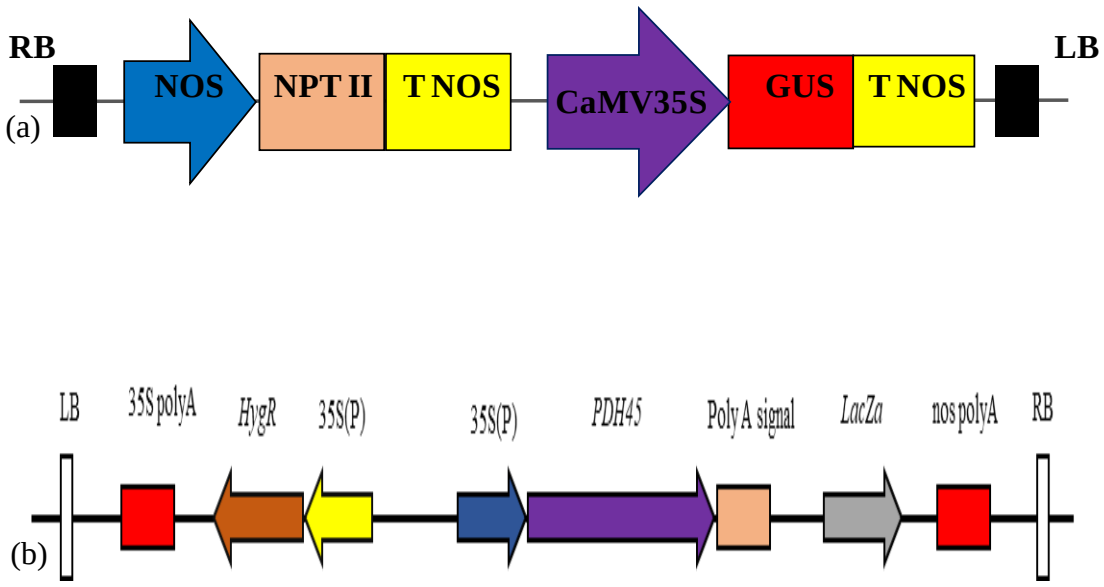

Fig. 3 (a-b): Diagrammatic representation of the linear partial map of two types of *Agrobacterium* strain (Right border and left border indicate T-DNA region). (a) *Agrobacterium* strain LBA4404 harboring the plasmid pBI121 and (b) *Agrobacterium tumefaciens* strain EHA105 harboring the plasmid pCAMBIA1301-PDH45.

2.3 Chemicals used for various purposes

2.3.1 Component of culture medium

Substance	Molecular weight	Company
MS Basal Salt Mixture	476 g/mol	DUCHEFA, Netherlands
Plant Agar	336.33 g/mol	DUCHEFA, Netherlands
Sucrose	180 g/mol	SIGMA, USA
Gelrite	500 kDa	SIGMA, USA

2.3.2 Antibiotics used for various purposes

Substance	Molecular weight (g/mol)	Company	Solvent
Carbenicillin	428.4	DUCHEFA, Netherlands	ddH_2O
Hygromycin	527.52	DUCHEFA, Netherlands	ddH_2O
Kanamycin	484.49	DUCHEFA, Netherlands	ddH_2O
Streptomycin	1457.4	DUCHEFA, Netherlands	ddH_2O
Rifampicin	822.94	DUCHEFA, Netherlands	ddH_2O

2.3.3 Plant growth hormones and additives

Substance	Molecular weight (g/mol)	Company	Solvent
BAP	225.3	DUCHEFA, Netherlands	1[N] NaOH
IAA	175.18	DUCHEFA, Netherlands	1[N] NaOH
IBA	203.2	DUCHEFA, Netherlands	1[N] NaOH
GA_3	346.4	DUCHEFA, Netherlands	1[N] NaOH
Zeatin	219.25	SIGMA, USA	1[N] NaOH
Acetosyringone	196.2	ROTH, Germany	DMSO

2.3.4 GUS-assay buffer

(i) 100 mM sodium phosphate buffer (pH 7.0)

(ii) 0.5 mM potassium ferrocyanide

(iii) 10 mM EDTA

(iv) 1 mM (0.5 mg/ml) X-GLUC (dissolved in DMSO before adding it to GUS buffer)

2.3.5 DNA markers

DNA marker	Concentration	Company
Gene Ruler^{TM} 100 bp DNA ladder	0.5 mg/ml	MBI Fermentas
Gene Ruler^{TM} 1 kbp DNA ladder	0.5 mg/ml	New England Biolab

2.3.6 Solvent, sterilizers and others with their sources

Compound	Company
Dimethyl sulfoxide (DMSO)	SERVA, Germany
KOH	Carl Roth, Germany
NaOH	Carl Roth, Germany

2.3.7 Following primers (with their sequence information) were used for various PCR analysis during this study.

Primer	Sequence (5'-3')	Product	Company
GUS-F GUS-R	5'-CATGAAGATGCGGACTTACG-3' 3'-ATCCACGCCGTATTCGGCGT-5'	750 bp	Macrogen, South Korea
nptII-F nptII-R	5'-TAGCTTCTTGGGTATCTTTAAAATA-3' 3'-CCAGTTACCTTCGGAAAAA GAGTT-5'.	720 bp	Macrogen, South Korea
PDH-F PDH-R	5'-ATGGCGACAACTTCTGTGG-3' 5'-GAGTCTAGATTATATAAGATCACCA ATATC-3'	1200 bp	Macrogen, South Korea
hptII-F hptII-R	5'- CGAAGAATCTCGTGCTTTCAGC-3' 5'- AGCATATACGCCCGGAGTCG-3'	750 bp	Macrogen, South Korea

2.4 Chemicals used for isolation of plant genomic DNA

All the solutions were made with deionised, sterile water and autoclaved.

(a) CTAB-buffer:

3	%	CTAB
1.4	M	NaCl
0.2	%	ß-Mercaptoethanol
20	mM	EDTA

100 mM Tris-HCl pH 8.0

0.5 % PVP- 40 (soluble)

Add CTAB and ß-Mercaptoethanol after autoclaving

(b) 24:1 CI Mix

24 ml Chloroform

1 ml Isoamylalcohol

(c) Wash buffer (WB)

76 % Ethanol Abs.

10 mM Ammonium acetate

(d) RNAs A: 10µg/µl Stock sol. in ddH_2O

(e) 7.5 M NH_4-Acetate

(f) 0.5 M EDTA (pH 8)

(g) TE-buffer + RNAseA

10 mM Tris-HCl, pH 8.0

1 mM EDTA

10 µg/ml RNAseA.

The volume was adjusted to 100 ml and stored at room temperature.

2.5. Materials for polymerase chain reaction (PCR)

Following components were required for the PCR amplification reaction:

a) PCR Reaction Flexi Buffer (5x Green Go Taq)

i. 500 mM KCl.

ii. l00 mM Tris-HCl (pH-8.3 at room temperature).

iii. 0.1% Gelatin.

b) 25 mM MgCl in water for PCR.

c) l0 mM of dTTP, dATP, dCTP & dGTP (pH-7.35)

d) Taq DNA polymerase.

e) DMSO (20%)

20% DMSO was prepared by mixing 20 ml of DMSO in 80 ml of deionized sterile water and stored at -20°C.

f) DNA template

g) TE Buffer : Buffer was filter sterilized and kept at -20°C for PCR use.

All the chemicals used for the preparation of these reagents were of Molecular biology grade and water used was ultra-pure.

2.6. Chemicals needed for Agarose gel electrophoresis

(a) Electrophoresis buffer (50XTAE) 500ml

Tris base	121 g
Acetic acid	26.8 ml
0.5 M EDTA, pH 8.0	50 ml

(b) Ethidium-bromide

10 mg/ml stock solution, store at 4°C.

2.7. Materials for stress analysis

2.7.1. Chemicals for the isolation of Chlorophyll

(a) 100% Acetone

(b) 80% Acetone

For the preparation of 80% 100 ml Acetone, 80 ml 100% Acetone was added to 20 ml DH_2O.

2.7.2 Chemicals for the analysis of Proline content

(a) PBS stock solution

(i) 200 mM Na_2HPO4 stock solution

Dissolved 53.65 gm $Na_2HPO4.7H_2O$ in 1000 ml DH_2O (Kept in 4°C before using).

(iii) 200 mM NaH_2PO_4 stock solution

Dissolved 27.8 gm NaH_2PO_4 in 1000 ml DH_2O (Kept in 4°C before using).

(b) Preparation of 100 mM of 200 ml PBS (P^H 7.0)

Added 61.0 ml of 200 mM Na_2HPO4 stock solution and then add 39.0 ml of NaH_2PO_4 stock solution. Finally added 100 ml DH_2O.

(c) 2.5 % acid-ninhydrin

Dissolved 1.25 gm ninhydrin in 30 ml glacial acetic acid and 20 ml of 100 mM PBS; P^H 7.0. (Kept in 4°C before using)

(d) 3 % Sulphosalicylic acid

Dissolved 3.496 gm 5-Sulphosalicylic acid dehydrate in 100 ml DH_2O.

2.7.3 Chemicals for the determination of H_2O_2 content

(a) 0.1% w/v TCA (Trichloroacetic acid)

Dissolved 0.1 gm TCA in 100 ml DH_2O.

(b) 1M Potassium Iodide (KI)

Dissolved 16.6 gm KI in 100 ml DH_2O.

(c) 10 mM phosphate buffer

Added 30.5 ml 200 mM Na_2HPO_4 and 19.5 ml 200 mM NaH_2PO_4 from stock solution and then add 50 ml DH_2O.

3. METHODS

3. METHODS

The experiments were carried out in the Plant Breeding and Biotechnology Laboratory, Department of botany, University of Dhaka. All the procedures carried out are described under the following heads.

3.1 Preparation of stock solutions for different culture media

Different stock solutions were prepared for the preparation of medium used in different steps of plant regeneration and selection of putative transformants. The stock solutions of various constituents of the medium were prepared for ready use during the preparation of various medium.

3.1.1 Stock solution A (Macro nutrients) for MS medium

This stock solution was made in such a way that its strength was 40 times more than the final strength of the medium in 1000 ml distilled water. For this purpose, 40 times the weight of different salts required for 1 litre of medium were weighed accurately and were sequentially dissolved one after another in a 1000 ml volumetric flask with 650 ml of distilled water. By further addition of distilled water the final volume of the solution was made up to 1000 ml. To remove all the solid contaminants like dust, cotton etc. the solution was filtered through Whattman No.1 filter paper and was poured into a clean plastic container. The solution was stored in a refrigerator at 4°C for several weeks after labelling.

3.1.2 Stock solution B (Micro nutrients) for MS medium:

Two different stock solutions were prepared for this constituent of the medium:

(i) Stock solution B_1 (all micro-nutrients except iron source)

Except FeSO4.7H20 and Na2-EDTA all the micro-nutrients was added to make this stock solution. This was made 100 times the final strength of necessary components in 500 ml of distilled water as described for the preparation of stock solution A. The prepared solution was filtered and was stored at 4°C.

(ii) Stock solution B_2 (Iron chelate solution)

This stock solution was made 100 times the final strength of $FeSO4.7H_20$ and Na_2-EDTA in 500 ml distilled water in a conical flask and heated slowly at a low temperature until the salts were dissolved completely. Finally the solution was filtered and stored in a refrigerator at 4°C for several weeks.

3.1.3 Stock solution C (Organic constituents) for MS medium

It was also made 100 times of the final strength of the medium in 500 ml of distilled water. This solution was also filtered and stored at 4°C for future use.

3.1.4 Preparation of MS medium

The following steps were carried out successively to prepare one litre medium,:

(i) For the preparation of MS, 4.405 g MS powder was dissolved in 750 ml of distilled water in a beaker. Dissolution was done using a magnetic stirrer (Stuart CB161, UK).

(ii) 30 g of sucrose (SIGMA, USA) was added to this 500 ml solution and dissolved well.

(iii) The required amount of hormonal supplements were taken from the stock solution of hormone and added to the medium and were mixed properly.

(iv) The whole mixture was then volume up to 1.0 litre with distilled water in a measuring cylinder.

(v) With the help of 1N NaOH and 1N HCl pH of the medium was adjusted to 5.8 with a digital pH meter (JENWAY). The pH meter was calibrated with two buffer solutions having pH 4.01 and 6.86 respectively before that.

(vi) To solidify the medium, either 8.0 g (at 0.8%) of phytoagar (DUCHEFA, Netherlands) or 2.5 g (at 0.25%) of gelrite (DUCHEFA, Netherlands) was added to the medium. To dissolve the solidifying agent the whole mixture was heated in a microwave oven.

(vii) The last step (vi) of media preparation was omitted to make a liquid medium.

3.1.5 Preparation of MS medium for plants using MS powder

Occasionally commercially available dry powdered MS medium (Duchefa Biocheme, Netharlands) containing all the constituents of MS medium (inorganic salts, vitamins and amino acids) except sucrose and agar were used to prepare medium for *in vitro* regeneration of plants. Appropriate amount of media powder is dissolved in distilled water (10% less than the final volume of the medium), and after adding sucrose (3%), the pH was adjusted to 5.8. To prepare agar solidified media, 0.8% agar was added.

3.1.6 Stock solutions for growth regulators

The growth regulators are naturally produced in higher plants. For the growth, differentiation and organogenesis of tissue, the growth regulators (auxins, cytokinins and additives) are important in tissue culture. In the present investigation the following different supplements and growth regulators were used:

I. Auxins

It causes cell division, cell elongation, swelling of tissues and the formation of adventitious roots. The auxins are:

i. Indole-3-acetic acic (IAA)

ii. Indole-3-butyric-acid (IBA)

II. Cytokinins

Cytokinins concerned with cell division and modification of shoot differentiation in tissue culture. It induces the adventitious shoot formation. The most frequently used cytokinins is 6-benzyl amino purine (BAP).

III. Gibberellic Acid

Gibberellic acid is a simple gibberellin, promoting growth and elongation of cells. The most commonly used gibberrelic acid is GA_3.

For preparation of any of the above mentioned hormonal stock solution, certain amount of hormone was weighted and dissolved in required amount of appropriate solvent. Then poured into clean sterilized plastic falcon tube and the final volume of the solution was made by addition of distilled water (stored in a refrigerator at 4ºC for future use).

3.1.7 Preparation of stock solution of Acetosyringone

For the preparation of 10 mM Acetosyringone stock solutions, 19.62 mg of Acetosyringone (molar mass- 196.19 g/mol) was dissolved in 1.0 ml of chloroform and then the final volume of the solution was made up 10 ml by addition of distilled water. After filter sterilization, this solution was stored in 1.5 ml eppendorf tubes at - 20ºC in the dark as stock.

3.1.8 Preparation of stock solutions of antibiotics

For the preparation of the above mentioned antibiotic stock solutions, 1.0 gm of each antibiotic was separately dissolved in 10 ml of deionized water or DMSO (Serva, Germany) in case of rifampicin. After filter sterilization, these solutions were stored in 1.5 ml Eppendorf tubes at -20 ºC in the dark as stock.

3.1.9 Preparation of *Agrobacterium* culture medium

Solid YEP (Yeast Extract Peptone) plates were used as culture and maintenance media for different strains of *Agrobacterium tumefaciens*.

3.1.10 Preparation of YEP (Yeast Extract Peptone) medium

For the growth of *Agrobacterium tumefaciens*, YEP medium was prepared in the following manner:

Components	Amount needed for 500 ml medium
Bactopeptone	5.0 g
Bacto Yeast extract	5.0 g
NaCl	2.5 g

All of these ingredients of the medium were added in 250 ml distilled water and mixed properly. Then the final volume was made up to 500 ml by adding distilled water. The pH was adjusted to 7.0-7.2.

For the preparation of bacterial maintenance medium, 7.5 g agar was added to the medium before autoclaving. Filter sterilized antibiotic kanamycin (100 mg/l), streptomycin (50 mg/l) and rifampicin (25 mg/l) were added to the autoclaved maintenance medium when the medium was cooled down enough. The medium was then poured into Petri plates. After solidification, the media were ready for bacterial culture. The plates were stored at 4°C for further use.

3.1.11 Preparation of bacterial suspension media

1.0 litre medium for suspending *Agrobacterium tumefaciens* for transformation was prepared using 4.405 g MS powder and 90 g sugar without any hormonal supplement. pH of the medium was adjusted to 5.8 and then autoclaved.

3.1.12 Preparation of Co-culture media

Co-culture medium was prepared using MS powder containing 100 μM acetosyringone (ROTH, Germany) without any hormonal supplement.

3.1.13 Preparation of medium for selection of putatively transformed plantlets

The *Agrobacterium* strains I contains *nptII* (Kanamycin resistance) gene. Therefore Kanamycin was used for selection of transformed tissue and plantlets. The *Agrobacterium* strains II contains *hptII* (Hygromycin resistance) gene. Therefore, hygromycin was used for selection of transformed tissue and plantlets. Carbenicillin was also used to control the overgrowth of bacteria. After preparation, the regeneration medium was autoclaved. The medium was cooled down to 50 and appropriate antibiotics were added at the desired concentration to a particular regeneration medium inside a laminar flow cabinet. The medium was then poured into a suitable culture vial and allowed to solidify.

3.2 Explants preparation and *in vitro* regeneration

3.2.1 Sprouting of potato tuber

Potato seed tubers were kept at 4°C Refrigerator for 15 days for cold treatment to break dormancy. Then the tubers were kept in dark chamber at 25±1°C for 15 days for sprout formation. This sprouts were used as explants in current experiment for growing plants both *in vitro* and *ex vitro*.

3.2.2 Sterilization of sprouts used for *in vitro* regeneration of shoot

For surface sterilization, the sprouts were dipped into 70% alcohol for one minute then washed with autoclaved distilled water for 3 times and then sterilized with 0.1 % $HgCl_2$ solution for 7-8 minutes. Afterwards the sprouts were washed with sterilized distilled water for 3 times. Then the surface sterilized sprouts were inoculated in the flasks containing MS medium.

3.2.3 Explant preparation

In potato, nodal segmet (NS), microtuber discs (MD), internodal segment (INS) and leaf explants (L) were used in this investigation. Nodal segment, internodal segment and leaf explants were excised from *in vitro* grown 15-20 days old shoots. For microtuber formation, shoots were cultured on MS with 3-12 % sucrose. Explants were then cultured on MS media supplemented with different concentrations and combinations of BAP, IAA, 2,4-D, Kn, GA_3, and Zeatin for *in vitro* regeneration of shoots.

3.2.4 Shoot regeneration and elongation

The explants were cultured on MS containing hormonal supplements, singly or in combinations for shoot regeneration. During culturing all the dissecting instruments (which were autoclaved earlier) namely, blades, scalpels and forceps were dipped in 70% alcohol and flamed over a spirit lamp before each time of use. The floor of the cabinet and hands were rinsed with 70% alcohol before starting the procedure. The culture tube and the conical flasks containing explants were incubated under fluorescent light of 2500-3,000 lux intensity. The light period of the culture room was maintained at 16/8 (dark/light) hours and temperature was 25 ± 2°C. For shoot initiation and development, MS medium (Murashige and Skoog 1962) supplemented with various combinations and concentrations of growth regulators viz. BAP (6- Benzyl amino purine), IAA (Indole-3-acetic acid), IBA (Indole-3- butyric acid), GA_3 (Gibberellic acid) were used.

3.2.5 Subculture of regenerated explants

Two weeks old regenerated explants were transferred into flasks or small bottles containing fresh media. Explants were subcultured regularly at an interval of 15-21 days for maintenance and were routinely examined.

3.2.6 Root induction of regenerated shoots

Half strength and full strength of MS medium supplemented with various concentrations of auxins, namely, IBA (Indole-3-butyric acid) and without auxin were used for induction of roots from the base of the *in vitro* grown shoots,. In potato, 1.0-2.0 cm long regenerated shoots were excised and transferred to test tubes (25×150 mm) containing freshly prepared MS medium as well as half strength of MS medium with different combinations and concentrations of IBA.

3.2.7 Transplantation and acclimatization of plantlets

After development of sufficient root system the plantlets were taken out from the culture vessels and to remove gelling agents the roots were washed under running tap water. The plantlets were then transplanted to small pots containing sterilized soil. Pots were then covered with transparent perforated polythene bags. The inner side of these bags was moistened with water to prevent desiccation. These plantlets were exposed to the external environment for 2-8 hours daily and again placed in growth room for another week. They were transferred to larger pots for further growth and to get tubers from those regenerated plants when the regenerated plants were fully established in the small pots,.

3.3 *Agrobacterium*-mediated Genetic Transformation

3.3.1 Hygromycin sensitivity assay

To determine the concentration of hygromycin for the effective selection of transformed plants, 15 days old shoot-derived nodal segment explants were cultured on MS (pH 5.8) supplemented with different concentrations (5, 10, 15, 20, 25 and 30 mg/l) of hygromycin and plant growth regulators (BAP and IAA). Nodal segment explants cultured on MS with BAP and IAA devoid of hygromycin were kept as control. The percentage of survived explant was calculated in both the varieties (Asterix and Diamant) after 28 days

of culture. In another set of experiment, 15 days old shoots of Asterix and Diamant variety were cultured on MS (pH 5.8) contaianing different concentrations (5, 10, 15, 20, 25 and 30 mg/l) of hygromycin and plant growth regulators (BAP and IAA). After 28 days of culture the percentage of responsive explants was calculated.

3.3.2 Preparation of *Agrobacterium* cells for transformation

Glycerol stocks (kept at -86ºC) of *Agrobacterium tumefaciens* LBA4404PBI121 and EHA105/pCAMBIA1301-PDH45 were used to prepare bacterial plates (YEP plates) by streak method. The plates were incubated at 28ºC for 48 hours in a dark chamber and then the bacterial growth was observed. After achieving suitable growth, another fresh YEP plate was streaked with bacterial cells from the plates. The plate was incubated in the same conditions as mentioned above and then used for transformation.

3.3.3 Preparation of bacterial suspension

After observing proper growth, a single colony of *Agrobacterium tumefaciens* strain LBA4404 harboring pBI121 (Construct I) and EHA105 harboring pCAMBIA1301 (Construct II) was scraped from freshly grown plates and suspended in 25 ml MS medium containing 9% sucrose with acetosyringone. With the help of a spectrophotometer the optical density (O.D.) of this suspension was determined at 600 nm. Bacterial suspension of O.D. 0.5-0.8 was preferred for transformation. Before 30 minutes of the infection process this bacterial suspension was prepared.

3.3.4 Infection and co-cultivation of explants with bacterial suspension

The explants collected from 15-20 days old shoots were dipped in the bacterial suspension and incubated at 28ºC for 15-45 min for construct I and 5-25 min for construct

II in 150 ml flask with a gentle rotation for every 2 min throughout the infection under sterile condition. The explants were co-cultured on regeneration medium (MS supplemented with BAP and IAA) containing acetosyringone after infection and incubation. To remove excess bacterial suspension explants were blotted on sterile Whatman no.1 filter papers for a short period of time and transferred onto the co-cultivation medium. The cultures were co-cultivated in dark at 25±2ºC for 2, 3 and 4 days.

3.3.5 Sub-culture of *Agrobacterium* infected explants and selection of putative transformed plants

To remove the surface bound *Agrobacterium* cells following co-cultured, explants were washed with distilled water three to four times until no opaque suspension was seen, then washed for 15 minutes with distilled water containing 300 mg/l carbenicillin. The explants were then gently soaked on a sterile Whatman no. 1 filter paper and transferred to selection medium (MS supplemented with BAP and IAA) with 300 mg/l Carbenicillin, 200 mg/l kanamycin for *Agrobacterium* construct I and 20 mg/l hygromycin for construct II. All the cultures were maintained at 25±2ºC in growth room and some infected explants were taken for GUS histochemical assay in case of construct I.

Transient GUS expression was calculated using the following formula:

$$\text{Transient GUS expression (\%)} = \frac{\text{Number of explants showing gus express ion}}{\text{Total number of } \textit{Agrobacterium} \text{ infected explants}} \times 100$$

At an interval of 20-22 days sub-culture of infected explants was done regularly on selection medium. Green calli were separated from brown dead explants after 65-70 days and sub-cultured on MS medium supplemented with BAP, IAA and GA_3. The multiple small shoots and shoot buds were transferred on same medium for shoot elongation after 95-100 days of inoculation. The elongated shoots were excised and transferred to root induction medium. The plantlets were transplanted to small plastic pots containing soil

after root formation. As control, non-infected explants were cultured on normal regeneration medium with selective agent. Control explants were sub-cultured on selection medium to detect the effect of selection agents on this. These controls were maintained with each set of transformation experiments to perform various comparative studies.

3.3.6 GUS (β-glucuronidase) histochemical assay:

Randomly selected co-cultured explants from each of the treatments were examined for GUS histochemical assay to calculate the transient expression of *GUS* reporter gene in Asterix variety of potato. The cultures of various stages after infection (nodal segments at initial stage, shoot, leaf and root tissues from completely regenerated plantlet, a whole plant, microtubers and germinated microtubers of T_0 plants) were incubated with X-gluc substrate (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) solution and incubated at 37ºC for 24-48 hrs. The explans were washed throughly with ethanol (70%) for 15-20 min each to remove chlorophyll, and were then examined for the presence of *GUS* gene.

3.3.7 Reagents for histochemical GUS assay:

0.5 M morphinoethane sulphonic acid (MES), pH 5.6:

9.76 gm of MES was dissolved in 80 ml ddH_2O. pH was adjusted to 5.6 with NaOH and volume was made. This was stored at room temperatures.

(i) Fixation solution, pH 5.6:

Component	Stock concentration	Final concentration
Formaldehyde (40%)	0.75% (v/v)	0.3%
0.5 M MES	0.002% (v/v)	10.0 mM
Manitol	5.46% (w/v)	0.3 M

This was stored at room temperature.

(ii) 50 mM solution of Na_3PO_4, pH 7.0:

Stock solutions:

A. 50 mM solution of $NaH_2PO_4.2H_2O$ (0.78 gm in 100 ml)

B. 50 mM solution of Na_2HPO_4 (0.71 gm in 100ml)

For the preparation of 100 ml 50 mM solution of Na_3PO_4 (pH 7.0), 39 ml of stock solution A and 61.0 ml of B was mixed and the pH was checked for 7.0.

(iii) GUS -Buffer / X-Gluc solution:

For the preparation of 10 ml X-gluc solution, 10 mg of X-gluc (5-Bromo-4-Chloro-3-indolyl β-D glucuronide) was dissolved in DMSO (dimethyl sulphonic acid) in a pyrex tube. The final volume was done with 50 mM solution of Na_3PO_4. This was stored at -20°C.

3.3.8 GUS (β-glucuronidase) histochemical assay of transient explants

Agrobacterium strain LBA4404 was used in the present study contains *gus (uidA)* reporter gene. It allows transformed tissues to be screened histochemically. From each batch of explants following each transformation experiment, randomly selected co-.cultured tissues were examined for GUS histochemical assay. Co-cultured explants, different parts and whole plantlets of putative transformants were immersed in X-gluc (5-Bromo-4-Chloro-3-indolyl-β-D glucuronide) solution and were incubated at 37℃ for overnight.

A characteristic indigo blue color would be the expression of GUS (β-glucuronidase) gene in the plant tissue. In each experiment non-transformed explants was used as control. Explants were transferred to 70% alcohol for degreening after X-Gluc treatment. Degreened explants were observed under stereomicroscope (Olympus, Japan).

3.3.9 Anatomical study:

Explant showing GUS expression following X-gluc treatment were transferred to 70% alcohol for degreening. Blue color containing explants were transferred to 1N NaOH into fresh glass vessels after degreening. These glass vessels were incubated for 20 min at 60ºC for adequate softening of the tissue. After cooling these materials were taken on a clean and oil free slide having a drop of 50% aqueous solution of glycerol (v/v) and clean cover slip was placed on each material and gentle pressure was given on the cover slip with a needle to spread the tissue uniformly. These slides were observed under Nikon P-III photomicroscope (Nikon, Japan).

3.3.10 Specimen preparation:

Explants showing GUS expression following X-gluc treatment were transferred to 70% alcohol for degreening. After degreening, blue color containing explants were transferred to 1N NaOH into fresh glass vessels. These glass vessels were inoculated for 10 minutes at 65ºC for adequate softening of the tissue. After cooling these explants were taken on a clean and oil free slide having a drop of 50% aqueous solution of glycerol (v/v) and clean cover slip was placed on each material and gentle pressure was given on the cover slip with a needle to spread the tissue uniformly. These slides were observed under Nikon P-III Micro photomicroscope.

3.3.11 Selection and direct plant regeneration of transformants

The nodal segments producing kanamycin and hygromycin resistant shoots were sub-cultured on MS supplemented with BAP, IAA and GA_3. The *in vitro* regenerated shoots (3-4 cm long) recovered from selection medium were sub-cultured onto MS rooting medium with appropriate selection agents. Rooted plantlets were hardened in pots

containing soil and established in the green house up to minituber formation. Stable transformation frequency was measured as

$$\text{Stable transformation frequency (\%)} = \frac{\text{Number of independent transgenic events}}{\text{Total number of } Agrobacterium \text{ infected explants}} \times 100$$

3.4 Collection of leaf sample

To extract genomic DNA, leaf samples from putative transformants of T_0, TG_1 and TG_2 generation of previously developed transgenic potato plants were collected and instantly placed in liquid nitrogen. The scissor was swabbed with 70% alcohol to avoid contamination of genomic DNA.

3.5 Preparation of Stock Solutions and Working Solutions used for DNA Isolation

For conducting the isolation procedures, the following stock solutions and working solutions were prepared.

3.5.1 1 M stock solution of Tris-HCl pH 8.0 (100 ml)

In 75 ml of distilled water12.14 gm of Tris base was dissolved. The pH of this solution was adjusted to 8.0. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. After autoclaving it was stored at 4ºC Refrigerator.

3.5.2 0.5 M stock solution of EDTA pH 8.0 (100 ml)

18.61 gm of EDTA was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. To adjust the final pH to 8.0 approximately 2 gm of NaOH pellets were added. With sterile de-ionized distilled water the final volume was adjusted to 100 ml. After autoclaving it was stored at 4ºC Refrigerator.

3.5.3 5 M stock solution of NaCl (100 ml)

29.22 gm of sodium chloride (NaCl) was dissolved in 75 ml of distilled water. Then it was volume up to 100 ml with distilled water. After autoclaving it was stored at 4ºC Refrigerator.

3.5.4 β-Mercaptoethanol

β-Mercaptoethanol was available at the strength of 14.4 M solution and was stored in a dark bottle at room temperature.

3.55 Chloroform: Isoamyl alcohol (24:1) (100 ml)

24 ml of Chloroform and 1 ml of Isoamyl alcohol were mixed properly using vortex mixture. This experiment was done only in a fume hood.

3.5.6 70% Ethanol (100 ml)

70 ml absolute ethanol was added to 30 ml double distilled water.

3.5.7 Stock solution of TE (Tris-HCl EDTA) buffer pH 8.0 (100 ml)

1.0 ml of 1 M Tris-HCl was added with 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. After autoclaving it was stored at 4ºC Refrigerator.

3.5.8 CTAB Buffer

Following components were used for preparing DNA extraction buffer.

Name of the chemical	Molecular Weight	Stock con.	Reference conc. /working conc.	Working Volume	
				100 ml	1000 ml
CTAB		10%	3%	3 ml	30 ml
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (pH 8)	372.24	0.5 M	20 mM	4 ml	40 ml
Trisbase (pH 8)	121.1	01 M	100 mM	10 ml	100 ml
β-Mercaptoethanol		100%	0.2%	0.2ml	2 ml

The following steps were performed for the preparation of Extraction Buffer (100 ml):

(i) 10 ml of 1 M Tris-HCl (autoclaved, pH 8.0) was taken in a 250 ml conical flask.

(ii) 28 ml of 5 M NaCl (autoclaved) was added to it.

(iii) 4 ml of 0.5 M EDTA (autoclaved, pH 8.0) was added next.

(iv) Then 0.5 gm PVP and 2/3gm CTAB was added freshly and kept it at 60ºC preheated water bath to dissolve the PVP.

(v) 200 µl β-mercaptoethanol was added prior to use the buffer and mixed it properlyby shacking.

3.6 Isolation of genomic DNA using the CTAB method

Plant genomic DNA was isolated according to Doyle and Doyle (1990). The steps of DNA isolation are as follows:

(i) 200-250 mg leaf materials of required source were taken and ground in liquid nitrogen (sometimes directly) using mortar and pestle.

(ii) 800 µl of CTAB buffer was added and ground the leaf until it became a homogenous paste.

(iii) The paste was transferred to 2 ml centrifuge tube and incubated at 60ºC water bath for 30 min.

(iv) 700 µl of chloroform: Isoamyl alcohol (24:1) was added to the extract and centrifuged at room temperature for 10 min at 13000 rpm. Sometimes this process was repeated twice to obtain a clear sample.

(v) Supernatant was collected and DNA was precipitated with 2/3 volume chilled Isopropanol and kept the sample overnight in -20ºC.

(vi) Then the suspension was centrifuged for 10 min at 13000 rpm at room temperature.

(vii) Supernatant discarded and the pellet was washed with 70% ethanol for 3 times.

(viii) DNA was then resuspended in 40-50 µl of TE buffer.

3.6.1 Quantification of Isolated DNA

Measurement of isolated DNA concentration was done by comparing DNA with the standard DNA on agarose gel electrophoresis.

3.7 Preparation of stock solutions used for Gel Electrophoresis

3.7.1 50X TAE (Tris-acetate-EDTA) Buffer (pH 8.0) (1 litre)

242 g Tris base was dissolved into 900 ml of sterile de-ionized distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (pH 8.0) was added in it. The pH of the solution was adjusted with concentrated HCl at pH 8.0. The final volume of the solution was adjusted to 1000 ml.

3.7.2 Ethidium Bromide Solution

For 1.0 ml solution, 10 mg of Ethidium Bromide was dissolved in 1.0 ml of sterile deionized distilled water. It was mixed by shaking. The solution was transferred to a dark bottle and was stored at room temperature.

3.8 Agarose Gel Electrophoresis

The standard method used to separate, identify and purify DNA fragments through electrophoresis was followed according to the method described by Sharp *et al.* (1973).

(i) 1.0 gm of agarose was melted into 100 ml of 1X TAE buffer. Ethidium Bromide was added (0.8 µg/ml) and poured into gel-tray fixed with appropriate combs.

(ii) After the gel was solidified it was placed into gel-electrophoresis apparatus containing 1X TAE buffer.

Digested plant DNA solutions were loaded with 6x gel loading dye and electrophoresis was continued until DNA fragments were separated well.

3.9 Polymerase Chain Reaction (PCR)

3.9.1 Preparation of purified Taq DNA polymerase

- 2 µl of purified Taq DNA Polymerase was transferred to an eppendorf (fresh and autoclaved).
- 4 µl of storage buffer was added.
- Dilution of the enzyme was 50 fold.

Storage Buffer:

Components	Molecular weight	Concentration
Tris	121.14	50.0m M
KCL	74.50	50.0mM
EDTA	372.20	0.1 mM
DTT	154.20	1.0mM
PMSF	174.20	0.5m M
Glycerol	---------	50.0%

- 0.0174 g of PMSF was first dissolved in minimal volume of isopropanol (~lml). Then 1.21 g of Tris, 0.745 g of KCL, 0.0074 g of EDTA and 0.03084 g of DTT were added and mixed thoroughly with deionized water after adjusting the pH to 7.9. The final volume was made 75ml with ddH2O and filter sterilized through 0.2/µm Millipore.

- Meanwhile, 80% glycerol was prepared from commercially available glycerol (98%) and autoclaved. 125 ml of the 80% glycerol was added to 75ml of the mixture of storage buffer and stored at 4°C.

3.9.2 Preparation of dNTPs mixture

100 µl each of dATP, dGTP, dCTP, dTTP [their concentrations being l0 mM each] were mixed in a fresh autoclaved eppendorf tube and the final volume was made l000 µl by adding 600 µl of TE solutions (l0 mM Tris-HCI, 0.l mM EDTA, pH 8.0) and dispensed as aliquots in tubes and stored at -20°C. The final concentration of each of the nucleotide in the above mixture was 1.0 mM. 2.0 mM of dNTPs mixture was also prepared.

3.9.3 Dilution of the DNA template

Since the isolated DNA varieties were highly concentrated and unsuitable for PCR use, the DNA varieties were diluted at different concentration with TE solution before use. The working concentration of the template DNA was 100 ng for each sample.

3.9.4 Preparation of PCR Reaction Mixture:

The following components were used to prepare PCR reaction mixture. The total volume of PCR reaction mixture was 375 µl for 15 samples.

Component of PCR Reaction Mixture (for 15 reactions):

Sl. No.	Reagents	Amount per sample	Total
1	Sterile de-ionized distilled water	17.95	269.25 µl
2	*Taq* Buffer A 10X (Tris with 15 mM $MgCl_2$)	2.5 µl	37.5 µl
3	Primer F	1.0 µl	15 µl
4	Primer R	1.0 µl	15 µl
5	dNTPs 2.5 mM	0.5 µl	7.5 µl
6	*Taq* DNA Polymerase	0.05 µl	0.75 µl
7	Sample DNA	2.0 µl	30 µl
	Total	**25.0 µl**	**375 µl**

During the experiment, PCR buffer, dNTPs, Primers and DNA sample solution were thawed from frozen stocks, mixed well by vortexing and kept on ice.

3.9.5 Thermal Cycling Profile used in PCR

The thermal cycling profile that were employed in the PCR for the amplification of inserted gene are tabulated below.

The thermo cycler was run with the temperature profile (for *gus*) specified below

Step	Temperature	Time	No. of Cycle
Initial denaturation	95°C	5 min	1 (first)
Denaturation	94°C	1 min	30
Annealing	56°C	1 min	
Elongation	72°C	1 min	
Final elongation	72°C	10 min	1 (last)

The thermo cycler was run with the temperature profile (for *npt II*) specified below

Step	Temperature	Time	No. of Cycle
Initial denaturation	95°C	5 min	1 (first)
Denaturation	94°C	1 min	30
Annealing	55°C	1 min	
Elongation	72°C	1 min	
Final elongation	72°C	10 min	1 (last)

The thermo cycler was run with the temperature profile (for PDH-45) specified below

Step	Temperature	Time	No. of Cycle
Initial denaturation	95°C	8.0 min	1 (first)
Denaturation	95°C	30 sec	35
Annealing	52°C	30 sec	
Elongation	72°C	2.0 min	
Final elongation	72°C	10 min	1 (last)

The thermo cycler was run with the temperature profile (for *hpt II*) specified below

Step	Temperature	Time	No. of Cycle
Initial denaturation	95°C	5 min	1 (first)
Denaturation	94°C	1 min	30
Annealing	52°C	30 sec	
Elongation	72°C	1.3 min	
Final elongation	72°C	5 min	1 (last)

3.9.6 Preparation of the Master Mixture

Master mixture was prepared by mixing all of the PCR components e.g. Dream tag buffer, dNTPs, Primer- F and R except the component against which the optimization strategy was intended. In each reaction, the volume of PCR buffer was used $1/10^{th}$ of the total reaction volume which was 25 µl. After mixing and momentary spin of the master mixture, it was transferred to different PCR tubes. The PCR component in question was then added. The final volume was made 25 µl by adding varying amounts of sterilized ultra-pure water. *Taq* DNA Polymerase was added just before starting the reaction. Finally, the tubes were subjected to momentary spin and transferred to thermocycler for the amplification reaction.

3.9.7 Visualizing the PCR Product

3 µl of DNA dye was added to the PCR amplified DNA. After a momentary spin, the PCR products were loaded in wells of 0.80% agarose gel containing ethidium bromide (0.8 µg/ml). Electrophoresis was accomplished at 90 volts and the PCR products were visualized under UV transilluminator and photographs were taken for documentation.

3.10 Morphometric evaluation of control under stress condition in green house

The *ex vitro* rooted control plants of both the varieties (Asterix and Diamant) were transplanted into small 6.0 inch plastic pot containing sand with cocopeat and allowed to grow under greenhouse conditions with different concentrations (0, 50, 100,150, 200 and 300 mM) of salt. These plants were supplemented with ¼ th strength of Hoagland solutio. Plants were carefully uprooted after 28 days and were washed gently with water. Growth parameters in terms of shoot length, root volume and leaf number were measured. In another experiment, the plants were grew till maturity and yield attributes in terms of total yield and total dry matter was recorded.

3.11 Morphometric evaluation of transgenic lines under stress condition

Different experiments were conducted to analyse growth, photosynthetic ability and yield attributes in the transgenic lines under stress conditions (200 mM NaCl). The *ex vitro* rooted TG_2 transgenic plants were transplanted into small 6.0 inch plastic pot containing sand with cocopeat and allowed to grow under greenhouse conditions. These plants were supplemented with ¼ th strength of Hoagland solution. Plants were carefully uprooted after 28 days and were washed gently with water. Growth parameters in terms of shoot length, root volume and leaf number were measured.

3.12 Biochemical evaluation of transgenic lines under stress condition

Different experiments were conducted to analyse the biochemical or physiological changes occured in the transgenic lines under stress conditions (200 mM NaCl). The *ex vitro* rooted TG_2 transgenic plants were transplanted into small 6.0 inch plastic pot and salt stress (200 mM NaCl) was applied for 28 days. After 28 days, the level of chlorophyll and carotenoid, H_2O_2 content and proline content were determined.

3.12.1 Leaf Relative Water Content (RWC)

The RWC was measured for both control and stressed samples of wild type control and second transgenic tuber (TG_2) generation of potato incase of var. Asterix and Diamant. The fully expanded leaves of the plants were excisedt and their fresh weight (FW) was measured immediately. Then leaf samples were soaked in distilled water in a Refrigerator (4) for 6.0 hrs to obtain their turgid weight (TW). Leaf samples were then oven dried for 48 hrs at 70 and finally dry weight (DW) were measured. The relative water content (RWC) was calculated by the following formula:

RWC (%) = [(FW-DW)/(TW-DW)] x 100; where FW is fresh weight, TW is turgid weight and DW is the dry weight.

3.12.2 Determination of level of chlorophyll and carotenoid

From transgenic and control plants of two potato varieties (Asterix and Diamant) the chlorophyll and carotenoid levels were evaluated spectrophotometrically. After grinding of leaf samples in 0.5 ml of 100% acetone, 1.0 ml of 80% acetone was added. After centrifugation at 12000 rpm for 15 min the supernatant obtained was adjusted to 2.0 ml with acetone 80%. The content of Chlorophyll a, chlorophyll b and carotenoid was calculated by measuring the absorbance at 663, 645 and 450 nm respectively. By the formula of Arnon (1949) the total chlorophyll level was determined.

3.12.3 Determination of proline content

The proline assay was performed according to Bates *et al*. (1973). Samples were ground in sulphosalicylic acid (3%). The mixtures were incubated for 1 hr at 100 in a water bath after adding glacial acetic acid and ninhydric acid. To stop the reaction the tubes were then transferred on ice. At 520 nm the absorbance of the toluene fraction was measured after adding 2 ml tolune. The concentration of proline was calculated using a calibration curve and is expressed as µmol proline/g FW.

3.12.4 Determination of H_2O_2 content

By the method of Loreto and Velikova (2001) the H_2O_2 concentration in both transgenic and control was measured. At 4℃ approximately 0.1 gm of samples was ground in 2.0 ml TCA 0.1% (w/v). At 4℃ the mixture was centrifuged for 15 min at 12000×g. Then, 0.5 ml supernatant was taken and 1.0 ml of 1M potassium iodide (KI) and 0.5 ml of 10 mM phosphate buffer (KP; pH 7.0) were added. With a standard calibration curve the H_2O_2 level was calculated by comparing its absobance at 390 nm. The H_2O_2 content is expressed as µmol/g FW.

3.12.5 Leaf discs senescence test

Leave discs were excised from healthy and fully expanded non-transgenic and transgenic (TG_3) potato leaves of similar age. The discs were floated in a 20 ml solution containing 0, 100 and 200 mM NaCl for 3 days. The experiment was carried out at 25℃. After the treatment the leaf pieces were blotted with Whatman no. 1 filter paper and weighed before measuring chlorophyll.

3.13 Statistical Analysis

The data were analyzed using analysis of variance (ANOVA) to determine the effects of NaCl on shoot and root length using SPSS 25. DMRT (Duncan Multiple Range Test) was also performed to look for statisticaly significant differences in different types of explants as well as treatments. A p value <0.05 was considered as threshold for significant differences between various data points.

4. RESULTS

4. RESULTS

The main objective of the present investigation was to transfer salt and drought tolerant gene to potato (*Solanum tuberosum* L.) varieties growing in Bangladesh through *Agrobacterium*-mediated genetic transformation. The whole research work was carried out mainly in two phases. The first phase of the investigation was to establish an efficient transformation compatible *in vitro* regeneration system for potato. In the second part, experiments were carried out for the stable integration of markers as well as salt and drought tolerant genes into locally grown potato varieties through *Agrobacterium*-mediated genetic transformation. The results of the present investigation obtained from different experiments are presented in the following sections.

4.1 *In vitro* regeneration of shoots

Genotype of plant materials, source of explants and growth conditions were considered to be important factors for *in vitro* regeneration of plantlets. In this study responses of four varieties of potato (*Solanum tuberosum* L.), namely, Asterix (BARI Alu-25), Diamant (BARI Alu-7), Granola (BARI Alu-13) and Lady Rosseta (BARI Alu-28) were used for *in vitro* regeneration of shoots from different explants on MS medium supplemented with different concentrations and combinations of auxins, cytokinins and Gibberellic Acid. A series of experiments were carried out to regenerate shoots directly from the explants or *via* callus induction. Experiments were also conducted to induce *in vitro* functional roots. The effects of various concentrations and combinations of growth regulators were also investigated for this purpose.

4.1.1 Determination of suitable explants for shoot induction

Various types of explants, namely, nodal segments (NS), inter nodal segment (INS), microtuber discs (MD) and leaf were used to examine their respective regeneration potential and to find out suitable explant for *in vitro* regeneration. During the present investigation MS media supplemented with two different hormonal combinations, namely, 1.0 mg/l BAP, 0.1 mg/l GA_3 and 4.0 mg/l BAP and 1.0 mg/l IAA were used for *in vitro* shoot regeneration following the report of Sarker *et al*. (2002) and Borna *et al*. (2010). Among the various explants nodal segment and microtuber discs exhibited positive responses towards multiple shoot regeneration through direct organogenesis. Regeneration capacity of other two explants, namely, internodal segment (INS) and leaf (L) via callus induction was also examined on different media composition. These experiments had been carried out in two phases. MS media supplemented with various concentrations and combinations of BAP, IAA and GA_3 were employed for callus induction and subsequent shoot regeneration from Asterix, Diamant, Granola and Lady Rosseta variety.

For induction of shoots nodal segments from Asterix, Diamant, Granola and Lady Rosseta were cultured on MS medium supplemented with (2.0-5.0) mg/l BAP + (0.5-1.0) mg/l IAA. Shoots were initiated from the explants after 8-12 days of culture on MS with 4.0 mg/l BAP and 1.0 mg/l IAA. The number of multiple shoots were increased upon subsequent subculturing on the same media composition. Approximately, 7.97±0.13 elongated shoots per explant were developed from nodal segments explant of var. Asterix (Table 1).

Table 1. Responses of various explants of potato towards multiple shoot formation

		No.of explants inoculated	No. of responsive explants	% of responsive explants	Time to start regeneration (days)	No. of shoot per explant (mean± SD)
Asterix	NS	150	147	98	8-10	7.97 ± 0.13^{a}
	MD	150	120	80	10-12	4.51 ± 0.16^{d}
Diamant	NS	150	145	96.67	8-10	6.90 ± 0.28^{b}
	MD	150	110	73.33	10-12	3.74 ± 0.38^{e}
Granola	NS	150	118	78.66	8-10	5.70 ± 0.38^{c}
	MD	150	78	52	10-12	4.07 ± 0.15^{e}
Lady Rosseta	NS	150	102	68	8-10	5.40 ± 0.43^{c}
	MD	150	62	41.33	10-12	3.8 ± 0.38^{e}

NS = Nodal segment and MD = Microtuber discs

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$.

Callus derived from inter nodal segments and leaf of Asterix, Diamant, Granola and Lady Rosseta were cultured for shoot induction on MS medium supplemented with (2.0-5.0) mg/l BAP, (0.5-1.0) mg/l IAA and 0.5 mg/l GA_3. In this combination, shoots were initiated after 55-65 days of culture (Table 2). The number of regenerated shoots was found to increase following the maintenance of these shoots on the same medium.

Table 2. Responses of various explants of potato towards multiple shoot formation via callus induction

Variety	Explant	No. of explants inoculated	No. of responsive explants	% of responsive Explants	Days required for callus induction	Time to start regeneration (days)	No. of shoot per explant (mean± SD)
Asterix	INS	135	118	78.67	25-30	55-60	5.79 ± 0.44^{a}
	L	135	98	65.33	30-35	60-65	3.84 ± 0.27^{c}
Diamant	INS	135	107	71.33	25-30	55-60	5.70 ± 0.46^{a}
	L	135	95	63.33	30-35	60-65	3.71 ± 0.47^{c}
Granola	INS	135	97	64.66	25-30	55-60	4.77 ± 0.46^{b}
	L	135	79	52.66	30-35	60-65	2.56 ± 0.43^{d}
Lady Rosseta	INS	153	83	53.33	25-30	55-60	3.76 ± 0.24^{c}
	L	135	72	48	30-35	60-65	1.82 ± 0.28^{e}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$.

4.1.1.2. Effects of various combinations of BAP and IAA on direct regeneration and multiple shoot proliferation using nodal segments and microtuber discs explants

Different concentrations and combinations of BAP and IAA were tried to observe the effect of growth hormones on the induction of shoots and their subsequent development from nodal segments and microtuber discs explants of four varieties of potato. The results of these experiments are presented in Tables 3, 4, 5 and 6.

It was observed from the table that the maximum number of shoots after 40 days (an average no. of 8.85±0.13 shoots per nodal segments and 4.79±0.26 shoots per microtuber discs explants) were observed on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA in case of variety Asterix (Table 3). In case of variety Diamant, maximum number of shoots after 40 days (an average of 7.84±0.16 shoots per nodal segments and an average of 4.25±0.28 shoots per microtuber discs explants) were observed on the same

media combination (Table 4). However, in case of variety Granola and Lady Rosseta the maximum number of shoots after 40 days (an average of 7.58±0.13 and 5.20±0.10 shoots per nodal segment and 4.09±0.19 and 3.13±0.11 shoots per microtuber discs explants respectively) were observed on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA (Table 5 & 6). Different stages of shoot regeneration are presented in Figs. 4 (a-d), 5 (a-d) and 6 (a-f).

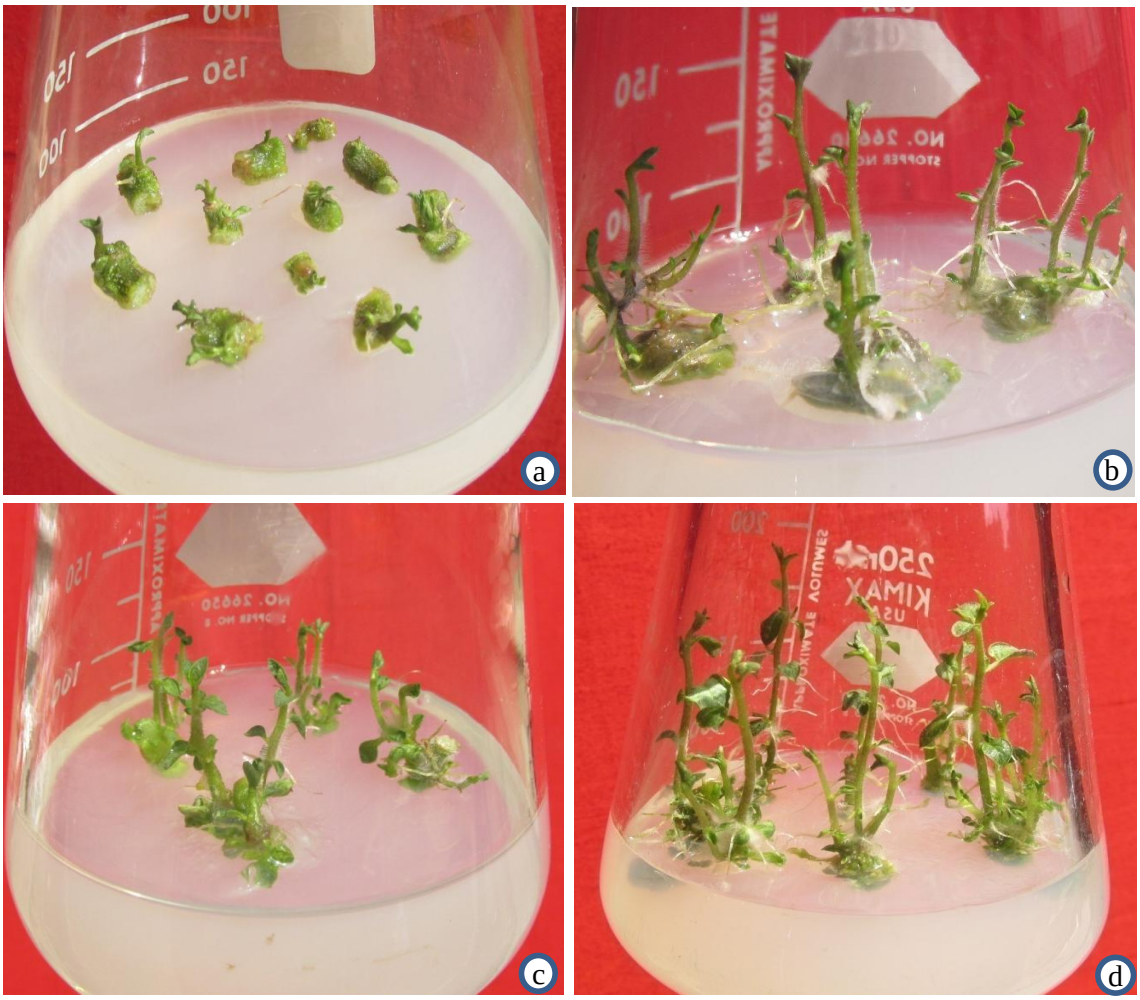

Fig. 4 (a-d): *In vitro* regeneration of shoots on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA in case of variety Asterix. (a-b) Initiation of shoots from nodal segment and microtuber discs explants; (c) Multiple shoots formation and (d) Formation of elongated shoots.

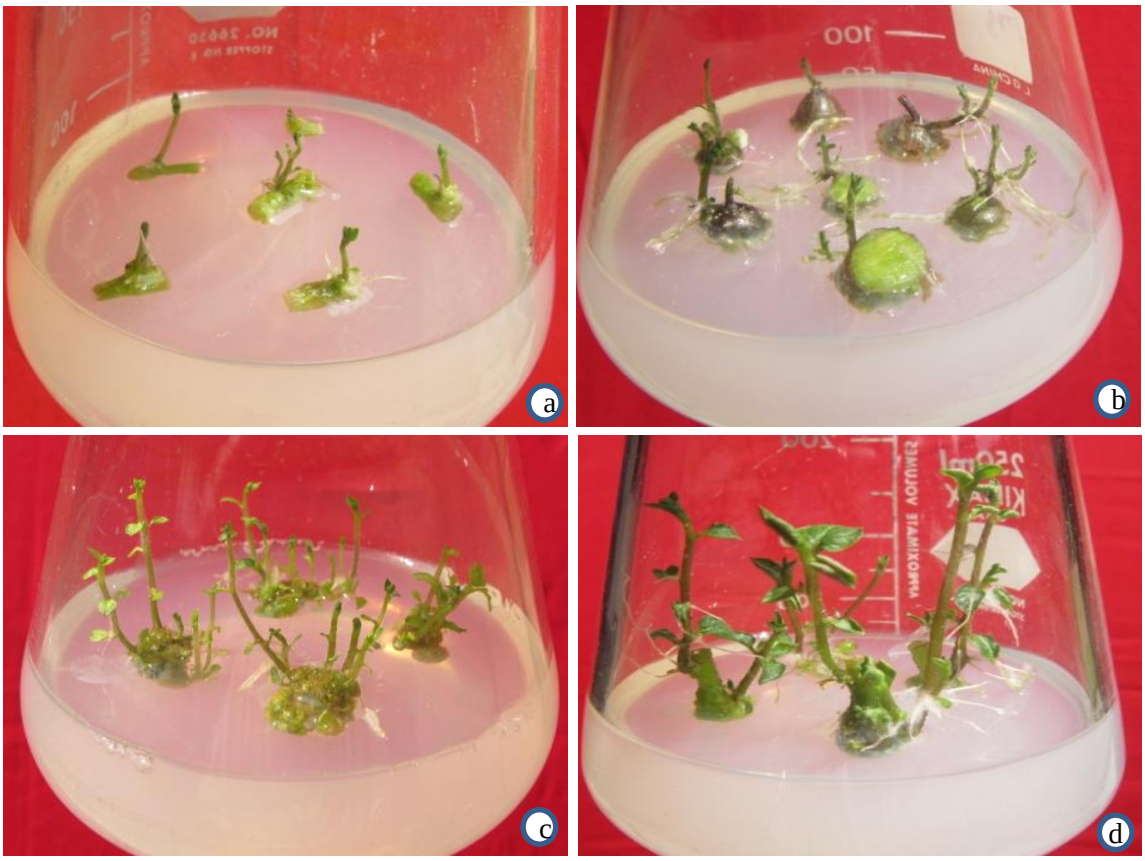

Fig. 5 (a-d): *In vitro* regeneration of shoots on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA in case of variety Diamant. (a-b) Initiation of shoots from nodal segment and microtuber slice explants; (c) Multiple shoots formation and (d) Formation of elongated shoots.

Table 3. Effects of MS supplemented with various combinations of BAP and IAA on regeneration and proliferation of multiple shoots from nodal segments and microtuber discs explants of potato (var. Asterix)

Explants	Hormonal supplement (mg/l)		No. of explants Inoculated	Percentage of responsive explants towards multiple shoot regeneration	No. of shoots/ explants after 40 days of inoculation (mean±SD)	Mean length of shoot/plant (cm) after 40 days of inoculation (mean±SD)	Mean no of node/plant after 40 days of inoculation (mean±SD)	Mean no of leaf/plant after 40 days of inoculation (mean±SD)
	BAP	IAA						
Nodal segmets	0.0	0.0	45	29	2.01 ± 0.13^{i}	7.70 ± 0.24^{a}	3.74 ± 0.16^{gh}	6.51 ± 0.24^{f}
	2.0	0.5	45	38	3.51 ± 0.18^{e}	6.94 ± 0.21^{bc}	4.01 ± 0.11^{fg}	6.45 ± 0.13^{f}
	2.0	1.0	45	42	3.93 ± 0.20^{d}	6.80 ± 0.19^{cd}	4.18 ± 0.23^{ef}	$7.04\pm0.08d^{e}$
	3.0	0.5	45	49	4.94 ± 0.07^{c}	6.23 ± 0.28^{bc}	4.68 ± 0.09^{cd}	6.65 ± 0.23^{ef}
	3.0	1.0	45	51	4.95 ± 0.06^{c}	6.88 ± 0.09^{bc}	4.35 ± 0.40^{de}	6.46 ± 0.37^{f}
	4.0	0.5	45	63	5.82 ± 0.16^{b}	6.47 ± 0.16^{ef}	5.83 ± 0.27^{a}	7.95 ± 0.15^{c}
	4.0	1.0	45	99	8.85 ± 0.16^{a}	5.92 ± 0.06^{hi}	5.69 ± 0.07^{a}	10.75 ± 0.21^{a}
	5.0	0.5	45	54	3.94 ± 0.06^{d}	7.26 ± 0.08^{b}	4.26 ± 0.34^{de}	7.24 ± 0.21^{d}
	5.0	1.0	45	53	3.04 ± 0.12^{fg}	7.68 ± 0.08^{a}	4.68 ± 0.18^{cd}	6.44 ± 0.43^{f}
Microtuber discs	0.0	0.0	45	24	2.28 ± 0.28^{i}	6.55 ± 0.15^{def}	3.72 ± 0.05^{gh}	4.80 ± 0.08^{i}
	2.0	0.5	45	31	2.77 ± 0.25^{gh}	6.15 ± 0.06^{gh}	3.51 ± 0.45^{h}	5.62 ± 0.22^{h}
	2.0	1.0	45	33	2.72 ± 0.050^{h}	7.00 ± 0.07^{bc}	4.03 ± 0.12^{gh}	5.94 ± 0.18^{gh}
	3.0	0.5	45	37	2.94 ± 0.07^{fgh}	6.95 ± 0.14^{bc}	3.75 ± 0.12^{gh}	6.25 ± 0.27^{fg}
	3.0	1.0	45	45	3.16 ± 0.17^{f}	6.55 ± 0.17^{de}	4.29 ± 0.23^{gh}	7.43 ± 0.44^{d}
	4.0	0.5	45	69	3.85 ± 0.23^{d}	5.62 ± 0.23^{ij}	4.89 ± 0.04^{bc}	8.28 ± 0.34^{c}
	4.0	1.0	45	82	4.79±0.26c	5.33 ± 0.04^{j}	5.15 ± 0.04^{b}	9.30 ± 0.39^{b}
	5.0	0.5	45	62	3.15±0.14f	6.64 ± 0.09^{cd}	4.58 ± 0.22^{cd}	5.85 ± 0.14^{gh}
	5.0	1.0	45	58	2.91±0.13fgh	6.84 ± 0.10^{cd}	3.76 ± 0.27^{gh}	5.59 ± 0.24^{h}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 4. Effects of MS supplemented with various combinations of BAP and IAA on regeneration and proliferation of multiple shoots from nodal segments and microtuber discs explants of potato (var. Diamant)

Explants	Hormonal supplement (mg/l)		No. of explants Inoculated	Percentage of responsive explants towards multiple shoot regeneration	No. of shoots/ explants after 40 days of inoculation (mean±SD)	Mean length of shoot/plant (cm) after 40 days of inoculation (mean±SD)	Mean no of node/plant after 40 days of inoculation (mean±SD)	Mean no of leaf/plant after 40 days of inoculation (mean±SD)
	BAP	IAA						
Nodal segmets	0.0	0.0	45	27	1.87±0.20^{j}	7.94±0.09^{a}	3.94±0.18^{f}	6.77±0.30^{ef}
	2.0	0.5	45	36	3.43±0.13^{ef}	6.91±0.08^{b}	4.07±0.16d^{e}	6.91±0.22^{de}
	2.0	1.0	45	40	3.68±0.18^{e}	6.73±0.27^{b}	4.48±0.45^{bc}	6.99±0.19^{de}
	3.0	0.5	45	45	4.81±0.22^{c}	6.48±0.35^{b}	4.81±0.17^{b}	6.28±0.25^{gh}
	3.0	1.0	45	48	4.92±0.07^{c}	5.98±0.06^{c}	4.88±0.19^{b}	6.71±0.22^{ef}
	4.0	0.5	45	62	6.15±0.73^{b}	5.71±0.29^{c}	5.94±0.24^{a}	7.26±0.27^{d}
	4.0	1.0	45	96	7.84±0.16^{a}	5.75±0.12^{c}	5.82±0.20^{a}	9.57±0.24^{a}
	5.0	0.5	45	52	3.69±0.12^{e}	6.44±0.47^{b}	4.54±0.43b^{c}	6.42±0.36^{fh}
	5.0	1.0	45	51	2.95±0.18^{gh}	5.96±0.08^{c}	4.97±0.28^{b}	6.26±22^{gh}
Microtuber discs	0.0	0.0	45	21	2.64±0.32^{hi}	6.66±0.12^{b}	3.85±0.15^{f}	4.53±0.21^{k}
	2.0	0.5	45	29	2.47±0.08^{i}	6.54±0.28^{b}	3.78±0.21^{f}	5.77±0.25^{i}
	2.0	1.0	45	30	2.77±0.20^{hi}	6.804±0.31^{b}	4.01±0.17^{ef}	5.94±0.07^{hi}
	3.0	0.5	45	33	3.00±0.14^{gh}	6.60±0.16^{b}	3.74±0.23^{f}	6.26±0.13^{gh}
	3.0	1.0	45	42	3.29±0.22^{ef}	5.99±0.05d^{c}	4.77±0.48^{bc}	7.14±0.12^{de}
	4.0	0.5	45	65	3.78±0.26^{e}	5.18±0.18c^{d}	4.28±0.28^{cd}	8.37±0.34^{c}
	4.0	1.0	45	75	4.25±0.28^{d}	5.61±0.16^{c}	5.71±0.34^{a}	9.05 ±0.52^{b}
	5.0	0.5	45	59	3.56±0.37^{e}	6.56±0.22^{b}	3.92±0.12^{f}	5.81±0.16^{hi}
	5.0	1.0	45	55	2.82±0.16^{gh}	6.59±0.41^{b}	3.91±0.41^{f}	5.22±0.22^{j}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 5. Effects of MS supplemented with various combinations of BAP and IAA on regeneration and proliferation of multiple shoots from nodal segments and microtuber discs explants of potato (var. Granola)

Explants	Hormonal supplement (mg/l)		No. of explants inoculated	Percentage of responsive explants towards multiple shoot regeneration	No. of shoots/ explants after 40 days of inoculation (mean±SD)	Mean length of shoot/plant (cm) after 40 days of inoculation (mean±SD)	Mean no of node/plant after 40 days of inoculation (mean±SD)	Mean no of leaf/plant after 40 days of inoculation (mean±SD)
	BAP	IAA						
Nodal segmets	0.0	0.0	45	25	1.86 ± 0.28^{j}	7.77 ± 0.27^{a}	3.33 ± 0.12^{hi}	6.18 ± 0.17^{fg}
	2.0	0.5	45	33	3.27 ± 0.29^{f}	6.89 ± 0.10^{b}	3.55 ± 0.13^{gh}	6.81 ± 0.21^{de}
	2.0	1.0	45	39	3.77 ± 0.25^{e}	6.77 ± 0.21^{b}	4.19 ± 0.21^{e}	6.42 ± 0.36^{ef}
	3.0	0.5	45	42	4.30 ± 0.27^{d}	6.12 ± 0.11^{cd}	4.40 ± 0.25^{de}	6.45 ± 0.28^{ef}
	3.0	1.0	45	47	4.91 ± 0.13^{c}	6.35 ± 0.19^{cd}	4.83 ± 0.15^{c}	6.95 ± 0.07^{d}
	4.0	0.5	45	60	5.37 ± 0.25^{b}	5.93 ± 0.03^{b}	5.91 ± 0.08^{a}	7.37 ± 0.09^{c}
	4.0	1.0	45	71	7.58 ± 0.13^{a}	5.87 ± 0.13^{fg}	5.77 ± 0.18^{a}	9.44 ± 0.40^{a}
	5.0	0.5	45	51	3.83 ± 0.23^{e}	5.62 ± 0.99^{gh}	4.16 ± 0.19^{e}	6.25 ± 0.28^{fg}
	5.0	1.0	45	48	2.42 ± 0.36^{hi}	6.38 ± 0.17^{c}	4.54 ± 0.13^{cd}	6.14 ± 0.15^{fg}
Microtuber discs	0.0	0.0	45	19	2.35 ± 0.09^{i}	6.09 ± 0.09^{de}	3.28 ± 0.29^{hi}	4.87 ± 0.14^{j}
	2.0	0.5	45	26	2.31 ± 0.16^{i}	6.82 ± 0.21^{b}	3.43 ± 0.17^{hi}	5.22 ± 0.20^{ij}
	2.0	1.0	45	29	2.72 ± 0.24^{gh}	6.21 ± 0.19^{cd}	3.81 ± 0.16^{fg}	5.84 ± 0.17^{h}
	3.0	0.5	45	31	3.0 ± 0.11^{fg}	6.75 ± 0.05^{b}	3.35 ± 0.30^{hi}	6.0 ± 0.06^{gh}
	3.0	1.0	45	39	3.06 ± 0.12^{fg}	6.32 ± 0.07^{cd}	4.12 ± 0.10^{ef}	7.15 ± 0.13^{cd}
	4.0	0.5	45	41	3.31 ± 0.13^{f}	6.03 ± 0.07^{ef}	4.20 ± 0.19^{e}	8.33 ± 0.30^{b}
	4.0	1.0	45	48	4.09 ± 0.09^{de}	5.16 ± 0.16^{i}	5.21 ± 0.19^{b}	9.26 ± 0.24^{a}
	5.0	0.5	45	40	3.10 ± 0.09^{fg}	5.53 ± 0.09^{h}	3.50 ± 0.16^{gh}	5.32 ± 0.28^{i}
	5.0	1.0	45	37	2.79 ± 0.20^{g}	6.01 ± 0.05^{ef}	3.13 ± 0.14^{i}	5.18 ± 0.19^{ij}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 6. Effects of MS supplemented with various combinations of BAP and IAA on regeneration and proliferation of multiple shoots from nodal segments and microtuber discs explants of potato (var. Lady Rosseta)

Explants	Hormonal supplement (mg/l)		No. of explants inoculated	Percentage of responsive explants towards multiple shoot regeneration	No. of shoots/ explants after 40 days of inoculation (mean±SD)	Mean length of shoot/plant (cm) after 40 days of inoculation (mean±SD)	Mean no of node/plant after 40 days of inoculation (mean±SD)	Mean no of leaf/plant after 40 days of inoculation (mean±SD)
	BAP	IAA						
Nodal segments	0.0	0.0	45	19	1.85 ± 0.16^{j}	7.48 ± 0.42^{a}	3.60 ± 0.34^{ghi}	6.43 ± 0.39^{f}
	2.0	0.5	45	23	2.43 ± 0.14^{hi}	6.58 ± 0.51^{bc}	3.50 ± 0.34^{hi}	7.44 ± 0.41^{de}
	2.0	1.0	45	27	2.74 ± 0.10^{efg}	6.41 ± 0.43^{cde}	3.78 ± 0.21^{fgh}	6.33 ± 0.30^{fg}
	3.0	0.5	45	33	3.06 ± 0.06^{d}	6.27 ± 0.36^{de}	3.81 ± 0.22^{fgh}	6.32 ± 0.21^{fg}
	3.0	1.0	45	42	3.99 ± 0.12^{b}	6.42 ± 0.36^{cde}	4.74 ± 0.16^{bc}	6.54 ± 0.22^{f}
	4.0	0.5	45	58	4.08 ± 0.10^{b}	6.25 ± 0.23^{de}	4.96 ± 0.17^{bc}	7.84 ± 0.12^{d}
	4.0	1.0	45	69	5.20 ± 0.10^{a}	5.90 ± 0.10^{efg}	5.85 ± 0.16^{a}	10.14 ± 0.14^{a}
	5.0	0.5	45	40	3.69 ± 0.21^{c}	6.07 ± 0.10^{de}	4.07 ± 0.09^{efg}	6.20 ± 0.17^{fgh}
	5.0	1.0	45	32	3.03 ± 0.11^{de}	7.06 ± 0.12^{ab}	3.18 ± 0.32^{i}	5.39 ± 0.33^{ij}
Microtuber discs	0.0	0.0	45	16	1.24 ± 0.11^{k}	6.92 ± 0.07^{abc}	3.10 ± 0.08^{i}	5.03 ± 0.08^{j}
	2.0	0.5	45	20	1.93 ± 0.09^{j}	5.57 ± 0.41^{fgh}	3.92 ± 0.06^{fgh}	5.27 ± 0.24^{j}
	2.0	1.0	45	25	2.61 ± 0.32^{fgh}	6.93 ± 0.05^{abc}	4.0 ± 0.08^{efg}	5.83 ± 0.17^{hi}
	3.0	0.5	45	29	2.55 ± 0.14^{ghi}	6.32 ± 0.28^{de}	4.23 ± 0.30^{ef}	5.75 ± 0.27^{hi}
	3.0	1.0	45	32	2.88 ± 0.11^{de}	5.49 ± 0.17^{gh}	4.48 ± 0.42^{cd}	7.15 ± 0.14^{e}
	4.0	0.5	45	37	2.86 ± 0.12^{def}	5.15 ± 0.1^{h}	4.99 ± 0.011^{b}	8.41 ± 0.35^{c}
	4.0	1.0	45	42	3.13 ± 0.11^{d}	6.15 ± 0.15^{de}	4.44 ± 0.65^{de}	9.35 ± 0.34^{b}
	5.0	0.5	45	28	2.30 ± 0.29^{i}	6.53 ± 0.46^{bc}	3.84 ± 0.38^{fgh}	5.92 ± 0.06^{gh}
	5.0	1.0	45	21	1.26 ± 0.24^{k}	6.25 ± 0.45^{de}	3.85 ± 0.16^{fgh}	5.79 ± 0.20^{hi}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Fig. 6 (a-f): *In vitro* regeneration of shoots on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA in case of Granola (BARI Alu-13) and Lady Rosseta (BARI Alu-28) varieties. (a-b) Formation of multiple shoots from nodal segments of variety Granola and Lady Rosseta; (c) Formation of multiple shoots from microtuber discs of var. Granola; (d) Elongated shoots of var. Granola and (e-f) Multiple elongated shoots of var. Lady Rosseta.

4.1.1.3 Effects of different concentrations of BAP, IAA and GA_3 on callus induction and multiple shoot regeneration using internodal segment and leaf explants

MS medium supplemented with different concentrations of BAP, IAA and GA_3 were used to see their effect towards callus induction. It was observed that Asterix showed 79.0 % responses towards callus induction when intermodal segments explants were cultured on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA and 0.5 mg/l GA_3 (Table 7). In case of Diamant, 72.0 % response was observed when explants cultured on same media (Table 8). On the other hand same media, Granola and Lady Rosseta showed 65% and 54% responses, respectively on the same media composition (Table 9 and 10). Callii formed on this media were compact and green in nature. Callus induction and regeneration of shoots from callus were presented in Figs. 7(a-e). Different concentrations and combinations of BAP, IAA and GA_3 were tried to observe their effect on shoot induction and their subsequent shoot development from leaf explants (Table 7-10). Leaves were collected from 15 days old shoots cultured on MS medium. Leaf explants were placed on regeneration medium keeping the adaxial surface touched with the medium.

In case of Asterix, different concentrations of BAP (2.0-5.0 mg/l), 1.0 mg/l IAA and 0.5 mg/l GA_3 were used. Best response towards shoot regeneration was observed on MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3. In this case 66 % of the explants showed shoot regeneration and number of shoots per explants was 3.26±0.15 (Table 7). In case of Diamant, different concentrations of BAP (2.0 – 5.0 mg/l), IAA (0.5-1.0 mg/l) and 0.5 mg/l GA_3 were used. Among these, best response towards shoot regeneration was observed on MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3 (Figs. 8 a-e). In this case 64 % of the explants showed response and number of shoots per explants was 3.33±0.15 (Table 8).

In case of Granola and Lady Rosseta, maximum number of regenerated shoots was found on same media combination (Figs. 9 a-f). In this case, of the explants of Granola and Lady Rosseta showed 53 % and 49 % regeneration. The number of shoots per explant was 2.35±0.19 and 2.97±0.13 (Table 9 and 10), respectively.

Table 7. Effects of various combinations of BAP, IAA and GA_3 on regeneration and proliferation of multiple shoots from internodal segments and leaf explants of potato (var. Asterix)

Explants	Hormonal supplement (mg/l)			No. of explants Inoculated	Percentage of responsive explants towards multiple shoot regeneration	No. of shoots/ explants after 40 days of inoculation (mean±SD)	Mean length of shoot/plant (cm) after 40 days of inoculation (mean±SD)	Mean no of node/plant after 40 days of inoculation (mean±SD)	Mean no of leaf/plant after 40 days of inoculation (mean±SD)
	BAP	IAA	GA_3						
Inter nodal segmets	0.0	0.0	0.0	45	22	1.12 ± 0.13^{j}	7.17 ± 0.15^{a}	3.60 ± 0.34^{ghi}	6.41 ± 0.12^{def}
	2.0	0.5	0.5	45	27	1.65 ± 0.12^{hi}	6.57 ± 0.29^{cde}	3.50 ± 0.34^{hi}	6.38 ± 0.15^{def}
	2.0	1.0	0.5	45	36	2.46 ± 0.37^{h}	6.58 ± 0.17^{cde}	3.78 ± 0.21^{fgh}	6.56 ± 0.13^{cde}
	3.0	0.5	0.5	45	43	3.25 ± 0.14^{fg}	6.19 ± 0.18^{fg}	3.81 ± 0.22^{fgh}	6.58 ± 0.20^{cde}
	3.0	1.0	0.5	45	49	3.78 ± 0.13^{c}	6.39 ± 0.12^{ef}	4.74 ± 0.16^{bc}	7.01 ± 0.63^{bc}
	4.0	0.5	0.5	45	61	3.87 ± 0.15^{b}	5.99 ± 0.06^{g}	4.96 ± 0.17^{bc}	8.25 ± 1.41^{ab}
	4.0	1.0	0.5	45	79	4.67 ± 0.11^{a}	5.68 ± 0.08^{h}	5.85 ± 0.16^{a}	8.91 ± 1.41^{a}
	5.0	0.5	0.5	45	52	3.38 ± 0.12^{c}	6.11 ± 0.10^{fg}	4.07 ± 0.09^{efg}	7.03 ± 0.22^{bc}
	5.0	1.0	0.5	45	48	2.43 ± 0.13^{h}	5.98 ± 0.04^{g}	3.18 ± 0.32^{i}	5.93 ± 0.92^{efg}
Leaf	0.0	0.0	0.0	45	19	1.01 ± 0.17^{j}	6.91 ± 0.09^{b}	3.10 ± 0.08^{i}	4.97 ± 0.56^{g}
	2.0	0.5	0.5	45	[27]	2.43 ± 0.12^{i}	6.57 ± 0.24^{cde}	3.92 ± 0.06^{fgh}	5.50 ± 0.22^{efg}
	2.0	1.0	0.5	45	30	2.58 ± 0.13^{h}	6.82 ± 0.09^{bc}	4.0 ± 0.08^{efg}	5.54 ± 0.26^{efg}
	3.0	0.5	0.5	45	33	2.60 ± 0.13^{h}	6.71 ± 0.11^{bc}	4.23 ± 0.30^{ef}	5.89 ± 0.26^{efg}
	3.0	1.0	0.5	45	39	2.68 ± 0.17^{gh}	6.12 ± 0.11^{fg}	4.48 ± 0.42^{cde}	6.64 ± 0.58^{cde}
	4.0	0.5	0.5	45	48	3.12 ± 0.14^{g}	5.41 ± 0.03^{i}	4.99 ± 0.01^{b}	7.80 ± 0.75^{abc}
	4.0	1.0	0.5	45	66	3.26 ± 0.15^{fg}	5.38 ± 0.23^{i}	4.44 ± 0.65^{de}	8.84 ± 0.44^{a}
	5.0	0.5	0.5	45	54	3.46 ± 0.12^{b}	6.34 ± 0.12^{ef}	3.84 ± 0.38^{fgh}	7.93 ± 0.80^{abc}
	5.0	1.0	0.5	45	42	2.48 ± 0.13^{h}	6.54 ± 0.12^{de}	3.85 ± 0.16^{fgh}	5.36 ± 0.11^{fg}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 8. Effects of various combinations of BAP, IAA and GA_3 on regeneration and proliferation of multiple shoots from internodal segments and leaf explants of potato (var. Diamant)

Explants	Hormonal supplement (mg/l)			No. of explants inoculated	Percentage of responsive explants towards multiple shoot regeneration	No. of shoots/ explants after 40 days of inoculation (mean±SD)	Mean length of shoot/plant (cm) after 40 days of inoculation (mean±SD)	Mean no of node/plant after 40 days of inoculation (mean±SD)	Mean no of leaf/plant after 40 days of inoculation (mean±SD)
	BAP	IAA	GA_3						
Inter nodal segmets	0.0	0.0	0.0	45	21	1.14 ± 0.15^{l}	6.74 ± 0.06^{a}	3.56 ± 0.11^{fg}	6.25 ± 0.11^{hi}
	2.0	0.5	0.5	45	29	1.75 ± 0.25^{jk}	6.54 ± 0.08^{ab}	3.76 ± 0.07^{f}	6.53 ± 0.06^{fg}
	2.0	1.0	0.5	45	32	2.65 ± 0.17^{g}	6.21 ± 0.03^{cde}	4.24 ± 0.08^{de}	6.52 ± 0.15^{fg}
	3.0	0.5	0.5	45	39	3.63 ± 0.11^{cd}	6.12 ± 0.12^{cde}	4.63 ± 0.07^{c}	6.69 ± 0.12^{ef}
	3.0	1.0	0.5	45	48	3.78 ± 0.12^{c}	6.04 ± 0.09^{def}	4.41 ± 0.04^{cd}	6.80 ± 0.07^{e}
	4.0	0.5	0.5	45	62	4.26 ± 0.29^{b}	5.85 ± 0.15^{efg}	5.66 ± 0.16^{a}	7.43 ± 0.14^{d}
	4.0	1.0	0.5	45	72	4.97 ± 0.13^{a}	5.63 ± 0.11^{efg}	5.43 ± 0.23^{b}	9.38 ± 0.15^{a}
	5.0	0.5	0.5	45	47	2.60 ± 0.16^{de}	6.07 ± 0.09^{cde}	4.18 ± 0.17^{e}	6.47 ± 0.17^{fgh}
	5.0	1.0	0.5	45	44	1.59 ± 0.17^{g}	5.85 ± 0.13^{efg}	4.57 ± 0.10^{c}	6.38 ± 17^{gh}
Leaf	0.0	0.0	0.0	45	16	1.83 ± 0.15^{k}	6.33 ± 0.18^{bc}	3.63 ± 0.07^{fg}	4.53 ± 0.11^{l}
	2.0	0.5	0.5	45	23	1.99 ± 0.12^{ij}	5.25 ± 1.73^{efg}	3.48 ± 0.10^{g}	5.23 ± 0.09^{k}
	2.0	1.0	0.5	45	29	2.46 ± 0.17^{hi}	6.42 ± 0.12^{abc}	3.46 ± 0.17^{g}	5.66 ± 0.11^{j}
	3.0	0.5	1.0	45	31	2.95 ± 0.17^{g}	6.23 ± 0.14^{cde}	3.51 ± 0.09^{g}	6.03 ± 0.07^{i}
	3.0	1.0	0.5	45	39	3.05 ± 0.16^{f}	6.02 ± 0.06^{def}	4.26 ± 0.08^{de}	6.93 ± 0.09^{e}
	4.0	0.5	0.5	45	52	3.05 ± 0.18^{f}	5.15 ± 0.13^{g}	4.53 ± 0.14^{c}	8.30 ± 0.27^{c}
	4.0	1.0	0.5	45	64	3.31 ± 0.15^{e}	5.43 ± 0.07^{efg}	5.36 ± 0.06^{b}	9.10 ± 0.10^{b}
	5.0	0.5	0.5	45	48	3.02 ± 0.16^{f}	5.52 ± 0.18^{efg}	3.56 ± 0.14^{fg}	5.64 ± 0.15^{j}
	5.0	1.0	0.5	45	46	2.18 ± 0.18^{h}	5.64 ± 0.10^{efg}	3.45 ± 0.11^{g}	5.16 ± 0.18^{k}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 9. Effects of various combinations of BAP, IAA and GA_3 on regeneration and proliferation of multiple shoots from internodal segments and leaf explants of potato (var. Granola)

Explants	Hormonal supplement (mg/l)			No. of explants inoculated	Percentage of responsive explants towards multiple shoot regeneration	No. of shoots/ explants after 40 days of inoculation (mean±SD)	Mean length of shoot/plant (cm) after 40 days of inoculation (mean±SD)	Mean no of node/plant after 40 days of inoculation (mean±SD)	Mean no of leaf/plant after 40 days of inoculation (mean±SD)
	BAP	IAA	GA_3						
Inter nodal segmets	0.0	0.0	0.0	45	17	1.14 ± 0.12^{k}	7.23 ± 0.37^{a}	3.14±0.13h	6.21±0.06f
	2.0	0.5	0.5	45	25	1.56 ± 0.07^{j}	6.52 ± 0.22^{b}	3.47±0.09fg	6.16±0.17fg
	2.0	1.0	0.5	45	31	2.53 ± 0.57^{gh}	6.20 ± 0.18^{cd}	4.44±0.27cd	6.66±0.08e
	3.0	0.5	0.5	45	39	3.14 ± 0.12^{de}	6.25 ± 0.29^{cd}	4.55±0.08c	6.79±0.12e
	3.0	1.0	0.5	45	41	3.93 ± 0.07^{b}	6.08 ± 0.10^{def}	5.03±0.07b	6.75±0.11e
	4.0	0.5	0.5	45	57	4.14 ± 0.13^{b}	5.79±0.17fgh	5.31±0.11a	7.45±0.10c
	4.0	1.0	0.5	45	65	4.74 ± 0.06^{a}	5.48±0.12hi	4.44±0.13cd	8.99±0.14a
	5.0	0.5	0.5	45	49	3.49 ± 0.06^{c}	6.25±0.11cd	4.25±0.07de	6.17±0.09fg
	5.0	1.0	0.5	45	43	2.45 ± 0.08^{ghi}	5.90±0.15efg	3.33±0.09gh	6.04±0.09fg
Leaf	0.0	0.0	0.0	45	15	1.07±0.08k	6.37±0.10cd	3.44±0.14fg	4.49±0.14i
	2.0	0.5	0.5	45	19	2.18±0.17i	6.44±0.11bc	3.56±0.09fg	5.48±0.26h
	2.0	1.0	0.5	45	27	2.25±0.08hi	6.26±0.09cd	3.57±0.09f	5.92±0.09g
	3.0	0.5	0.5	45	29	2.46±0.07ghi	6.12±0.11cde	4.12±0.12e	6.14±0.10fg
	3.0	1.0	0.5	45	36	2.84±0.07ef	6.05±0.10def	4.40±0.13cd	7.18±0.09d
	4.0	0.5	0.5	45	38	3.03±0.07e	5.47±0.17hi	5.23±0.07ab	8.24±0.10b
	4.0	1.0	0.5	45	53	2.35±0.19cd	5.45±0.23i	3.39±0.14fg	8.92±0.11a
	5.0	0.5	0.5	45	39	2.97±0.03b	5.62±0.16ghi	3.12±0.04h	5.56±0.11h
	5.0	1.0	0.5	45	31	2.64±0.17fg	6.07±0.10def	2.79±0.16i	5.38±0.23h

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 10. Effects of various combinations of BAP, IAA and GA_3 on regeneration and proliferation of multiple shoots from internodal segments and leaf explants of potato (var. Lady Rosseta)

Explants	Hormonal supplement (mg/l)			No. of explants inoculated	Percentage of responsive explants towards multiple shoot regeneration	No. of shoots/ explants after 40 days of inoculation (mean±SD)	Mean length of shoot/plant (cm) after 40 days of inoculation (mean±SD)	Mean no of node/plant after 40 days of inoculation (mean±SD)	Mean no of leaf/plant after 40 days of inoculation (mean±SD)
	BAP	IAA	GA_3						
Inter nodal segmets	0.0	0.0	0.0	45	13	1.11±0.10	7.64±0.10a	3.28±0.10ij	6.44±0.13f
	2.0	0.5	0.5	45	18	1.84±0.18	6.70±0.05c	4.02±0.11cde	7.35±0.11e
	2.0	1.0	0.5	45	24	2.65±0.12	6.29±0.14de	3.80±0.16efg	6.40±0.17f
	3.0	0.5	0.5	45	29	2.81±0.12	6.21±0.10de	3.57±0.18ghi	6.61±0.15f
	3.0	1.0	0.5	45	38	2.97±0.13	6.28±0.07de	4.18±0.17c	6.55±0.08f
	4.0	0.5	0.5	45	49	3.40±0.17	6.49±0.33cd	4.57±0.10b	7.62±0.04d
	4.0	1.0	1.0	45	54	3.88±0.10	5.77±0.09f	5.73±0.17a	9.30±0.09b
	5.0	0.5	0.5	45	41	3.04±0.19	6.03±0.08f	3.69±0.38fgh	6.14±0.12g
	5.0	1.0	0.5	45	28	2.72±0.19	7.16±0.14b	3.39±0.10hij	5.33±0.13j
Leaf	0.0	0.0	0.0	45	10	1.01±0.12	6.69±0.21c	2.72±0.16k	4.74±0.15k
	2.0	0.5	0.5	45	18	1.34±0.11	6.44±0.14cd	3.20±0.24j	5.34±0.14j
	2.0	1.0	0.5	45	23	1.85±0.11	6.31±0.06de	3.73±0.05efg	5.76±0.07h
	3.0	0.5	0.5	45	27	1.96±0.13	6.18±0.03de	3.92±0.07def	5.82±0.13h
	3.0	1.0	0.5	45	30	2.24±0.11	6.11±0.10de	4.12±0.12cd	7.32±0.13e
	4.0	0.5	0.5	45	35	2.75±0.17	5.10±0.67de	4.55±0.18b	8.66±0.12c
	4.0	1.0	0.5	45	49	2.97±0.13	5.10±0.10g	4.48±0.10b	9.55±0.13a
	5.0	0.5	0.5	45	22	2.15±0.13	6.16±0.07de	4.07±0.06cd	5.65±0.08i
	5.0	1.0	0.5	45	19	1.05±0.18	6.75±0.11c	3.38±0.29hij	5.48±0.19i

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Fig. 7 (a-e): Different stages of *in vitro* shoot regeneration via callus formation in Asterix (a) Formation of shoots from callus of inter nodal segments on MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3; (b) Stereomicroscopic view of shoot initiation from green compact callus (arrows indicate the regeneration of shoots); (c) Initiation of shoots from leaf explants on MS supplemented with 3.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3; (d) Multiple shoots formation and (e) Elongated shoots on MS medium.

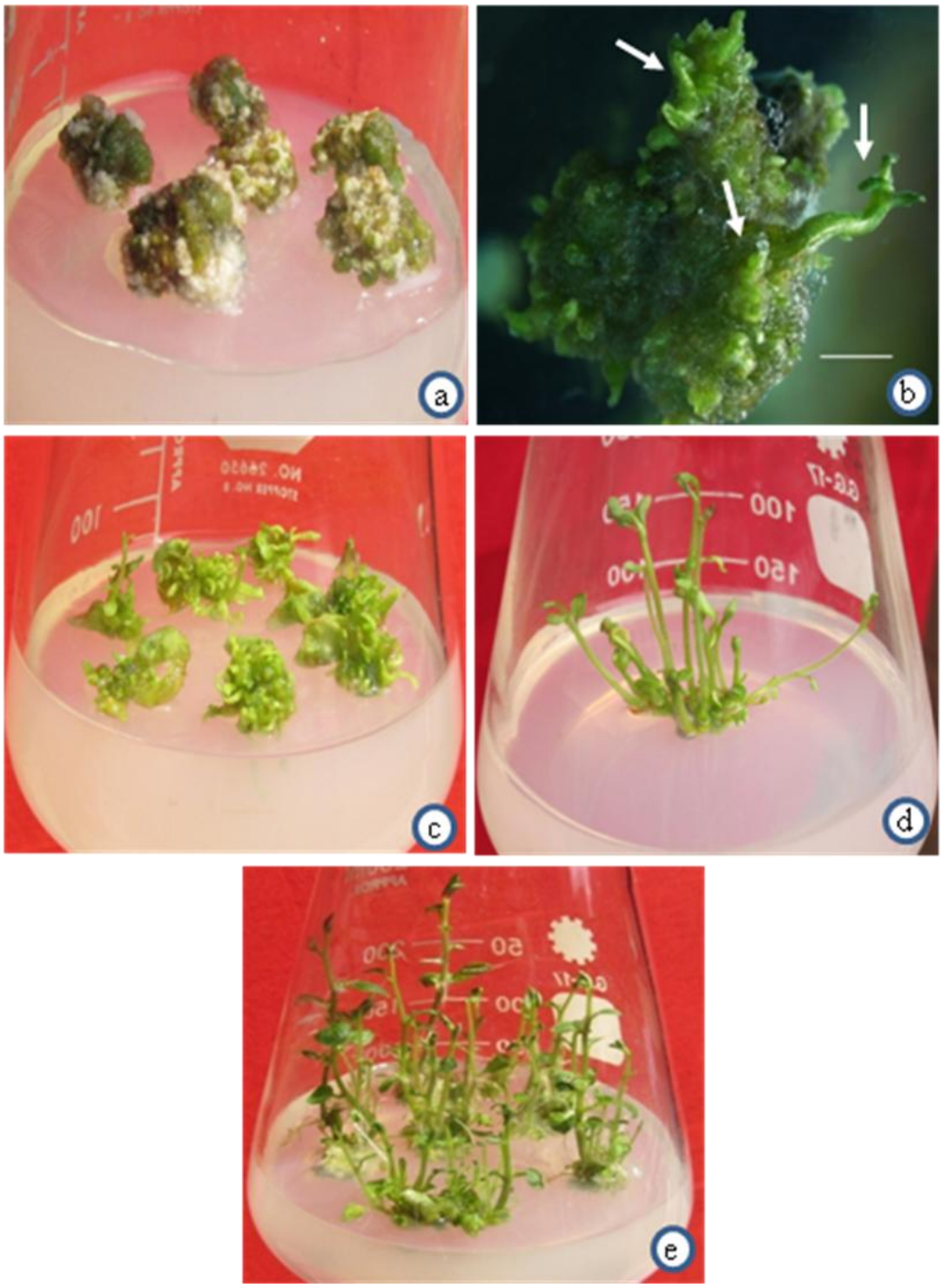

Fig. 8 (a-e): Different stages of *in vitro* shoot regeneration in Diamant. (a) Callus induction from inter nodal segments on MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3; (b) Stereomicroscopic view of shoot initiation from compact green callus (arrows indicate regeneration of shoots); (c) Initiation of shoots from leaf explants on MS supplemented with 3.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3; (d) Multiple shoots formation and (e) Elongated shoots on MS medium.

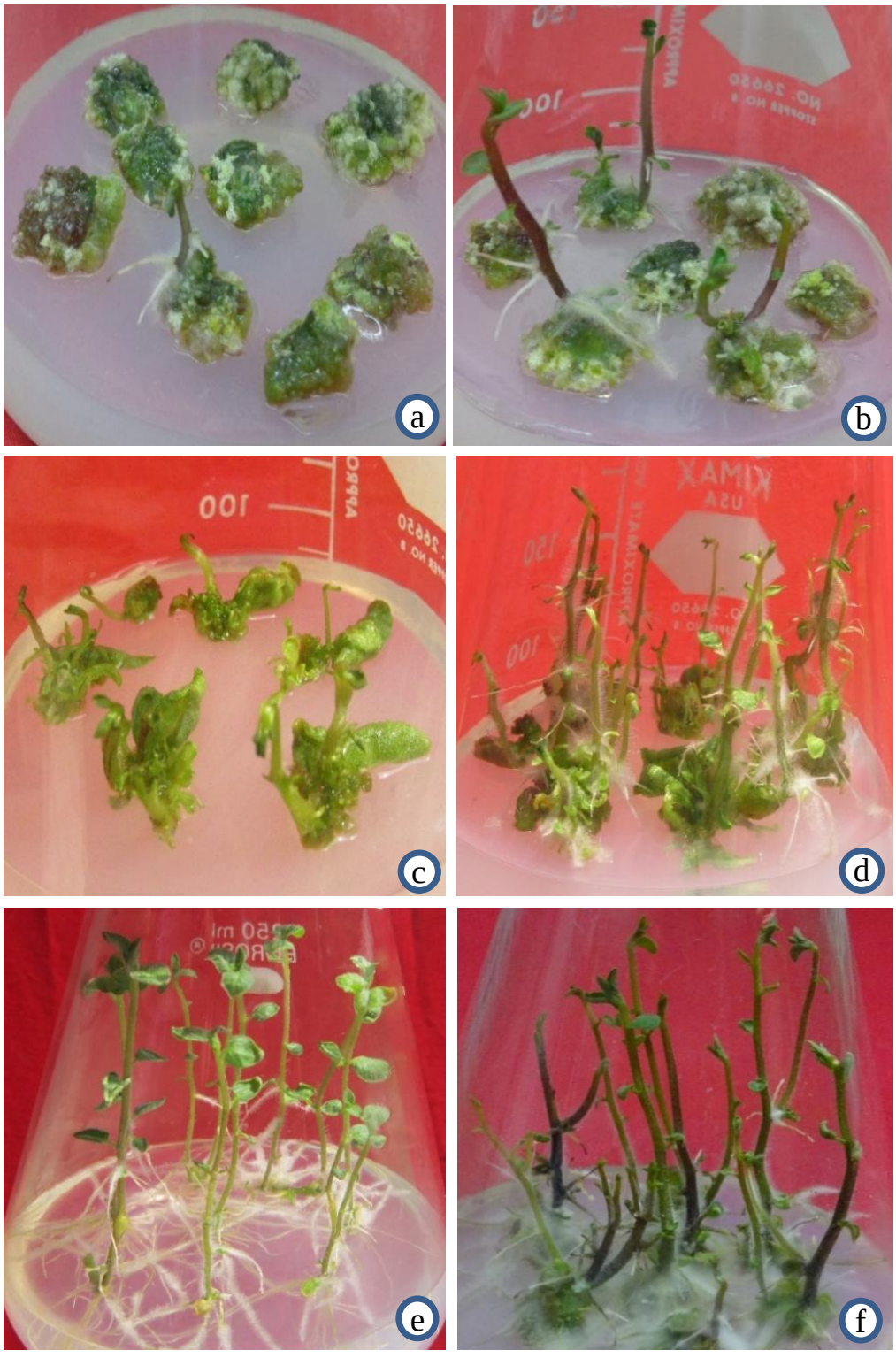

Fig. 9 (a-f): *In vitro* regeneration of shoots in Granola and Lady Rosseta on MS supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA and 0.5 mg/l GA_3. (a-b) Formation of multiple shoots from callus of internodal segments of var. Lady Rosseta; (c-d) Development of multiple shoots from callus of leaf of var. Granola; (d) Elongated shoots of var. Granola and (e) Elongated shoots of var. Lady Rosseta.

4.1.2 Induction of roots from *in vitro* regenerated shoots

Induction of roots from regenerated shoots is considered to be very important to obtain complete plantlet. In the present study most of the explants produced high number of roots on shoot induction medium (MS supplemented with BAP and IAA) and subsequently these roots were found to produce healthy effective root system when they were transferred to MS basal medium. After 3-4 weeks, well rooted shoots were carefully taken out from the culture tube and were successfully transplanted to plastic pots containing soil. About 95% transplanted plantlets survived which produced phenotypically normal minitubers.

4.1.2.1 Effect of half and full strength of MS on root induction in four varieties of potato

Agar solidified full and half strength of MS medium devoid of any hormonal supplement were used as root induction medium (Table 11). About 15 days old (2-3 cm in length) single shoots were used for root induction at the base of *in vitro* grown shoot. Four varieties of potato showed identical response towards root induction on MS medium and it took 6-8 days for root induction. Mean number of roots per shoot was 12.38±0.19, 11.98±0.23, 10.54±0.38, 10.23±0.25 in Asterix, Diamant, Granola and Lady Rosseta, respectively (Figs. 10-12).

4.1.2.2 Effects of different concentrations of auxins on root induction

Effects of different auxins, namely, IAA and IBA on root induction were monitored in case of *in vitro* grown shoots of four potato varieties. For this, 0.2 mg/l IAA and 0.5 mg/l IBA were added separately with MS medium to induce roots (Table 12). It is observed that percentage of roots were highest on MS medium supplemented with 0.2 mg/l IBA.

Mean number of roots per shoot was 20.52±0.45, 15.91±0.13, 15.20±0.20, 15.07±0.70 for Asterix, Diamant, Granola and Lady Rosseta, respectively.

Table 11. Effects of full MS and half strength of MS on root formation in four varieties of potato

Variety	Media	Number of inoculated Shoots	Days to initiate root	% of shoot forming roots	Mean number of roots per plant (±SD)	Mean length (cm) of roots per plant (±SD)	Days required to get well developed roots
Asterix	½ MS	30	6-8	86.67	10.19±0.42	7.8±0.43c	15-20
Diamant		30	6-8	83.33	9.92±0.27	6.7±0.21d	15-20
Granola		30	6-8	80	9.13±0.31	6.1±0.17e	15-20
Lady Rosseta		30	6-8	73.33	8.98±0.59	5.8±0.32f	15-20
Asterix	MS	30	6-8	93.33	12.38±0.19	8.1±01.07a	15-20
Diamant		30	6-8	90	11.98±0.23	7.9±0.29b	15-20
Granola		30	6-8	83.33	10.54±0.38	7.5±1.12c	15-20
Lady Rosseta		30	6-8	80	10.23±0.25	7.0±0.78c	15-20

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

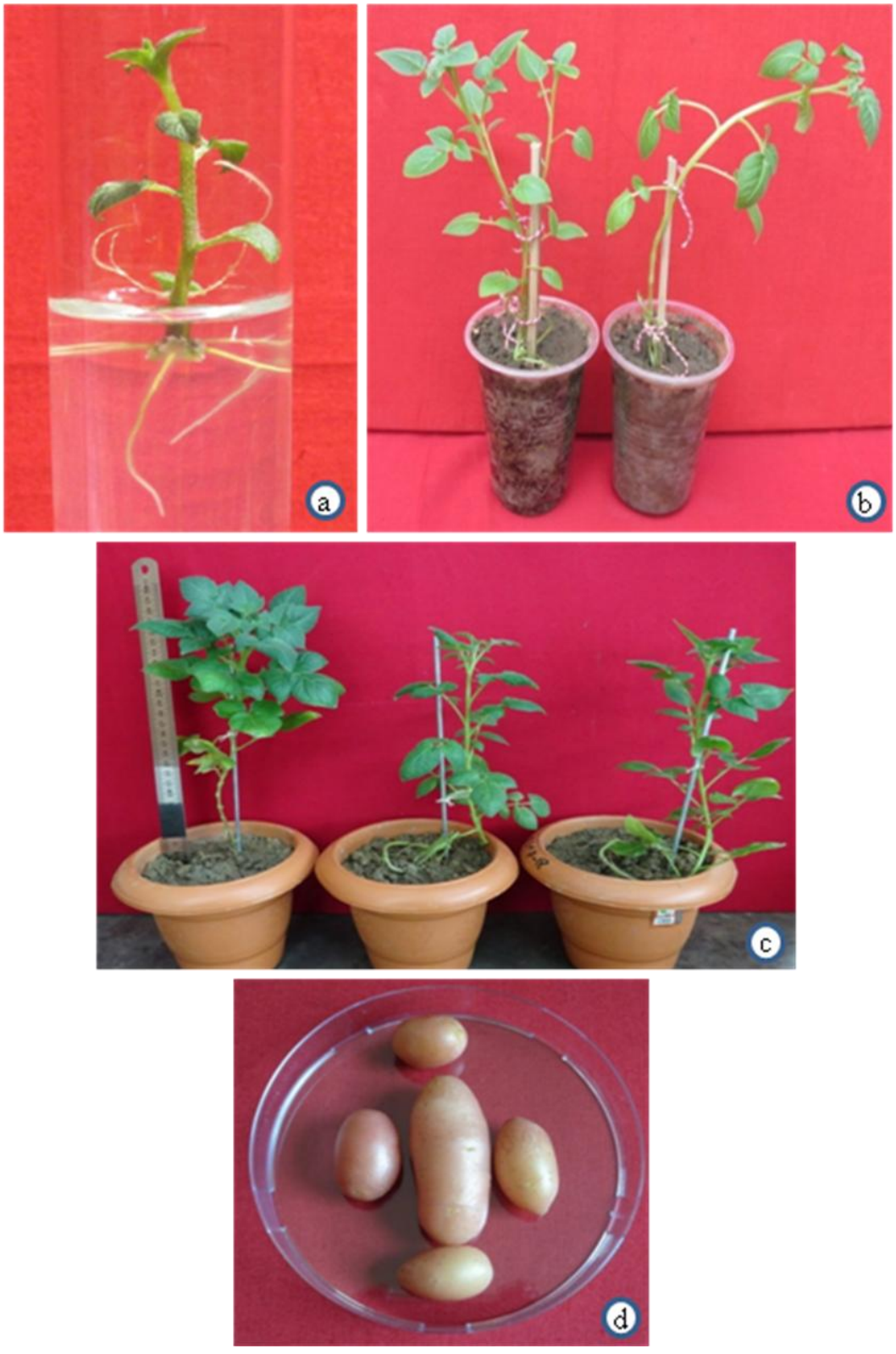

Fig. 10 (a-d): Transplantation of tissue culture derived plantlets of Asterix. **(a)** Fully developed rooted plantlet on MS medium; **(b)** Plantlets transferred to small pot containing soil; **(c)** Regenerated plants of Asterix after two months following transplantation and **(d)** Fully developed matured microtubers of tissue culture derived plant of Asterix.

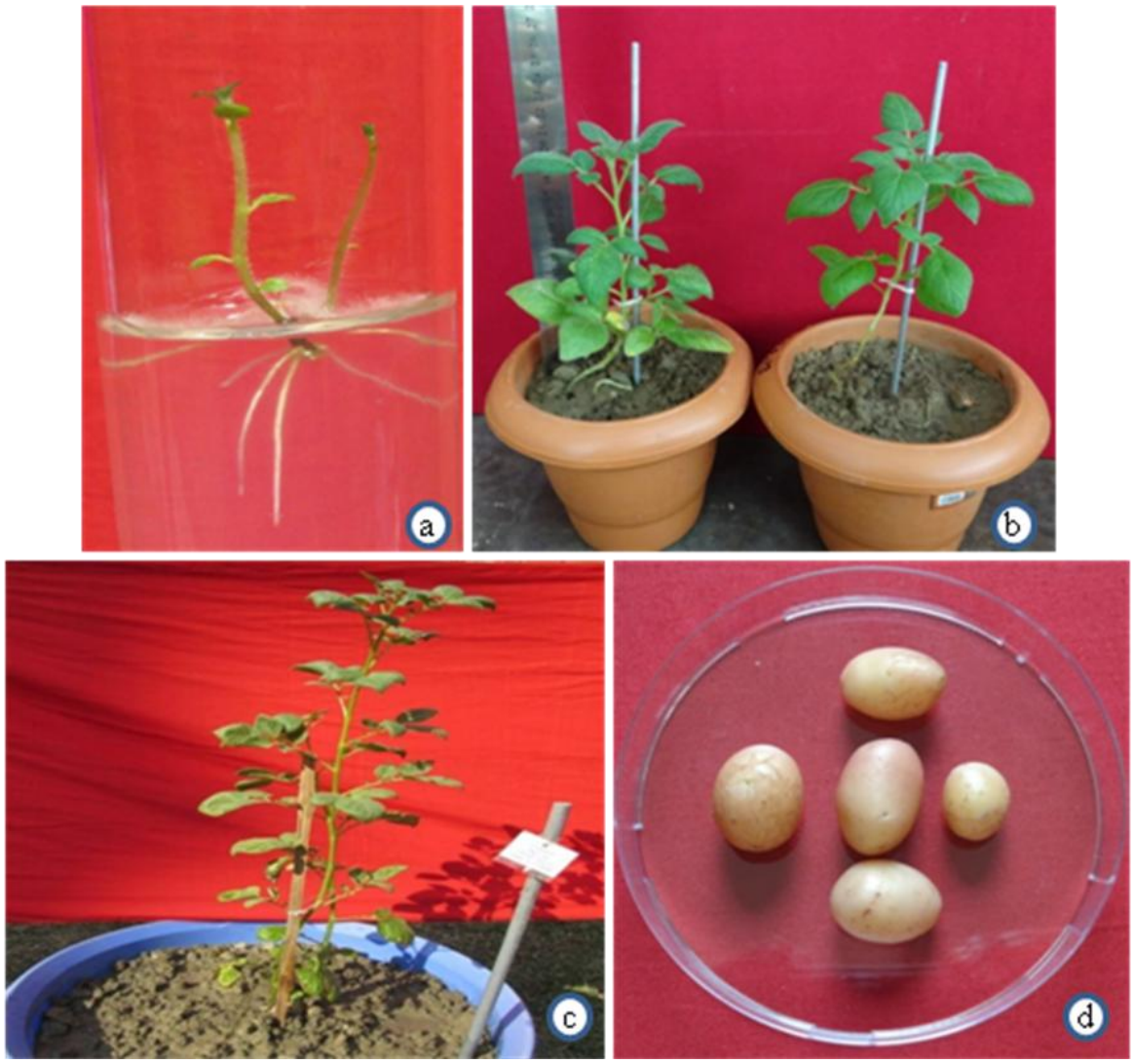

Fig. 11 (a-d): Transplantation of tissue culture derived plantlets of Diamant. **(a)** Fully developed rooted plantlet on MS medium; **(b)** Plantlets transferred to small pot containing soil; **(c)** Regenerated plant of Diamant after two months following transplantation and **(c)** Fully developed matured microtubers of tissue culture derived Diamant.

Fig. 12 (a-f): Transplantation of tissue culture derived plantlets of Granola and Lady Rosseta. **(a-b)** Fully developed rooted plantlet of Granola and Lady Rosseta on MS medium; **(c-d)** Regenerated plants of Lady Rosseta and Granola after two months following transplantation and **(e-f)** Fully developed matured minitubers of tissue culture derived plant of Lady Rosseta.

Table 12. Effects of MS with different concentrations of auxins (IAA and IBA) on root formation in four varieties of potato

Variety	Concentrations of growth regulators (mg/l)	Number of inoculated shoots	Days to initiate roots	% of shoots forming roots	Mean number of roots/ shoot (±SD)	Mean length (cm) of roots/plant (±SD)	Days required to get well developed roots
Asterix	0.2 IAA	30	6-8	63.33	10.38 ± 0.13^{h}	3.6 ± 0.11^{h}	15-20
	0.5 IAA	30	6-8	53.33	15.92 ± 0.16^{c}	4.5 ± 0.45^{b}	15-20
	0.2 IBA	30	6-8	76.67	20.52 ± 0.45^{a}	3.5 ± 0.34^{de}	15-20
	0.5 IBA	30	6-8	70	17.69 ± 0.51^{b}	4.1 ± 0.32^{c}	15-20
Diamant	0.2 IAA	30	6-8	56.67	14.45 ± 0.41^{ef}	3.4 ± 0.22^{de}	15-20
	0.5 IAA	30	6-8	43.33	14.56 ± 0.15^{ef}	4.7 ± 0.45^{b}	15-20
	0.2 IBA	30	6-8	73.33	15.91 ± 0.13^{c}	5.2 ± 0.31^{a}	15-20
	0.5 IBA	30	6-8	63.33	15.02 ± 0.16^{d}	4.8 ± 0.23^{b}	15-20
Granola	0.2 IAA	30	6-8	46.67	13.91 ± 19^{fg}	3.1 ± 0.33^{f}	15-20
	0.5 IAA	30	6-8	40	14.46 ± 0.19^{ef}	2.9 ± 0.45^{fg}	15-20
	0.2 IBA	30	6-8	70	15.20 ± 0.20^{d}	2.2 ± 0.28^{g}	15-20
	0.5 IBA	30	6-8	53.33	14.98 ± 0.11^{e}	2.7 ± 0.29^{fg}	15-20
Lady Rosseta	0.2 IAA	30	6-8	43.33	13.84 ± 0.15^{g}	3.1 ± 0.17^{f}	15-20
	0.5 IAA	30	6-8	36.67	14.28 ± 0.26^{fg}	4.5 ± 0.28^{b}	15-20
	0.2 IBA	30	6-8	63.33	15.07 ± 0.70^{d}	3.8 ± 0.44^{d}	15-20
	0.5 IBA	30	6-8	53.33	$15.43\pm0.0.40^{cd}$	4.6 ± 0.35^{b}	15-20

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$.

4.1.3 Establishment of plantlets

After sufficient development of roots, plantlets obtained from four potato varieties were successfully transplanted into small plastic pots containing autoclaved soil. For their further growth and establishment, the survived plantlets were transferred to large clay pots. In case of variety Asterix, Diamant, Granola and Lady Rosseta, survival rate of plants was 98.33, 96.67, 93.33 and 90 %, respectively (Table 13).

Table 13: Survival rate of tissue cultured derived plantlets of four potato varieties

Varieties	No. of rooted plantlets transferred to soil	No. of survival plants in soil	% of survival plants
Asterix	60	59	98.33
Diamant	60	58	96.67
Granola	60	56	93.33
Lady Rosseta	60	54	90

4.2 *Agrobacterium*-mediated genetic transformation

A genotype independent and effective plant regeneration protocol is a prerequisite for successful transformation system. During the present study plant regeneration was achieved through organogenesis either directly from the explant or through intervention of callus. Transformation experiments were undertaken using four different explants namely, nodal segment (NS), microtuber discs (MD), intermodal segments (INS) and leaf (L) of two potato varieties, namely, Asterix (BARI Alu-25) and Diamant (BARI Alu-7). In the present study, two different *Agrobacterium* strains LBA4404/pBI121 (construct I) and EHA105/pCAMBIA1301-PDH45 (construct II) were used for transformation. Results of these experiments are presented below in different headings.

4.2.1 Genetic Transformation using marker gene (construct I)

In the present study, genetically engineered *Agrobacterium* strain LBA4404/pBI121 (construct I) was used as marker strain which contains *GUS* gene as screenable marker and *nptII* gene as selectable marker conferring kanamycin resistance. Marker gene was used to investigate the transformation ability of various explants of different varieties of potato. Generally transient assay for such marker genes were routinely performed as a preliminary step to identify the conditions required for successful transfer of desired gene.

4.2.1.1 Determination of optimum kanamycin concentration for selection

To determine the optimum selection level, non infected explants and shoots (those served as negative control) were subjected to selection pressure. For this experiment the explants were transferred to suitable regeneration medium containing 0, 50, 100, 150 and 200 mg/l kanamycin. From this experiment it was observed that with increasing concentration of kanamycin, regeneration percentage from explants and survived shoots were found to decrease. Because of kanamycin, shoots first became albino and finally died. In this study it was observed that all the non-infected explants (negative control) died in presence of 200 mg/l kanamycin within 15 days of inoculation. Therefore, shoot developed and survived on regeneration medium with 200 mg/l kanamycin were considered as transformed.

4.2.1.2 Optimization of different parameters influencing transformation efficiency of explants

Available reports on *Agrobacterium*-mediated genetic transformation indicate that, transformation efficiency is influenced by several factors such as optical density (O.D.) of *Agrobacterium* suspensions, incubation and co-cultivation period of the explants. Optimization of these conditions were done by monitoring transient expression of the GUS reporter gene after co-cultivation of explants in *Agrobacterium* strains LBA4404 containing binary plasmid pBI121 (construct I).

4.2.1.3 Influence of optical density (O.D.), incubation period and age of explants on transformation of Asterix

Agrobacterium suspension prepared from the *Agrobacterium* culture was used to infect the explants. In this experiment, relationship between optical density and incubation period of *Agrobacterium* suspension as well as transformation efficiency of explants were studied. For this purpose, optical density (O.D.) was measured at 600 nm and bacterial

suspension with optical density of 0.5, 0.6, 0.8 and 1.0 having incubation period of 15, 20, 30 and 45 minutes were used in these experiments.

Maximum percentage of transformation was observed at OD_{600} of 0.6. Percentage of GUS positive explants was 90, whereas minimum transient GUS expression (10%) was recorded at OD_{600} of 1.0 in case of Asterix (Table 14). No significant GUS expression was observed when infection was allowed in potato variety at lower OD_{600} (0.2-0.4). Suspension in high density of bacteria (OD600 = > 0.6) also resulted in weak expression of transgene. Infection time of 5, 10, 15, 20 and 25 mins showed lower frequency of GUS expression in the potato variety tested. Lower expression of GUS gene was observed when nodal segment explants of Asterix were treated for a longer period (Table 14). Moreover, overgrowth of *Agrobacterium* was noticed when infection time was increased to 40 min. The current study revealed the responsiveness of the nodal segments to be optimal for regeneration and shoot proliferation on shoot regeneration medium after 30 min of infection with *Agrobacterium* culture. Nodal segments from shoots of different ages (10-30 days old) were assessed for their competency in *Agrobacterium*-mediated transformation and regeneration in this study. Infection of 10-12 days old explants for 30 min showed decreased frequency of GUS expression. In case of 15-20 days old nodal segments, mean transient GUS expression was significantly higher. Significant differences were found among the responses of nodal segments derived from 10-30 days old shoots. Asterix produced 90 % of mean transient GUS expression (Table 14). The results indicated that 15-20 days old nodal segments were more compatible for *Agrobacterium* infection. Nodal segments of 30 or more than 30 days old showed correspondingly decreased frequency of transient GUS expression in Asterix variety of potato.

The maximum percentage of GUS positive explants was observed at 30 minutes of incubation at OD_{600} of 0.6 for these variety. It was also observed that when duration of incubation period and optical density of the bacterial suspension was more than 45 minutes and 1.0, respectively, overgrowth of bacteria in the co-cultured plates was observed which hampered desired regeneration capability of the explants. In some cases, overgrowth of bacteria was noticed following one or two subcultures when the explants were treated with the above mentioned conditions. This undesired overgrowth of bacteria can be controlled when bacterial suspension had an OD_{600} of 0.6 with incubation period of 30 min. Therefore, the optimum incubation period for the explants was found to be 30 minutes with optical density of 0.6.

4.2.1.4 Influence of co-cultivation period on transformation

15 days old explants infected for 30 min with 0.6 at OD_{600} of *Agrobacterium* culture and co-cultivation for 1, 2, 3, 4 and 5 days separately showed varied frequencies of transient GUS expression and stable transformation in Asterix variety of potato. Mean transient GUS expression frequencies of 96% were obtained for Asterix on 3 days of co-cultivation (Table 15). However, co-cultivation for 4 days increases the over growth of *Agrobacterium* on nodal segments which was difficult to remove. Higher co-cultivation period of 4-5 days negatively affected both transient frequency and stable transformation in the potato varieties used. Thus, three days of co-cultivation was used for transformation of the potato variety with nodal segment explants.

Table 14. Influence of optical density (measured at 600 nm) of *Agrobacterium* suspension and effect of different incubation periods on *Agrobacterium*-mediated transformation of nodal segment explants of Asterix variety of potato analyzed through transient GUS histochemical assay

OD_{600}	Incubation period (min)	No. of explants infected	No. of explants assayed for GUS	No. of GUS +ve explants	% of GUS+ve explants
0.5	15	50	20	3	15
	30	50	20	4	20
	45	50	20	5	25
	60	50	20	7	35
0.6	15	50	20	12	60
	30	50	20	18	90
	45	50	20	16	80
	60	50	20	9	45
0.8	15	50	20	6	30
	30	50	20	9	45
	45	50	20	10	50
	60	50	20	8	40
1.0	15	50	20	7	35
	30	50	20	8	40
	45	50	20	5	25
	60	50	20	2	10

Table 15. Influence of different co-cultivation periods on transformation analyzed by transient GUS histochemical assay of potato var. Asterix

Variety	Co-cultivation period (days)	No. of explants assayed for GUS expression	No. of GUS +ve explants	% of GUS +ve explants
Asterix	1	25	12	48
	2	25	17	68
	3	25	24	96
	4	25	19	76
	5	25	8	32

4.2.1.5 Effect of acetosyringone on transformation of potato var. Asterix

Improvement of transformation frequency and stable transformation of Asterix was achieved using various concentrations (50-200 µM) of acetosyringone in the co-cultivation medium keeping other conditions optimized above, namely, 0.6 optical density of *Agrobacterium* culture, 30 min infection time and 3 days of co-cultivation. Addition of acetosyringone in the co-cultivation medium considerably enhanced transformation efficiency. Infected nodal segments cultured on co-cultivation medium containing 100 µM acetosyringone showed maximum frequency of transient GUS expression in Asterix (Table 16). Highest frequency of stable transformation was also obtained on this medium for this variety. Supplementation of acetosyringone both at lower (50 µM) and higher concentrations (150-200 µM) decrease the frequency of transient GUS expression and transformation. Very few numbers of transgenic shoots were found in the co-cultivation medium without acetosyringone.

Table 16. Effect of Acetosyringone concentrations on transformation analyzed by transient GUS histochemical assay of potato var. Asterix

Variety	Concentrations of acetosyringone (µM)	No. of explants assayed for GUS expression	No. of GUS +ve explants	% of GUS +ve explants
Asterix	0	25	4	16
	50	25	7	28
	100	25	22	88
	150	25	17	68
	200	25	10	40

4.2.1.5 Responses of various explants of potato towards *Agrobacterium*-mediated genetic transformation

Transformation ability of various explants, namely, nodal segments, microtuber discs, internodal segments and leaf segment of Asterix variety of potato was monitored through the expression of the GUS gene following incubation for a period of 30 min with *Agrobacterium* strain LBA4404 having an OD of 0.6 and co-cultivation for 3 days. GUS expression was detected by histochemical GUS assay and such expression was characterized by the formation of indigo blue colour within the transformed cells of the infected explants. Control explants were always maintained in each set of experiment and were subjected to GUS histochemical assay (Table 17). A good number of co-cultured explants showed positive expression to GUS staining. GUS positive regions were visualized mostly at the peripheral area of the cut surfaces as well as within the internal tissues of various explants. Prominent blue coloured (GUS+ve) zones within co-cultured explants were visualized under stereomicroscope. It was evident that, nodal segment explants showed the best response towards transformation with LBA4404 strain and the percentage of GUS positive nodal segment explants was 95 in case of var. Asterix (Table

17). Next to nodal segment explants leaf explants showed better responses towards transformation and in this case the percentage of GUS positive explants was found to be 81.67 for Asterix (Table 17). Transformation efficiency was comparatively low in case of microtuber discs explants and its regeneration capacity was also found to be lowest among the four different types of explants studied. Therefore, these explants were not included for further transformation experiments. Besides, internodal segment explant was not included in transformation experiments due to their poor regeneration responses, despite of showing better transformation efficiency. Thus, further transformation experiments were carried out only with nodal segment and leaf explants.

Table 17. Responses of various explants of Asterix towards GUS histochemical assay following co-cultivation.

Variety	Explants	No. of explants assayed for GUS	No. of GUS+ve explants	% of GUS+ve explants
Asterix	NS	60	57	95
	MD	60	35	58.33
	INS	60	42	70
	L	60	49	81.67

NS = Nodal Segment, MD = Microtuber discs, INS = Inter Nodal Segment, L = Leaf

4.2.1.5 *In vitro* regeneration and selection of putatively transformed plants of var. Asterix using kanamycin

For the selection of transformed tissues, antibiotic selection pressure was applied immediately after co-cultivation. Selection of transformed shoots was done using kanamycin. For direct selection, explants were transferred directly to selection medium (4.0 mg/l BAP, 1.0 mg/l IAA) with 300 mg/l carbenicillin and 200 mg/l kanamycin. Direct selection allows to obtain regeneration of only transformed shoots following co-cultivation. It was observed that when kanamycin was applied immediately after co-cultivation, non-transformed explants turned into albino or deep brown colour but the

transformed explants remain green or light brown. Non-transformed albino explants were discarded. Green and brown coloured explants were sub-cultured regularly in every 20-30 days. After 3 months, brown coloured explants started greening and formed green callus. Regeneration and proliferation of shoots was occurred after 15-20 days of callus induction on MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA, 0.5 mg/l GA_3, 300 mg/l carbenicillin and 200 mg/l kanamycin. After that regenerated shoots were sub-cultured on the same medium supplemented with 200 mg/1 kanamycin. In case of nodal segments, mean number of shoot per explants was 8.5±0.23 for Asterix. In this variety, mean transformation efficiency was 32.17 and 17.50 for nodal segments and leaf explants, respectively (Table 18). Non-transformed explants were also inoculated into selection medium as control. These explants were failed to survive and turned albino (Fig. 13).

Table 18. Effects of 200 mg/l kanamycin towards regeneration on MS medium containing 4.0 mg/l BAP and 1.0 mg/l IAA and 0.5 mg/l GA_3 after co-cultivation and analysis of transformation efficiency for potato var. Asterix

Variety	Explants Name	Total no. of explants infected	No. of responsive explants after six weeks	No. of regenerated explants produced shoots	Mean no. of shoots/explant after 95 days of inoculation (±SD)	Transformation efficiency (%)	Mean transformation efficiency (%)
Asterix	Nodal segment	235	190	82	8.5 ± 0.23^{b}	34.89	32.17
		190	160	55	10.5 ± 0.41^{a}	28.94	
		260	215	85	9.0 ± 0.17^{ab}	32.69	
	Leaf	210	165	38	5.1 ± 0.38^{c}	19.0	17.50
		160	95	27	4.8 ± 0.22^{d}	13.5	
		200	152	40	5.2 ± 0.32^{c}	20	

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

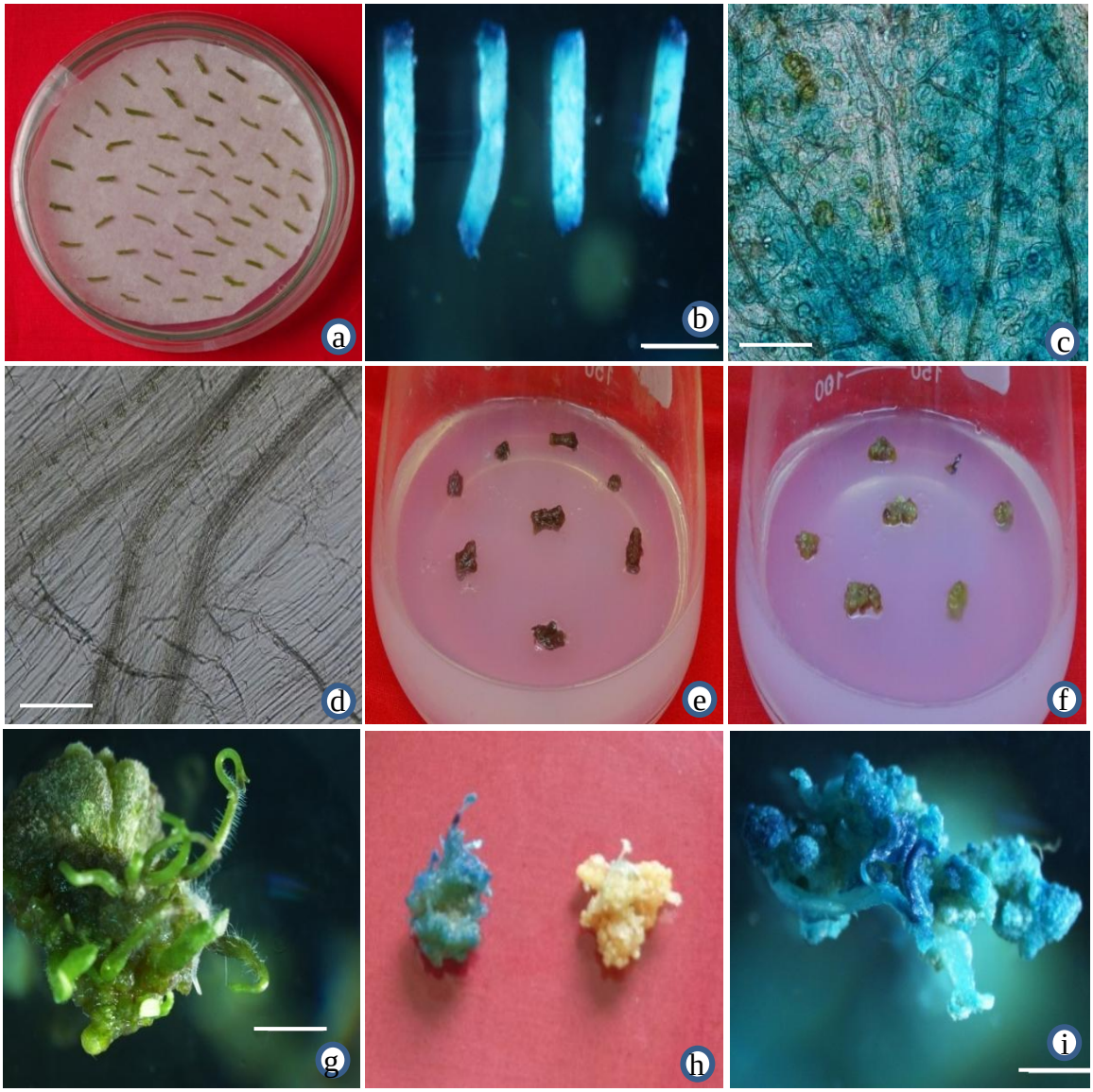

Fig. 13 (a-i): Putative transgenic plantlets development from nodal segment explants infected with *Agrobacterium* strain LBA4404 containing plasmid pBI121. (a) Co-culture of nodal segments of Asterix; (b) Stereoscopic view of nodal segment explants showing consecutive blue colour at cut end; (c) photomicrographs of nodal segment tissues showing expression of *GUS* (blue color); (d) photomicrographs of control nodal segment tissues didn't show any blue colour; (e) Negative control explants did not show any response towards regeneration after 3 weeks of inoculation; (f) Callus formation from brown nodal segments after 5-6 weeks of inoculation on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA with 200 mg/l Kanamycin and 300 mg/l Carbenicillin; (g) Stereomicroscopic view of initiation of shoots from callus of nodal segments after 7 weeks of infection; (h) *GUS* expression on callus of nodal segments explants and (i) Stereomicroscopic view of initiation of shoots from callus showing expression of *GUS* (blue color). Scale bar = 2.0 mm.

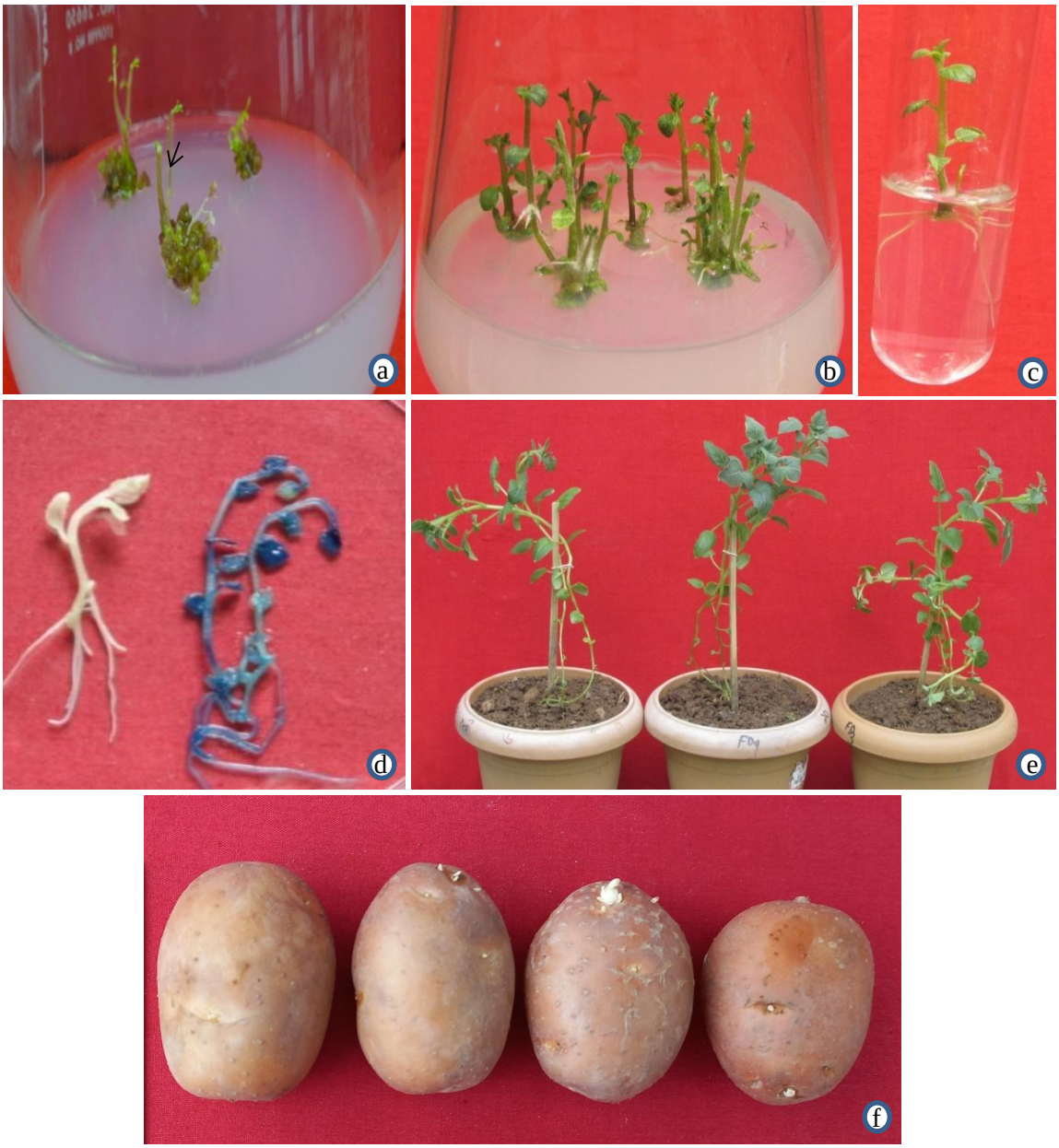

Fig. 14 (a-f): Transplantation of putatively transformed plants of var. Asterix. (a) Initiation of shoots from callus of nodal segments on MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3; (b) Multiple shoots formation on same medium; (c) *GUS* expression of putative transgenic plantlets with a control; (d) Fully developed roots of Asterix on MS medium; (e) Transplantation of regenerated plantlets in pots containing soil and (f) Microtubers formation from putatively transformed plants.

4.2.1.6 Establishments of putative transformed plantlets to soil

Putative transgenic shoots of 2.0-3.0 cm in length that regenerated from callus under the kanamycin selection pressure were separated and transferred to MS medium with 200 mg/l kanamycin and 100 mg/l carbenicillin for root induction. Root induction started within 6-8 days. For var. Asterix, altogether 75 shoots regenerated through direct selection were subjected for root induction. It was observed that 72 out of 75 shoots showed positive responses towards root induction. In case of var. Diamant, 67 shoots regenerated under selection were subjected for roots induction. Out of 67, 64 shoots showed response towards root induction. Following proper acclimatization plantlets were transplanted to soil (Fig. 14 a-f).

4.2.1.7 GUS assay of developed transformed plants

Histochemical GUS assay (Jefferson 1987) was performed to detect the stable expression of *GUS* gene. Leaves and small branches of the transformed plantlets were subjected to GUS histochemical assay. Such assay demonstrated the presence of blue coloured zones on selected shoots and leaves. Microtubers, germinated microtubers and microtuber discs were also subjected to GUS histochemical assay and they showed the presence of indigo blue coloured zones. Fully developed rooted plantlets also showed GUS positive blue colour (Figs. 15 a-c).

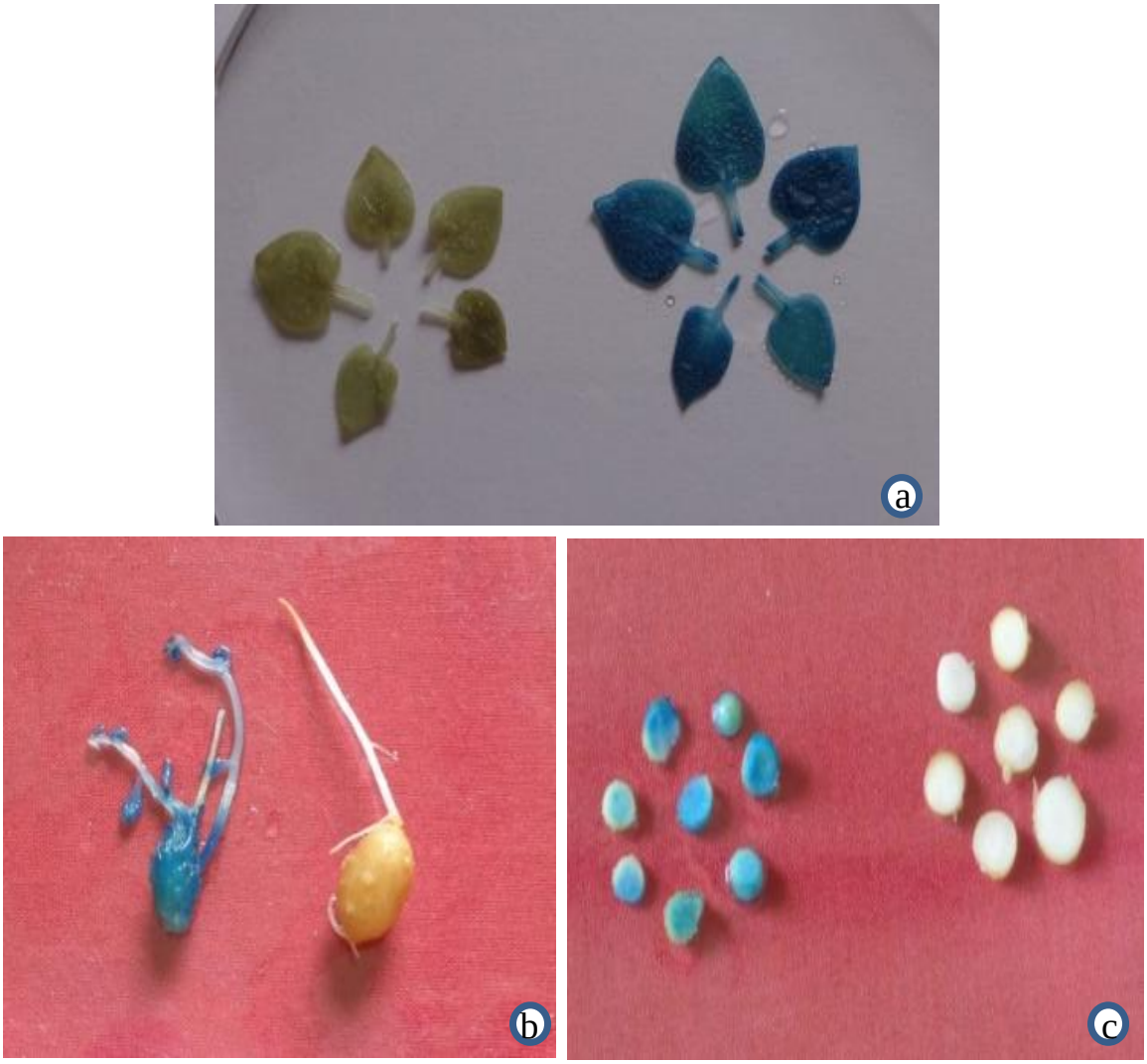

Fig. 15 (a-c). Histochemical localization of *GUS* activity of different parts of putative transgenic plants of Asterix. (a) leaf of T_0 plant showing conspicuous blue colour; (b) Germinated microtubers showing blue colour and (c) Microtuber slices from T_0 plant showing *GUS* expression with non-transformed control plant.

4.2.1.8 Analysis of transgene integration for var. Asterix through PCR

Transgenic nature of the putative transformed shoots was confirmed by amplification of *GUS* and *nptII* genes present within the genomic DNA of the transformed shoots. For this purpose, total genomic DNA was isolated from the leaf of putative transformants as well as control non-transformed potato plants. This isolated DNA was subjected to PCR for the amplification of *GUS* and *nptII* gene.

Specific primers for *GUS* and *nptII* genes were used for PCR amplification. LBA4404/pBI121 plasmid was used as positive control. The amplified DNA was analyzed through agarose gel electrophoresis. Presence of 750 bp band for *GUS* gene and 720 bp band for *nptII* gene in transformed shoots confirmed the presence of *GUS* and *nptII* genes (Fig. 16).

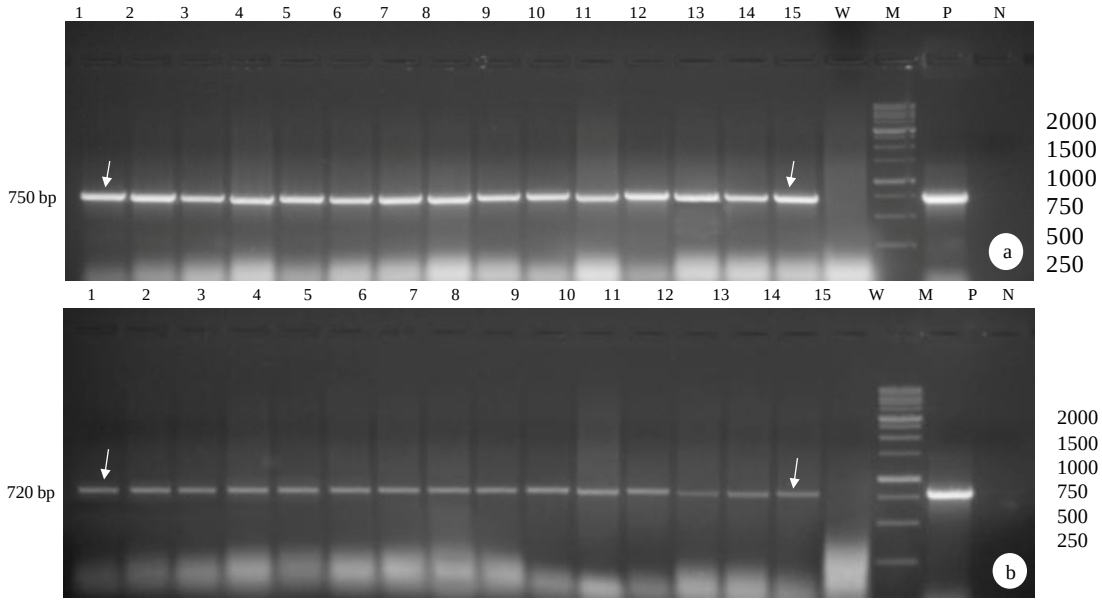

Fig. 16: Molecular confirmation of the putative transformants of var. Asterix. PCR amplification of *GUS* (a) and *nptII* (b) genes (lanes 1-18 genomic DNA of transformed shoots; lane W- wild; lane M-1kb ladder; lane P-plasmid DNA of LBA4404/pBI121 as positive control; lane N- negative control; arrows indicate the positive band of *GUS* and *nptII* genes).

4.2.2 Genetic transformation using salinity and drought tolerant gene construct

Agrobacterium-mediated genetic transformation was carried out using other genetically engineered *Agrobacterium* strain, namely, EHA105/pCAMBIA1301-PDH45 (construct II) to integrate salinity and drought tolerant gene in potato varieties.

4.2.2.1 Transformation of potato with EHA105/pCAMBIA1301-PDH45 strain (construct II)

Transformation experiments were carried out following the protocol that has been established with marker gene only with nodal segment explants from Asterix variety of potato.

4.2.2.1.1 Determination of optimum hygromycin concentration for selection of transformed shoots

For construct II hygromycin was used as selective agent as the construct contained *PDH45* and *hptII* genes which is resistant to hygromycin. Different concentrations (5.0-30 mg/l) of hygromycin were used to find out the appropriate concentration of selection agent. From this experiment it was revealed that with the increase of hygromycin concentration the percentage of regeneration of shoots from explants were found to decrease in case of two varieties (Asterix and Diamant) of potato (Fig.17 a-g). About 80 % of the explants showed necrosis and did not show any sign of regeneration at 5.0 mg/l hygromycin concentration. Further increase in the level of hygromycin led to browning of explants and caused almost inhibition of regeneration and explants failed to survive within 20 days of inoculation. Therefore, this concentration (20 mg/l hygromycin) was used for selection of shoots.

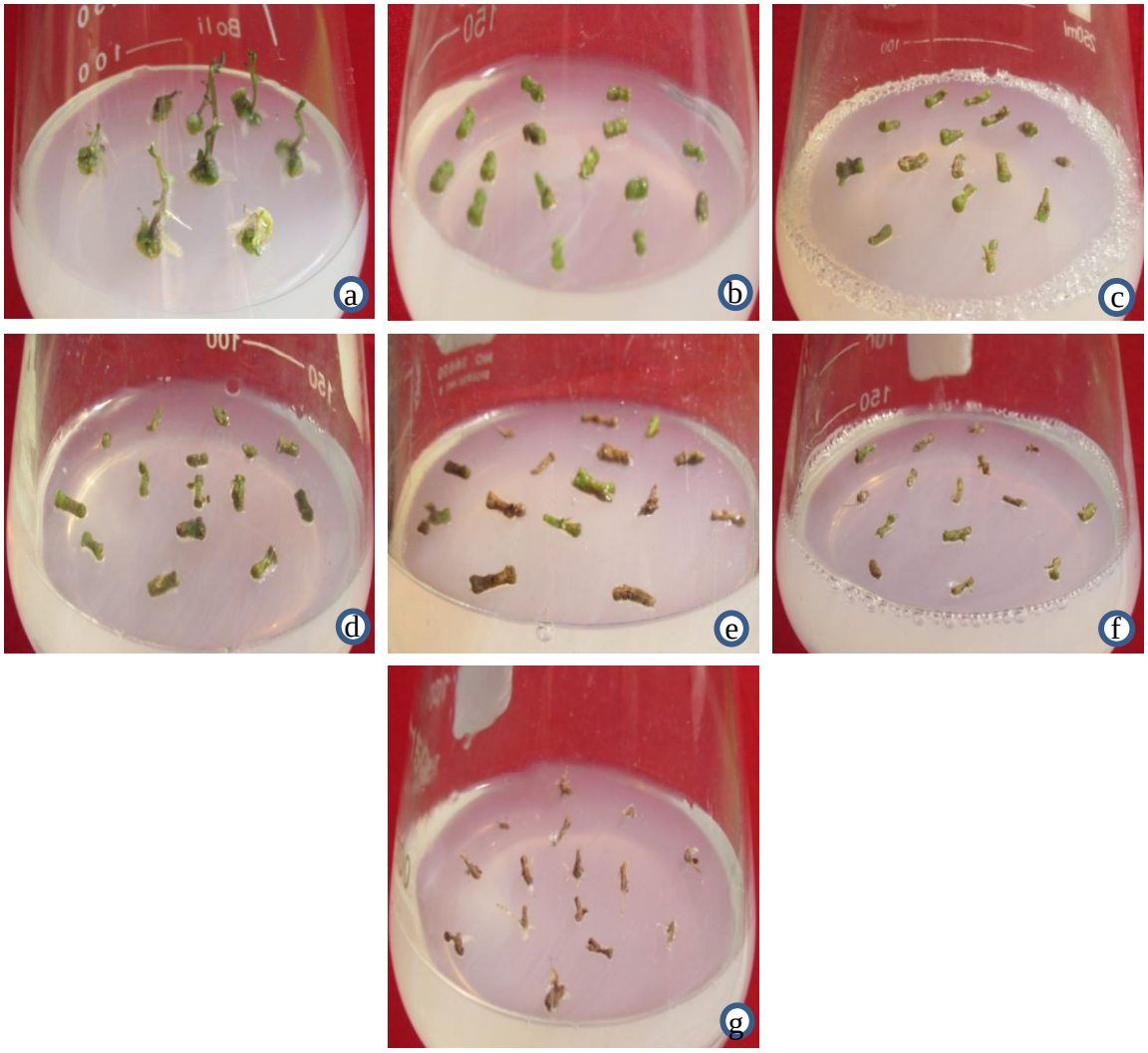

Fig. 17 (a-g): Hygromycin sensitivity test of nodal segments explants of Asterix on MS medium with different concentrations of hygromycin. (a) MS with 0; (b) 5; (c) 10; (d) 15; (e) 20; (f) 25 and (g) 30 mg/l hygromycin.

4.2.2.1.2 Effect of different parameters influencing transformation efficiency using *Agrobacterium* strain EHA105

In this experiment, the effect of optical density (OD) of *Agrobacterium* suspension, incubation and co-cultivation period of nodal segment explants with *Agrobacterium* suspension towards transformation were studied. For this purpose optical density (OD) was measured at 600 nm and bacterial suspension with optical density of 0.5, 0.6, 0.7 and 0.8 having incubation period of 10, 15, 20 and 25 min were used.

It was observed that more than 20 min of incubation with optical density of 1.0 or above of bacterial suspension resulting overgrowth of bacteria in the co-cultured plates and this kind of overgrowth hampered proper growth of the explants. Negligible overgrowth was observed when the explants were infected with the *Agrobacterium* suspension having an OD of 1.0 or less with incubation period of 20 min or less than this. Therefore, optimum incubation period was found to be 20 min with optical density of 0.5. At incubation period of 20 min the percentage of responsive explants towards multiple shoot regeneration was 92 and number of shoots per explant was 11.86±0.12 for Asterix (Table 19). In case of Diamant, percentage of responsive explants towards multiple shoot regeneration was 89 and number of shoots per explants was 10.24±0.10 (Table 20).

Duration of co-cultivation was also played an important role to recover explants after transformation. Four different co-cultivation periods ranging from 2-4 days with OD of 0.5 and were tried to find out the optimum co-culture period. It was observed that a co-cultivation for more than 2 days occasionally promoted overgrowth of bacteria and 4 days of co-cultivation period a remarkable overgrowth of bacteria was observed which covered the whole culture plate. Therefore, most of the explants in co-culture media suffered from poor health, became brown and failed to regenerate. Based on the above results it was demonstrated that 2 days of co-cultivation period with optical density of 0.5 is optimum for transformation with construct II (Table 21).

Table 19. Influence of optical density (measured at 600 nm) of *Agrobacterium* suspension and effect of different incubation periods on *Agrobacterium*-mediated transformation of var. Asterix

OD_{600}	Incubation period (min)	No. of explants infected	Percentage of responsive explants towards multiple shoot regeneration	Mean no. of shoots/explant **(±SD)**
0.5	15	50	74	10.43 ± 0.22^{b}
	20	50	92	11.86 ± 0.12^{a}
	25	50	64	9.55 ± 0.30^{c}
	30	50	26	8.81 ± 0.10^{d}
0.6	15	50	50	9.71 ± 0.24^{c}
	20	50	42	7.33 ± 0.25^{f}
	25	50	38	6.84 ± 0.19^{g}
	30	50	22	6.01 ± 0.13^{h}
0.7	15	50	32	8.17 ± 0.17^{e}
	20	50	28	7.59 ± 0.16^{f}
	25	50	24	6.20 ± 0.19^{h}
	30	50	22	5.18 ± 0.18^{i}
0.8	15	50	20	4.28 ± 0.14^{j}
	20	50	18	4.02 ± 0.08^{j}
	25	50	16	2.80 ± 0.13^{k}
	30	50	12	1.72 ± 0.10^{l}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 20. Influence of optical density (measured at 600 nm) of *Agrobacterium* suspension and effect of different incubation periods on *Agrobacterium*-mediated transformation of var. Diamant

OD_{600}	Incubation period (min)	No. of explants infected	Percentage of responsive explants towards multiple shoot regeneration	Mean no. of shoots/explant **(±SD)**
0.5	15	50	72	9.43 ± 0.31^{b}
	20	50	89	10.24 ± 0.10^{a}
	25	50	62	8.69 ± 0.16^{c}
	30	50	24	7.82 ± 0.17^{d}
0.6	15	50	49	8.44 ± 0.18^{c}
	20	50	38	6.23 ± 0.10^{g}
	25	50	36	5.49 ± 0.29^{h}
	30	50	20	5.58 ± 0.43^{h}
0.7	15	50	30	7.43 ± 0.26^{e}
	20	50	26	6.56 ± 0.24^{f}
	25	50	22	5.25 ± 0.21^{h}
	30	50	20	4.82 ± 0.23^{i}
0.8	15	50	18	3.90 ± 0.15^{j}
	20	50	16	3.31 ± 0.18^{k}
	25	50	14	2.48 ± 0.17^{l}
	30	50	10	1.44 ± 0.22^{m}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 21. Influence of different co-cultivation periods on transformation of potato var. Asterix and Diamant in case of *Agrobacterium* strain EHA105/1301-PDH45

Variety	Co-cultivation period (days)	No. of explants inoculated	Percentage of responsive explants towards multiple shoot regeneration	Mean no.of shoot/explants **(±SD)**
Asterix	1	60	33.33	10.25±0.13b
	2	60	**90.0**	**11.38**±0.25a
	3	60	**Overgrowth of bacteria occurred**	-
	4	60	**Overgrowth of bacteria occurred**	-
Diamant	**1**	60	**30.0**	**9.75**±0.22c
	2	60	**88.33**	**10.34**±0.17b
	3	60	**Overgrowth of bacteria occurred**	-
	4	60	**Overgrowth of bacteria occurred**	-

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$.

4.2.2.1.3 Regeneration and establishment of putative transgenic potato varieties (Asterix and Diamant)

For the selection of transformed tissues, antibiotic selection pressure was applied immediately after co-cultivation. Selection of transformed shoots was done using hygromycin, since hygromycin resistance *PDH45* and *hptII* genes were present in the plasmid of the engineered *Agrobacterium*. For direct selection, the explants were transferred directly to selection medium (4.0 mg/l BAP, 1.0 mg/l IAA) with 300 mg/l carbenicillin and 20 mg/l hygromycin. Direct selection allows to obtain regeneration of only transformed shoots following co-cultivation. It was observed that when hygromycin

was applied immediately after co-cultivation, non-transformed explants turned albino or deep brown colour. However, the transformed explants remained green or light brown. The non-transformed albino explants were discarded. Green and brown coloured explants were sub-cultured regularly in every 20-30 days. After 65-70 days, brown coloured explants started greening and formed green callus. Regeneration and proliferation of shoots was occurred after 15-20 days of callus induction on MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA, 0.5 mg/l GA_3, 300 mg/l carbenicillin and 20 mg/l hygromycin. After that regenerated shoots were sub-cultured on the same medium supplemented with 20 mg/1 hygromycin. Mean number of shoots per explant was 12.45±0.21 and 10.67±0.17 for Asterix and Diamant, respectively (Table 22). For control, non-transformed explants were also inoculated into selection medium. These explants were failed to survive and turned brown. Transformed explants produced hygromycin-resistant shoots in selection medium. These shoots induced healthy roots within 15 days after culturing on hormone free MS medium. Hygromycin-resistant plantlets successfully recovered from root induction medium and were grown to maturity after hardening and transfer to growth room condition. The mean plant survival frequency was 95%. No phenotypic difference in regenerated plantlets and microtuber formation were observed compared to control plants (Figs. 18 a-f and 19 a-f). Mean transformation efficiency for Asterix and Diamant was 31.30 and 29.50 %, respectively (Table 22). After sufficient development of roots, putative transgenic plantlets obtained from var. Asterix and Diamant were successfully transplanted to soil and healthy minitubers formation occurred in both the varieties of potato (Fig. 20 a-e).

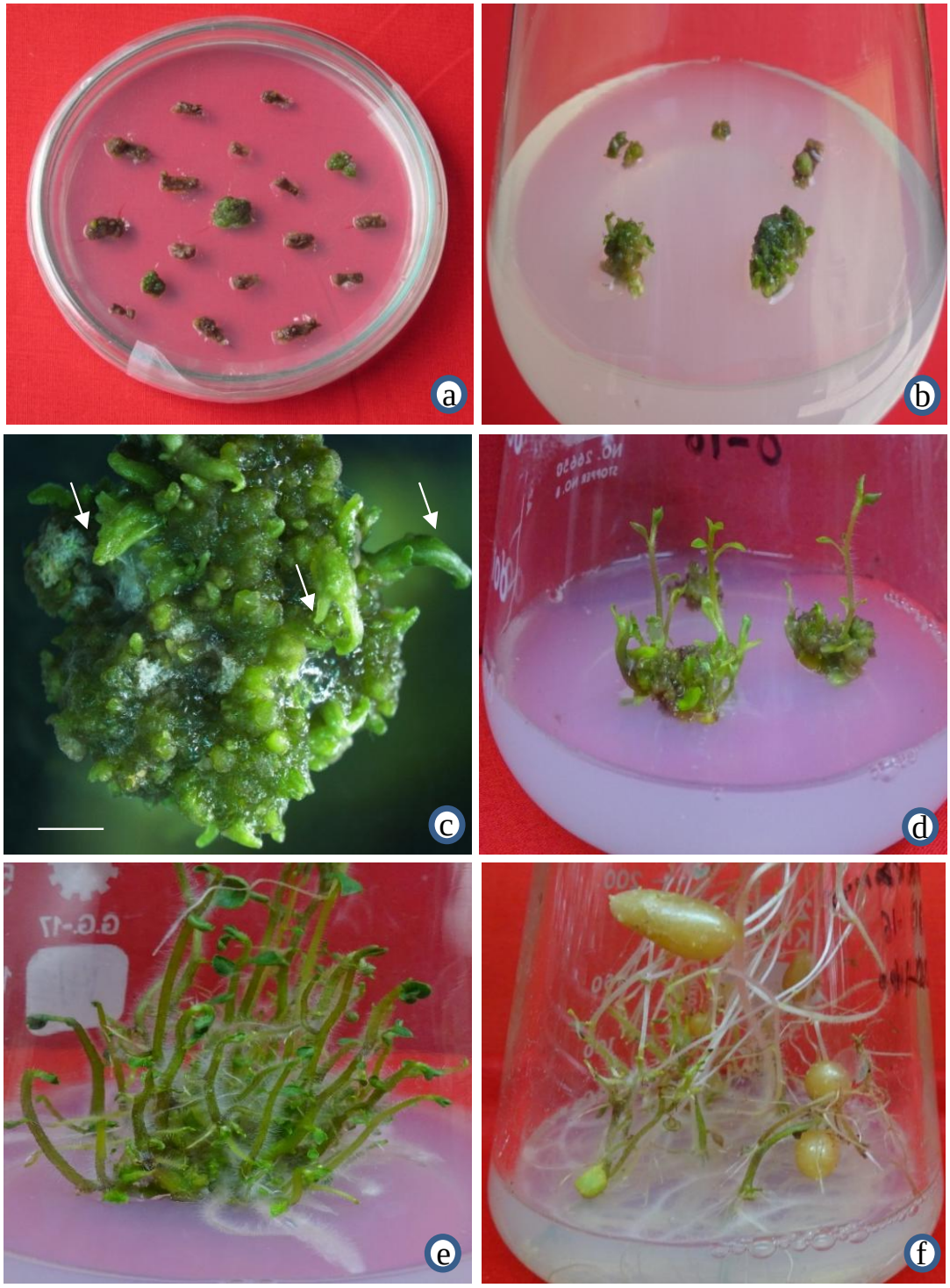

Fig. 18 (a-f): Different stages of transformation of Asterix with strain EHA105/pCAMBIA1301-PDH45. (a) Callus formation from brown nodal segments; (b) Initiation of shoots from callus of nodal segments; (c) Stereomicroscopic view of shoot initiation from callus (arrows indicate the regeneration of shoots); (d) Multiple shoots formation after 60 days of culture on MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3, 300 mg/l carbenicillin and 20 mg/l hygromycin; (e) Elongated shoots on same medium and (f) *In vitro* microtuber formation on MS with 9% sucrose. Scale bar = 2.0 mm.

Fig. 19 (a-f): Different stages of transformation of Diamant with strain EHA105/ pCAMBIA1301-PDH45. (a) Callus formation from brown nodal segments; (b) Stereomicroscopic view of green callus; (c) Shoot initiation from green callus on MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3, 300 mg/l Carbenicillin and 20 mg/l hygromycin; (d) Stereomicroscopic view of shoot initiation from green callus; (e) Formation of multiple shoots and (f) Elongated multiple shoots formation after 60 days of culture. Scale bar = 2.0 mm.

Fig. 20 (a-e): Rooting and transplantation of transgenic plantlets infected with EHA105/ PCAMBIA1301-PDH45. (a) Fully developed roots of Asterix on MS medium; (b) Transplantation of regenerated plantlets in small pots and (c) Plantlets transferred to large plastic pot; (d) Fully developed roots of Diamant on MS medium and (e) Transplantation of regenerated plantlets in small pots of var. Diamant.

Table 22. Effects of MS with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3 on regeneration and proliferation of multiple shoots from nodal segments of potato (var. Asterix and Diamant) after transformation

Variety	Total experiment done	Total no. of explants infected	No. of explants transferred to regeneration media	No. of shoots/explant after 95 days of inoculation (±SD)	No. of shoots transferred to microtuberization media
Asterix	9	2045	1996	12.45 ± 0.21^{a}	120
Diamant	7	1680	1610	10.67 ± 0.17^{b}	95

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 23. Transformation efficiency of nodal segment explants of Asterix and Diamant towards genetic transformation by *Agrobacterium* (Strain EHA105 containing the plasmid pCAMBIA1301-PDH45).

Variety	No. of explants inoculated	No. of responsive explants after 65-70 days of inoculation	No. of regenerated explants produced shoots	No. of shoots/explant after 95 days of inoculation (±SD)	Transformation efficiency (%)	Mean Transformation efficiency (%)
Asterix	250	130	75	12.85 ± 0.13^{a}	30.0	31.30
	200	120	67	10.23 ± 0.25^{c}	33.50	
	250	140	76	11.5 ± 0.36^{b}	30.40	
Diamant	255	125	70	9.75 ± 0.28^{d}	27.45	29.50
	198	140	65	11.23 ± 0.17^{b}	32.83	
	245	135	69	10.46 ± 0.29^{c}	28.16	

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

4.2.2.1.4 Molecular analysis for the integration of salt and drought tolerant genes

Molecular analysis through PCR amplification confirmed the presence of *PDH45* and *hptII* genes in putative transformants. DNA was isolated from putative transformed shoots as well as control plants for PCR amplification. Plasmid of pCAMBIA1301-PDH45 was used as template DNA. 87 (45 from Asterix and 42 Diamant) out of 300 surviving shoots recovered through selection pressure were subjected to PCR analysis. 72 survived shoots showed positive response towards the integration of *PDH45* and *hptII* genes. Therefore, percentage of transformation was about 31.3 in case of Asterix and 29.5 in case of Diamant.

Specifics primer for *PDH45* and *hptII* genes were used for PCR amplification. Amplification of 1200 bp band for *PDH45* gene and 750 bp band for *hptII* gene in transformed shoots confirmed presence of *PDH45* and *hptII* genes (Figs. 21-24).

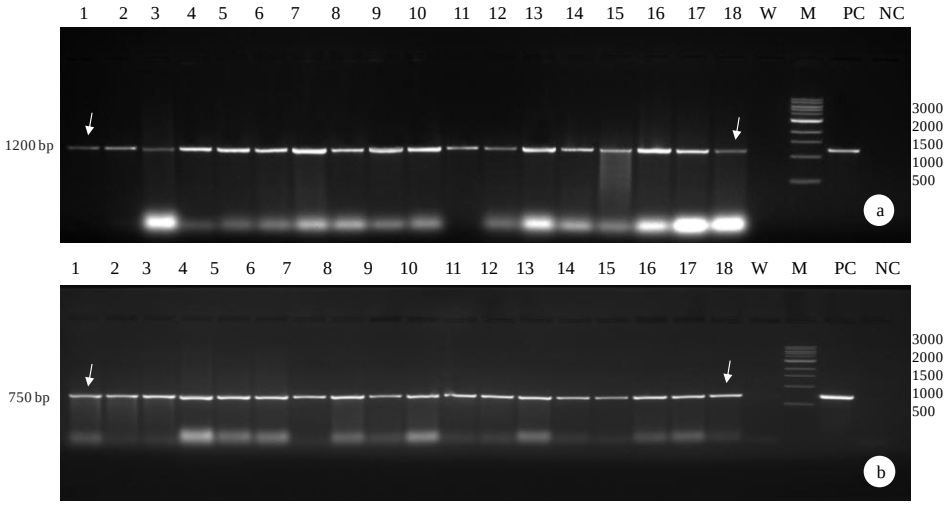

Fig. 21: Molecular confirmation of the putative transformants of var. Asterix. PCR amplification of *PDH45* (a) and *hptII* (b) genes (lanes 1-18 genomic DNA of transformed plants ; lane W- wild; lane M-1kb ladder; lane PC-plasmid DNA of EHA105/pCAMBIA1301-PDH45 as positive control; lane NC- negative control; arrows indicate the positive band of *PDH45* and *hptII* genes).

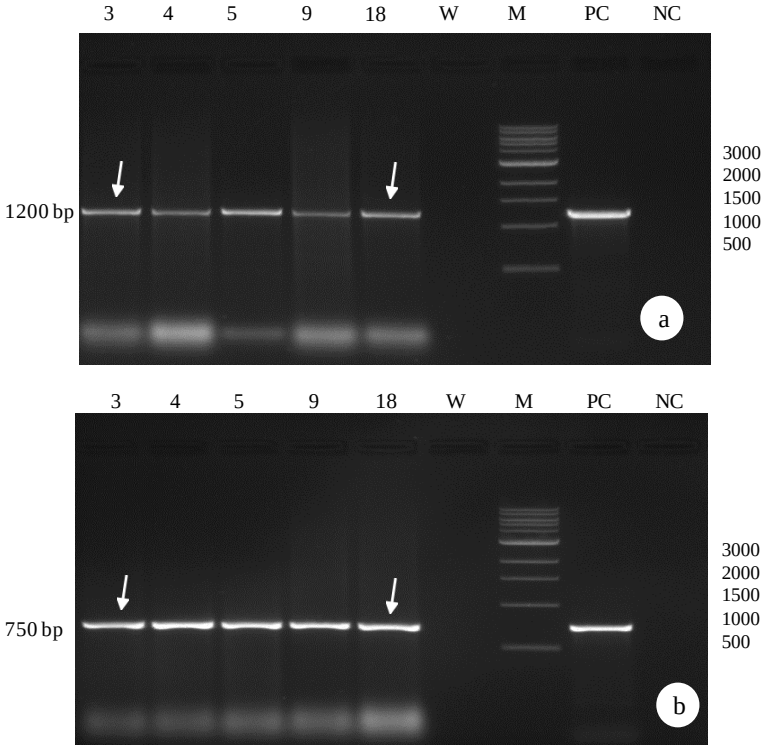

Fig. 22: Molecular confirmation of transformants of Asterix. PCR amplification of *PDH45* (a) and *hptII* (b) genes (lanes 1-5 genomic DNA of transformed plants (PA-3, 4, 5, 9, and 18); lane W- wild; lane M-1kb ladder; lane PC-plasmid DNA of EHA105/pCAMBIA1301-PDH45 as positive control; lane NC- negative control; arrows indicate the positive band of *PDH45* and *hptII* genes).

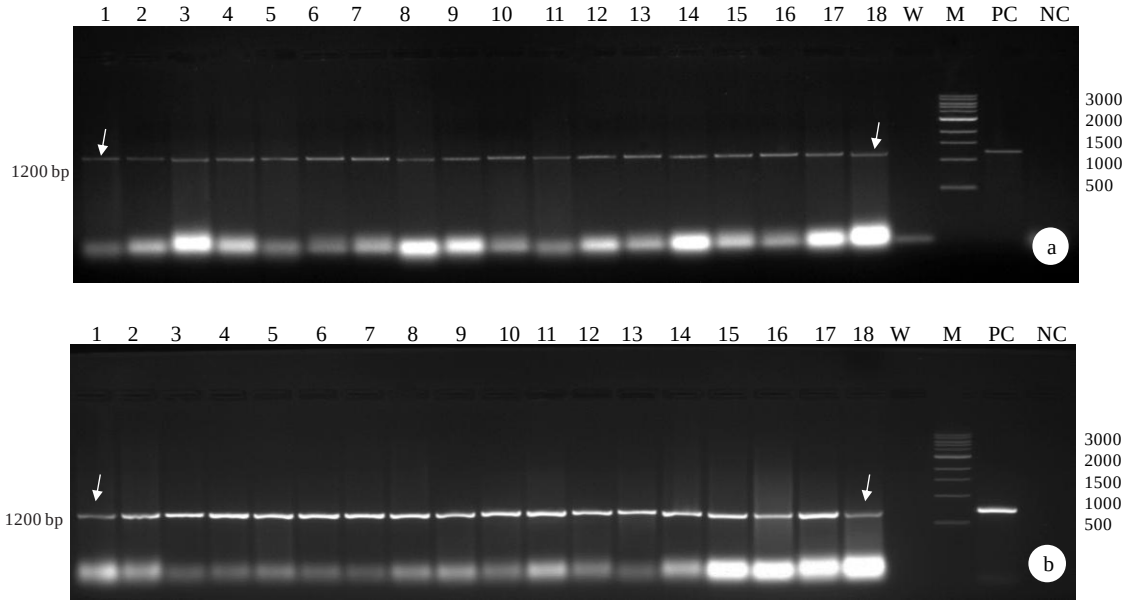

Fig. 23: Molecular confirmation of the putative transformants of Diamant. PCR amplification of *PDH45* (a) and *hptII* (b) genes (lane M-1kb ladder; lane PC-plasmid DNA of EHA105/pCAMBIA1301-PDH45 as positive control; lane NC-negative control; lanes 1-18 genomic DNA of transformed shoots; lane W- wild type control; arrows indicate the positive band of *PDH45* and *hptII* genes).

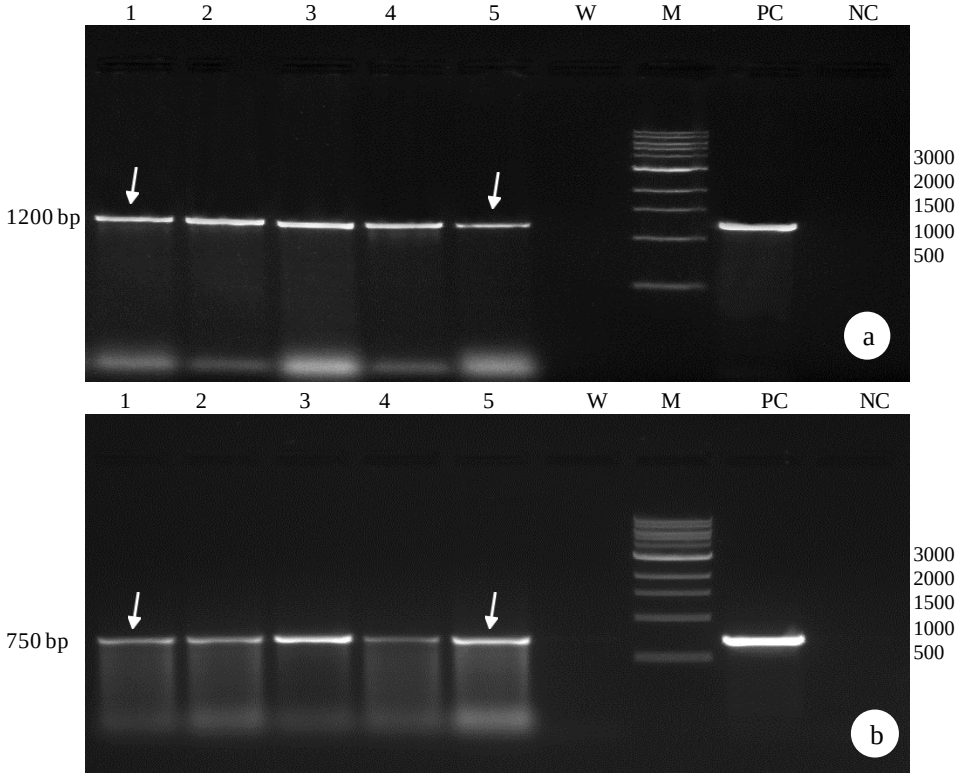

Fig. 24: Molecular confirmation of transformants of Asterix. PCR amplification of *PDH45* (a) and *hptII* (b) genes (lanes 1-5 genomic DNA of transformed plants (LD-1, 2, 3, 4, and 5); lane W- wild; lane M-1kb ladder; lane PC-plasmid DNA of EHA105/pCAMBIA1301-PDH45 as positive control; lane NC- negative control ; arrows indicate the positive band of *PDH45* and *hptII* genes).

4.2.2.1.5 Progeny analysis

Minitubers that developed on PCR positive T_0 plants were harvested and were subjected for germination in plastic pot containing sterile soil in green house along with controls (non-transformed). Minitubers of T_0 plants germinated and gave rise to TG_1 (Figs. 25 a-f and 26 a-d) plants as well as the TG_1 minitubers germinated and gave rise to TG_2 plants. These TG_2 minitubers germinated again and gave rise to TG_3 plants. DNA isolation and PCR amplification for *PDH45* and *hptII* gene was also done from the TG_1 plants (Figs. 27-31).

Fig. 25 (a-f): Tuber development of first transgenic generation (TG_1) of Asterix in green house. (a-b) transgenic plants in large pot; (c-e) Formation of flower and (f) Minitubers of transgenic plants.

Fig. 26 (a-d): Tuber development from first transgenic generation (TG_1) of Diamant in green house. (a-b) transgenic plants in large pot; (c) Formation of flower and (d) Minitubers of transgenic plants.

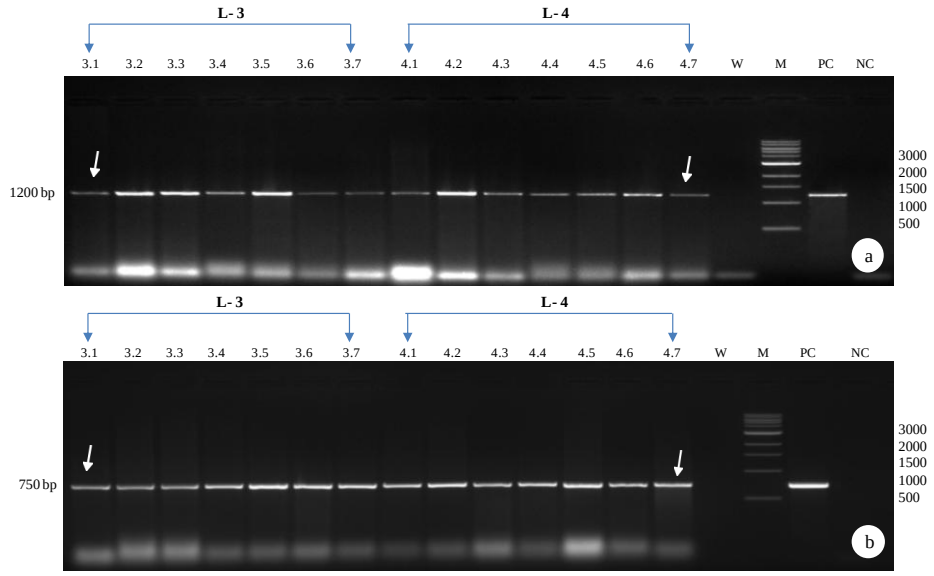

Fig. 27: Molecular confirmation of putative first transgenic tuber generation (TG_1) of Asterix (LA- 3 and 4). PCR amplification of *PDH45* (a) and *hptII* (b) gene (lane M-1kb ladder; lane PC-plasmid DNA of EHA105/pCAMBIA1301-PDH45 as positive control; lane NC- negative control; lanes 1-14 genomic DNA of transformed plants; lane W- wild type control; arrows indicate the positive band of *PDH45* and *hptII* genes).

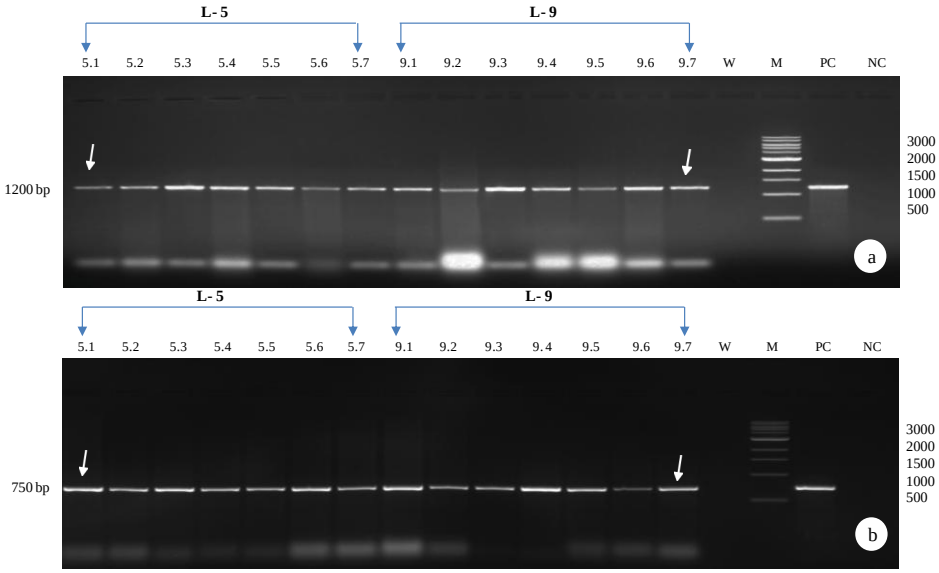

Fig. 28: Molecular confirmation of first transgenic tuber generation (TG_1) of Asterix (line: LA- 5 and 9). PCR amplification of *PDH45* (a) and *hptII* (b) gene (lane M-1kb ladder; lane PC-plasmid DNA of EHA105/pCAMBIA1301-PDH45 as positive control; lane NC- negative control; lanes 1-14 genomic DNA of transformed plants; lane W- wild type control; arrows indicate the positive band of *PDH45* and *hptII* genes).

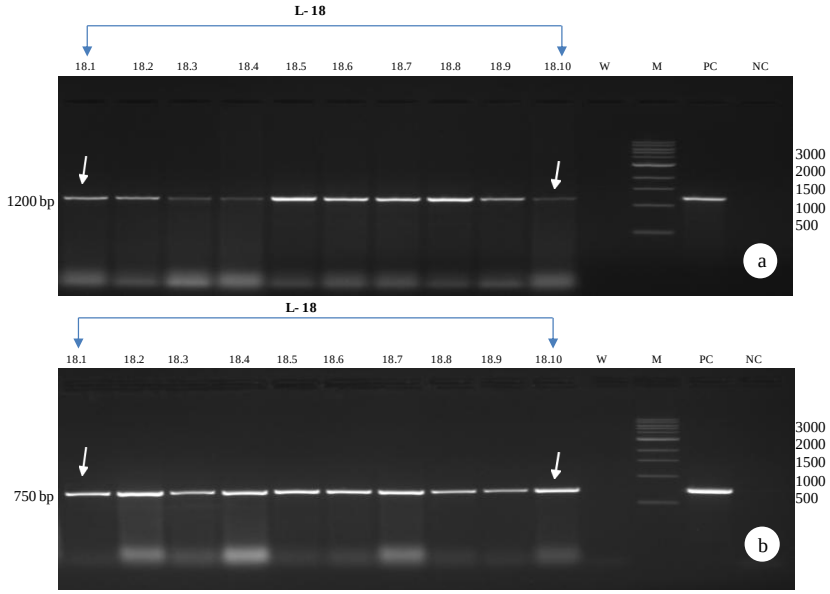

Fig. 29: Molecular confirmation first transgenic tuber generation (TG_1) of Asterix (line: LA- 18). PCR amplification of *PDH45* (a) and *hptII* (b) genes (lane M-1kb ladder; lane PC-plasmid DNA of EHA105/pCAMBIA1301-PDH45 as positive control; lane NC- negative control; lanes 1-10 genomic DNA of transformed plants; lane W- wild type control; arrows indicate the positive band of *PDH45* and *hptII* genes).

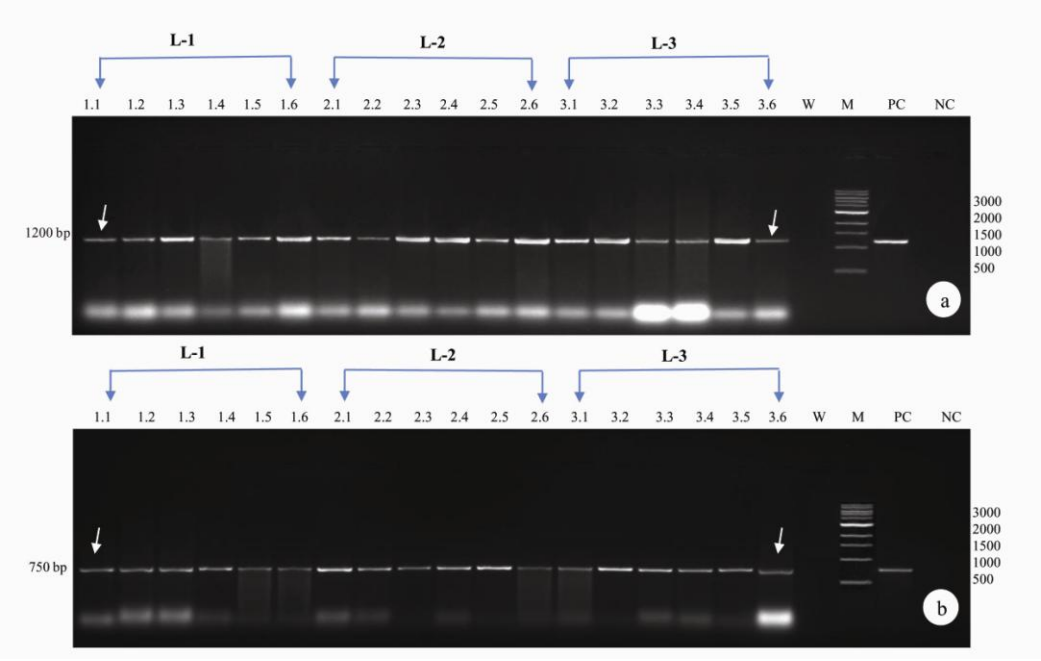

Fig. 30: Molecular confirmation of first transgenic tuber generation (TG_1) of Diamant (LD- 1, 2 and 3). PCR amplification of *PDH45* (a) and *hptII* (b) gene (lane M-1kb ladder; lane PC-plasmid DNA of EHA105/pCAMBIA1301-PDH45 as positive control; lane NC- negative control; lanes 1-18 genomic DNA of transformed plants; lane W- wild type control; arrows indicate the positive band of *PDH45* and *hptII* genes).

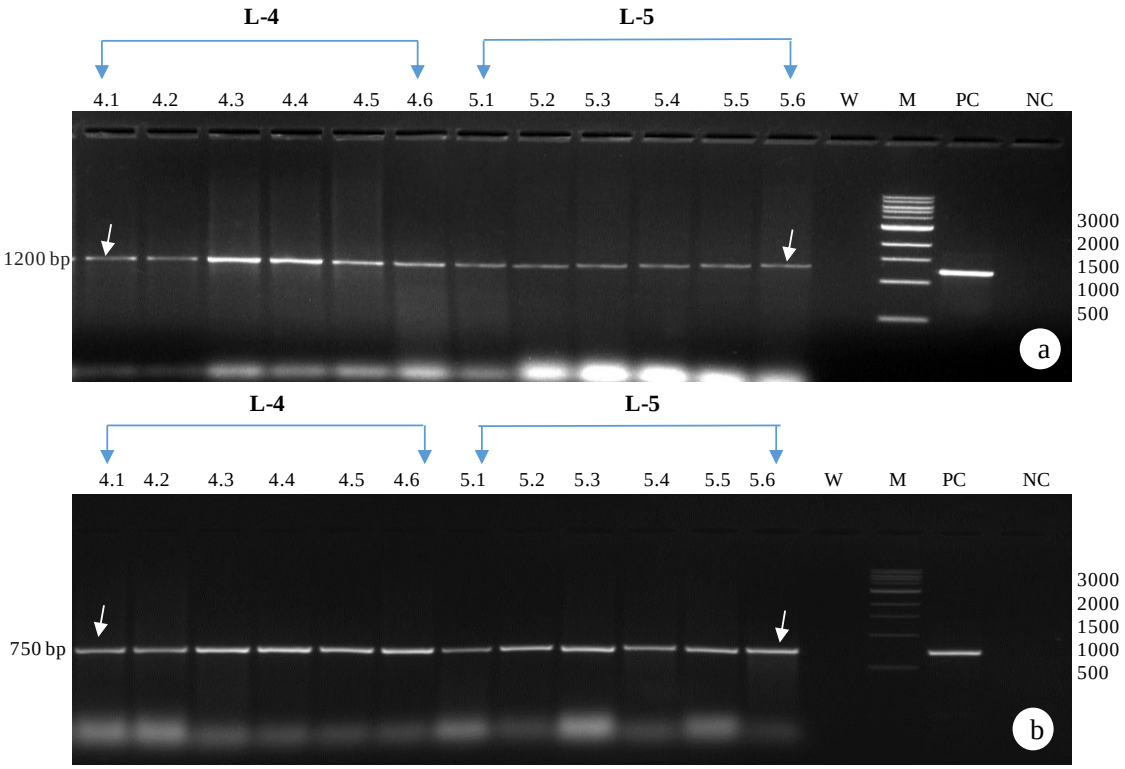

Fig. 31: Molecular confirmation of first transgenic tuber generation (TG_1) of Diamant (LD- 4 and 5). PCR amplification of *PDH45* (a) and *hptII* (b) gene (lane M-1kb ladder; lane PC-plasmid DNA of EHA105/pCAMBIA1301-PDH45 as positive control; lane NC- negative control; lanes 1-18 genomic DNA of transformed plants; lane W- wild type control; arrows indicate the positive band of *PDH45* and *hptII* genes).

4.2.3 Morphometric evaluation of control under stress condition in green house

30 days old greenhouse grown transgenic lines of Asterix and Diamant were used for *ex vitro* rooting. Apical shoots were cut approximately 5.0 cm below the top and dipped in 10 mM IBA for 5.0 min. Shoots were planted in wet sterile soil and stony sand with coco peat in small plastic pot. The pots were covered by transparent perforated polythene bag and placed in moist-shady places (Temperature below 25ºC and Relative humidity 65-70%). The plantlets were sprayed with water at weekly interval. After 15 days well developed rooted plantlets were carefully removed from the crushed stony sand with coco peat. Data on number of rooted shoots, total number of primary roots and root length were recorded. Well-developed rooted plantlets were subsequently transferred to large pot for further growth and to get tubers from those *ex vitro* regenerated plants. Survival rate and comparison of minituber formation from *in vitro* and *ex vitro* rooted plants was done. *Ex vitro* rooted plants performed well like *in vitro* rooted plants.

Ex vitro rooted control plants of both the varieties (Asterix and Diamant) were transplanted into small 6.0 inch plastic pot containing crushed stony sand with coco peat and allowed to grow under greenhouse conditions with different concentrations (0, 50, 100,150, 200 and 300 mM) of salt. Morphological differences under different concentrations of salt stress was shown in Table 24 and Fig. 32(a-f). These plants were supplemented with ¼ th strength of Hoagland solution. Plants were carefully uprooted after 28 days and were washed gently with water. Growth parameters in terms of shoot length, root length, root volume, leaf number and number of nodes were measured. In another experiment, the plants were grown till maturity and yield attributes in terms of total yield and total dry matter was recorded.

The control potato plants grew well on 50, 100 and 150 mM NaCl salt stress but the plants could not survive when treated with 200 and 300 mM NaCl at 40 and 32 days of

starting salinity stress. Under control condition (watered without NaCl) no significance difference existed in morphological appearance of control potato plants.

Table 24. Morphometric evaluation of control plants under different concentrations of salt (NaCl) stress in green house condition

Variety	Concentrations of NaCl (mM)	Average length of shoot (cm) (±SD)	Average length of root (cm) (±SD)	Average volume of root (mg) (±SD)
Asterix	0	27.29±0.44[a]	7.15±0.16[a]	2.47±0.19[a]
	50	24.46±0.35[c]	5.33±0.19[c]	1.62±0.17[b]
	100	20.05±0.11[e]	4.25±0.13[e]	1.38±0.17[c]
	150	16.12±0.15[f]	2.81±0.15[f]	1.27±0.18[c]
	200	5.6±0.16[i]	1.53±0.17[h]	0.50±0.12[d]
	300	5.1±0.10[j]	1.15±0.15[i]	0.36±0.12[d]
	400	4.03±0.17[k]	1.06±0.15[i]	0.22±0.15[e]
Diamant	0	26.18±0.31[b]	6.88±0.19[b]	2.44±0.15[a]
	50	23.12±0.11[d]	5.09±0.18[d]	1.68±0.12[b]
	100	20.15±0.13[e]	5.23±0.17[cd]	1.32±0.15[c]
	150	14.33±0.29[g]	4.17±0.15[e]	1.23±0.16[c]
	200	6.26±0.12h	2.46±0.19g	0.50±0.13d
	300	5.52±0.28i	1.39±0.12h	0.**31**±**0**.13d
	400	4.93±0.22j	1.07±0.16i	0.24±0.11e

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Fig. 32 (a-f): Shoot and root growth of control plants of var. Asterix on different concentrations of NaCl salt stress. (a) 0 mM; (b) 50 mM; (c) 100 mM; (d) 150 mM; (e) 200 mM and (f) 300 mM NaCl. Scale bar = 4.0 cm.

Minituber production of control potato varieties watered by salt stress 0, 50, 100 and 150 mM were recorded. The control plants were growing in salt stress (0-150 mM) for 60 days. After harvest, the number and weight of the tubers having a diameter greater than 1.0 cm was measured.

4.2.3.1 Morphometric evaluation of transgenic lines under stress condition

Different experiments were conducted to analyze growth, photosynthetic ability and yield attributes in the transgenic lines under stress condition (200 mM NaCl). The *ex vitro* rooted TG_2 transgenic plants were transplanted into small 6 inch plastic pot containing sand with coco peat and allowed to grow under greenhouse conditions. These plants were supplemented with ¼ th strength of Hoagland solution. Plants were carefully uprooted after 28 days and were washed gently with water. Growth parameters in terms of shoot length, root volume and leaf numbers were measured. In another experiment, plants were grown till maturity and yield attributes in mg of total yield was recorded both in the transgenic and wild type control plants for comparative analysis.

4.2.3.2 Morphometric evaluation of transgenic lines under 200 mM of salt (NaCl) stress in green house condition

In 200 mM NaCl salt stress condition, average shoot length of wild type control Asterix and Diamant was 5.01±0.19 and 4.95±0.17 respectively. On the other hand, transgenic lines of Asterix and Diamant average length of shoot was 19.17±0.15 - 20.84±0.24 and 18.06±0.12-20.22±0.52, respectively (Figs. 33-35 and Table 25). In another experiment, plants were grown till maturity and yield attributes was recorded. Three plants from each transgenic line was grown under 200 mM NaCl salt stress condition for 90 days. Minituber production of wild type control and transgenic plants were recorded after 60 and 90 days in green house. Average number of minitubers in case of Asterix wild type

control and transgenics were 2.54±0.15 and 8.29±0.34-9.18±0.17 (Table 26). In case of var. Diamant average number of minitubers were 2.60±0.17 and 7.02±0.12-8.78±0.12, respectively (Fig. 36- 37 and Table 26).

Table 25. Morphometric evaluation of transgenic lines under 200 mM of salt (NaCl) stress in green house condition

Variety	Transgenic lines	Average length of shoot (cm) (±SD)	Average length of root (cm) (±SD)	Average volume of root (mg) (±SD)
Asterix	Wild type control	5.01±0.19^{e}	1.35±0.21^{g}	0.89±0.16^{d}
	PA-3.3.1	20.0±0.15^{b}	7.81±0.17^{b}	2.22±0.28^{ab}
	PA-3.4.1	19.17±0.15^{c}	6.32±0.18^{e}	2.01±0.10^{bc}
	PA-4.1.2	20.13±0.13^{b}	5.28±0.18^{f}	1.90±0.16^{bc}
	PA-4.5.4	18.85±0.23^{c}	6.86±0.18^{d}	1.95±0.23^{bc}
	PA-18.3.3	20.84±0.24^{a}	7.18±0.19^{c}	1.99±0.13^{bc}
Diamant	Wild type control	4.95±0.17^{e}	1.21±0.16^{g}	0.66±0.12^{d}
	PD-1.1.1	20.05±0.13^{b}	8.45±0.18^{a}	1.82±0.13^{c}
	PD-1.2.3	18.06±0.12^{d}	7.14±0.14^{c}	1.94±0.24^{bc}
	PD-2.1.2	20.17±0.18^{b}	6.40±0.14^{e}	1.89±0.10b^{c}
	PD-2.2.2	**20.22±0.52^{b}**	7.06±0.12^{cd}	2.17±0.35^{ab}
	PD-3.2.3	19.18±0.18^{c}	6.31±0.19^{e}	2.41±0.18^{a}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Table 26. Formation of minitubers from different transgenic lines of Asterix and Diamant under salt stress (200 mM) in green house condition

Variety	Transgenic lines (TG_2)	Mean no. of microtubers/shoot (±SD)	Average weight (mg) of microtubers/shoot (±SD)
Asterix	Wild type control	1.00 ± 0.15^{g}	0.83 ± 0.19^{g}
	PA-3.3.1	8.29 ± 0.34^{d}	7.60 ± 0.23^{b}
	PA-3.4.1	9.12 ± 0.10^{ab}	7.89 ± 0.12^{a}
	PA-4.1.2	8.34 ± 0.20^{d}	7.31 ± 0.28^{cd}
	PA-4.5.4	9.18 ± 0.17^{a}	7.91 ± 0.18^{a}
	PA-18.3.3	8.84 ± 0.24^{ab}	7.5 ± 0.14^{bc}
Diamant	Wild type control	1.04 ± 0.17^{g}	0.89 ± 0.12^{g}
	PD-1.1.1	8.71 ± 0.21^{c}	7.05 ± 0.18^{ef}
	PD-1.2.3	8.78 ± 0.12^{bc}	6.92 ± 0.10^{f}
	PD-2.1.2	7.67 ± 0.12^{e}	7.18 ± 0.11^{de}
	PD-2.2.2	8.53 ± 0.31^{cd}	7.28 ± 0.18^{cd}
	PD-3.2.3	7.02 ± 0.12^{f}	6.82 ± 0.18^{f}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

Fig. 33 (a-f): After 35 days of NaCl (200mM) salt stress in a control and five transgenic TG_2 lines of var. Asterix. (a) Wild type control; (b) PA-3.3.1; (c) PA-3.4.1; (d) PA-4.1.2; (e) PA-4.5.4 and (f) PA-18.3.3; Scale bar = 4.0 cm.

Fig. 34 (a-f): Growth of shoot and root in control and 5 transgenic lines (TG_2) of var. Asterix after 25 days of 200 mM NaCl salt stress. (a) Wild type control; (b) PA-3.3.1; (c) PA-3.4.1; (d) PA-4.1.2; (e) PA-4.5.4 and (f) PA-18.3.3; Scale bar = 4.0 cm.

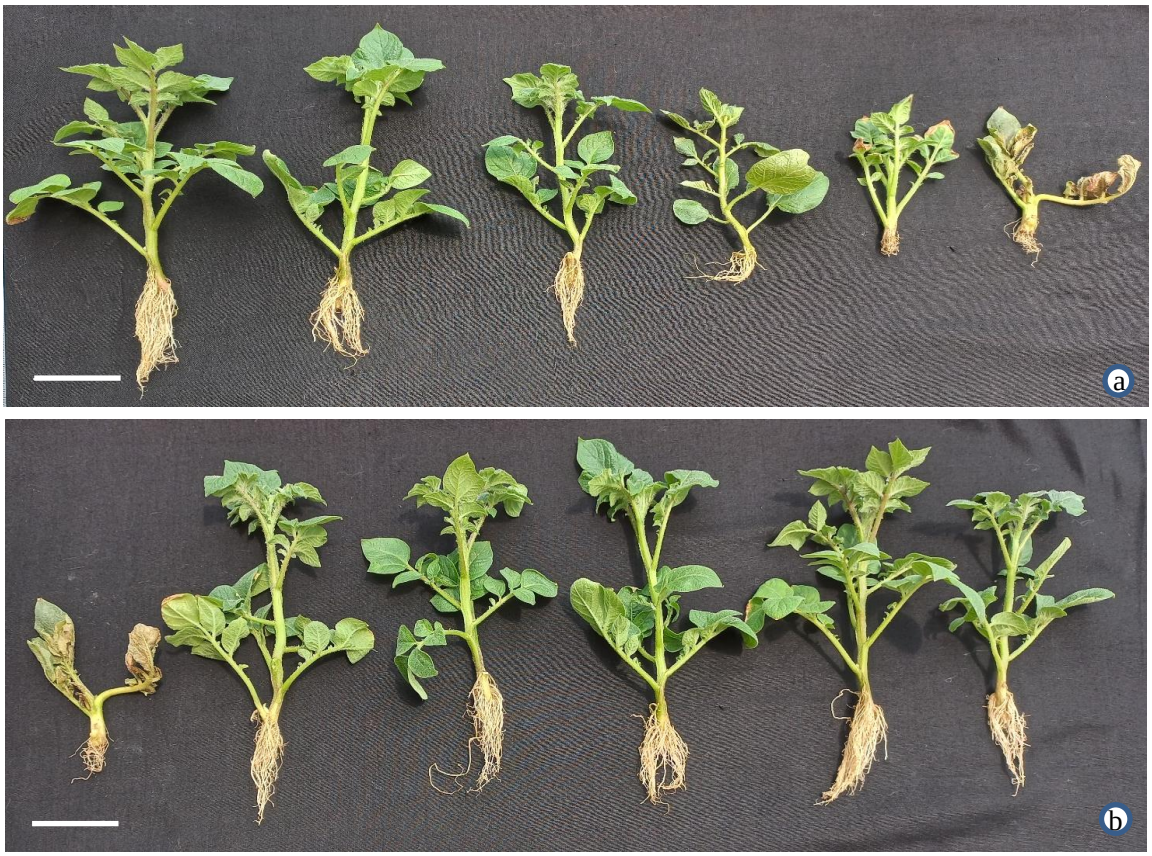

Fig. 35 (a-b): Effect of different concentrations of NaCl on control and transgenics TG_2 lines of var. Diamant. (a) Control plants in different concentrations of salt stress (0 mM, 50 mM, 100 mM, 150 mM, 200 mM and 300 mM NaCl) and (b) Wild type control plants and TG_2 transgenic lines (PD-1.1.1, 1.2.3, 2.1.2, 2.2.2 and 3.2.3) in 200 mM NaCl salt stress. Scale bar = 4.0 cm.

Fig. 36 (a-f): Minitubers of Control and transgenic TG_2 lines of var. Asterix in 200 mM NaCl salt stress condition. (a) Wild type control; (b) PA-3.3.1; (c) PA-3.4.1; (d) PA 4.1.2; (e) PA-4.5.4 and (f) PA- 18.3.3). Scale bar = 2.0 cm.

Fig. 37 (a-f): Minitubers of Control and transgenic TG_2 lines of var. Diamant in 200 mM NaCl salt stress condition. (a) wild type control; (b) PD-1.1.1; (c) PD-1.2.3; (d) PD-2.1.2; (e) PD-2.2.2 and (f) PD- 3.2.3). Scale bar = 2.0 cm.

4.2.6 Biochemical and physiological analysis of transgenic lines under stress condition in green house

Different experiments were conducted to analyse the biochemical and physiological changes in the transgenic lines under stress conditions (200 mM NaCl). *Ex vitro* rooted TG_2 transgenic plants were grown on hydroponics system (half strength of Hoagland solution) and subjected to salt stress (200 mM NaCl) for 20 days. Relative water content (RWC), chlorophyll and carotenoid content, Proline content and H_2O_2 content were determined.

4.2.6.1 Estimation of relative water content (RWC)

Physiological changes in transgenic potato plants mimicked salt stress were evaluated by comparing RWC in control and transgenic lines of both the varieties (Asterix and Diamant). RWC determines water status of the plant as a physiological consequence of cell water deficiency due to 200 mM NaCl stress. Under control condition, relative water content of control was higher than that of different transgenic lines (Table 27). However, NaCl treatment led to a decrease of RWC in control plants. A lower RWC decrease was obtained in transgenic lines compared to control.

Table 27. Measurement of Relative Water Content (RWC) under hydroponics condition containing half strength of Hoagland solution with 200 mM NaCl salt stress in green house

Variety	Transgenic TG_2 lines	Fresh weight (mg) FW (±SD)	Turgid weight(mg) TW (±SD)	Dry weight (mg) DW (±SD)	Relative Water Content (±SD)
Asterix	Wild type control	1648.33±3.51[a]	1689.59±7.63[a]	188.0±3.60[b]	69.28±0.66c
	PA-3.3.1	1523.66±6.02[f]	1583.23±8.85[e]	194.33±5.50[a]	83.98±0.85ab
	PA-3.4.1	1512.33±8.32[g]	1575.53±8.91[e]	182.66±9.45[c]	84.41±1.10a
	PA-4.1.2	1586.33±8.50[c]	1644.02±12.40[b]	191.66±5.68[a]	84.85±1.35a
	PA-4.5.4	1580.66±6.02[cd]	1619.84±5.70[c]	193.66±4.04[a]	85.64±0.59a
	PA-18.3.3	1552.0±4.0[e]	1603.45±7.67[d]	177.33±4.72[d]	85.75±0.88a
Diamant	Wild type control	1621.0±6.55[b]	1551.52±4.10[g]	181.0±4.35[c]	63.64±0.42d
	PD-1.1.1	1512.66±8.02[g]	1572.64±5.40[e]	170.66±6.02[e]	86.51±0.12a
	PD-1.2.3	1526.66±9.07[f]	1557.20±6.19[f]	170.33±3.51[e]	86.26±0.38a
	PD-2.1.2	1506.0±6.0[h]	1569.15±8.99[e]	186.0±7.21[b]	84.78±0.51a
	PD-2.2.2	1521.33±5.13[f]	1624.5±74.0[c]	188.33±3.05[b]	84.96±0.60a
	PD-3.2.3	1585.331±1.05[c]	1568.59±6.56[e]	172.66±5.50[e]	86.97±0.85a

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$

4.2.6.2 Determination of the level of chlorophyll and carotenoid

Chlorophyll plays most important role in photosynthesis for the plant development and biomass production. Evaluation of chlorophyll content was performed in transgenic potato lines and control potato plants of both the varieties (Asterix and Diamant). Under control condition, chlorophyll content of the control was higher than that of the different transgenic lines but significant loss in chlorophyll content was observed in control upon 200 mM NaCl treated condition. However, most of the transgenic TG_2 lines of Asterix and Diamant showed higher chlorophyll content in comparison to control plants (Fig. 38 and 39).

Total carotenoid content of transgenic plants showed similarity to chlorophyll in control condition. 200 mM NaCl treatment allows a generalized decrease in the concentration of the carotenoid pigment in control plants than transgenic TG_2 lines (Fig. 38 and 39).

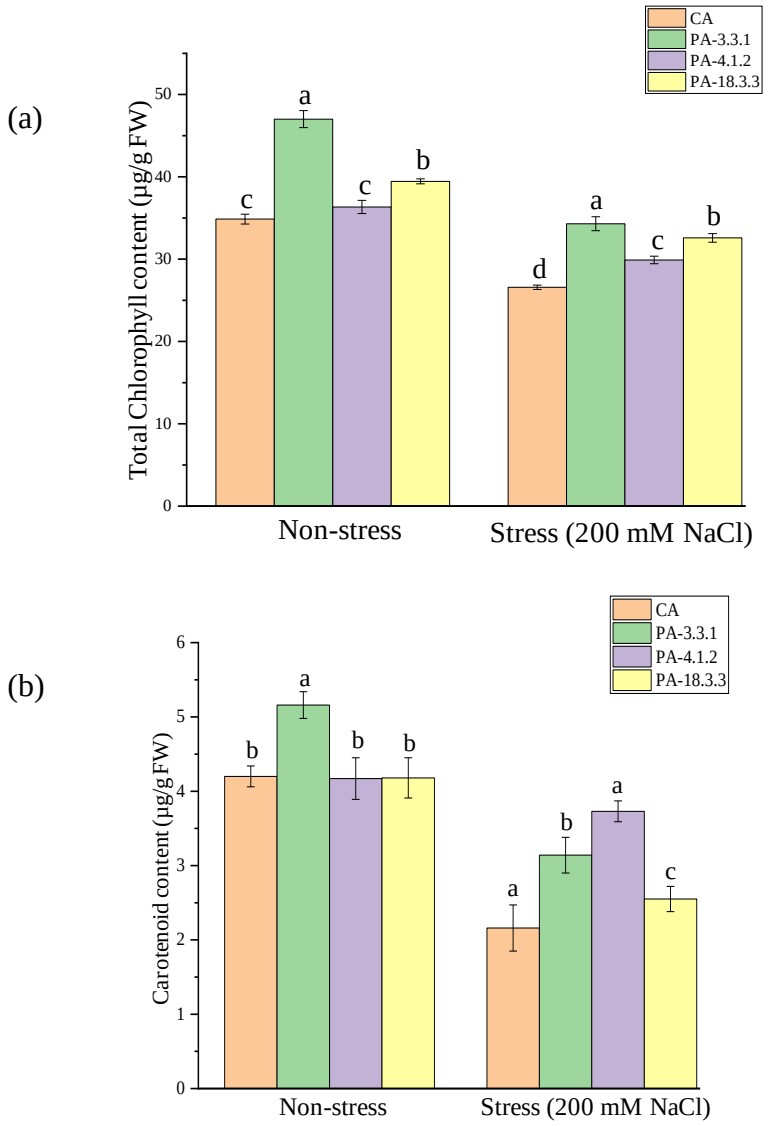

Fig. 38: Total Chlorophyll and Carotenoid contents under non-stress and 200 mM NaCl salt stress condition in wild type control (CA) and transgenic lines (PA-3.3.1, PA-4.1.2 and PA-18.3.3) of Asterix variety. (a) Chlorophyll content and (b) Carotenoid content. Different letters above error bar indicate significant differences at $P < 0.05$ and data were analysed by Duncan's multiple range test. The error bars indicate the mean ± SD.

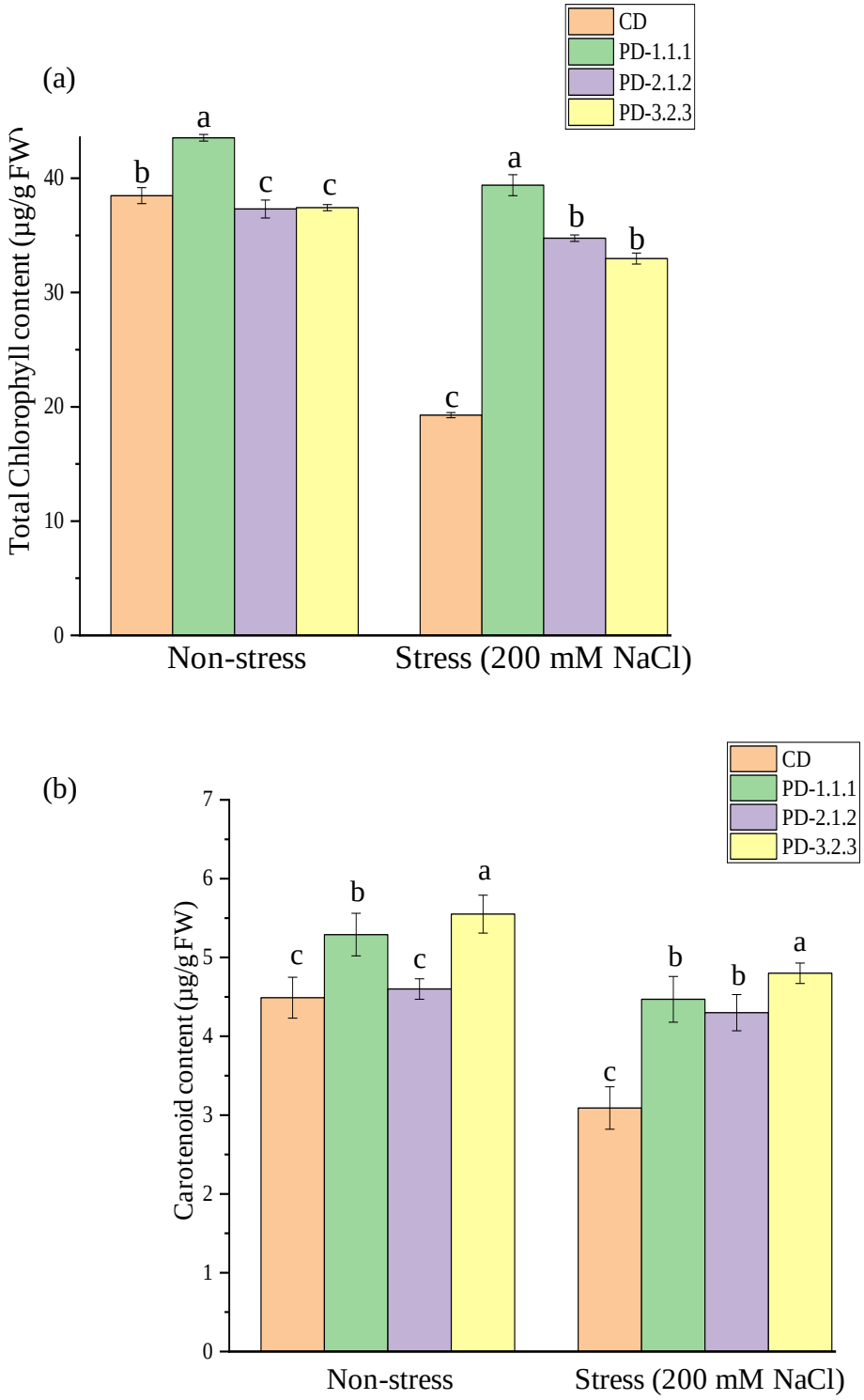

Fig. 39: Total Chlorophyll and Carotenoid contents under non-stress and 200 mM NaCl salt stress condition in wild type control (CD) and transgenic lines (PD-1.1.1, PD-2.1.2 and PD-3.2.1) of Diamant variety. (a) Chlorophyll content and (b) Carotenoid content. Different letters above error bar indicate significant differences at $P < 0.05$ and data were analysed by Duncan's multiple range test. The error bars indicate the mean ± SD.

4.2.6.3 Determination of Proline content

Proline accumulation enhances in response to salt stress. Proline content significantly increased in transgenic lines after exposure to salt stress in contrast to control plants (CA and CD) (Fig. 40).

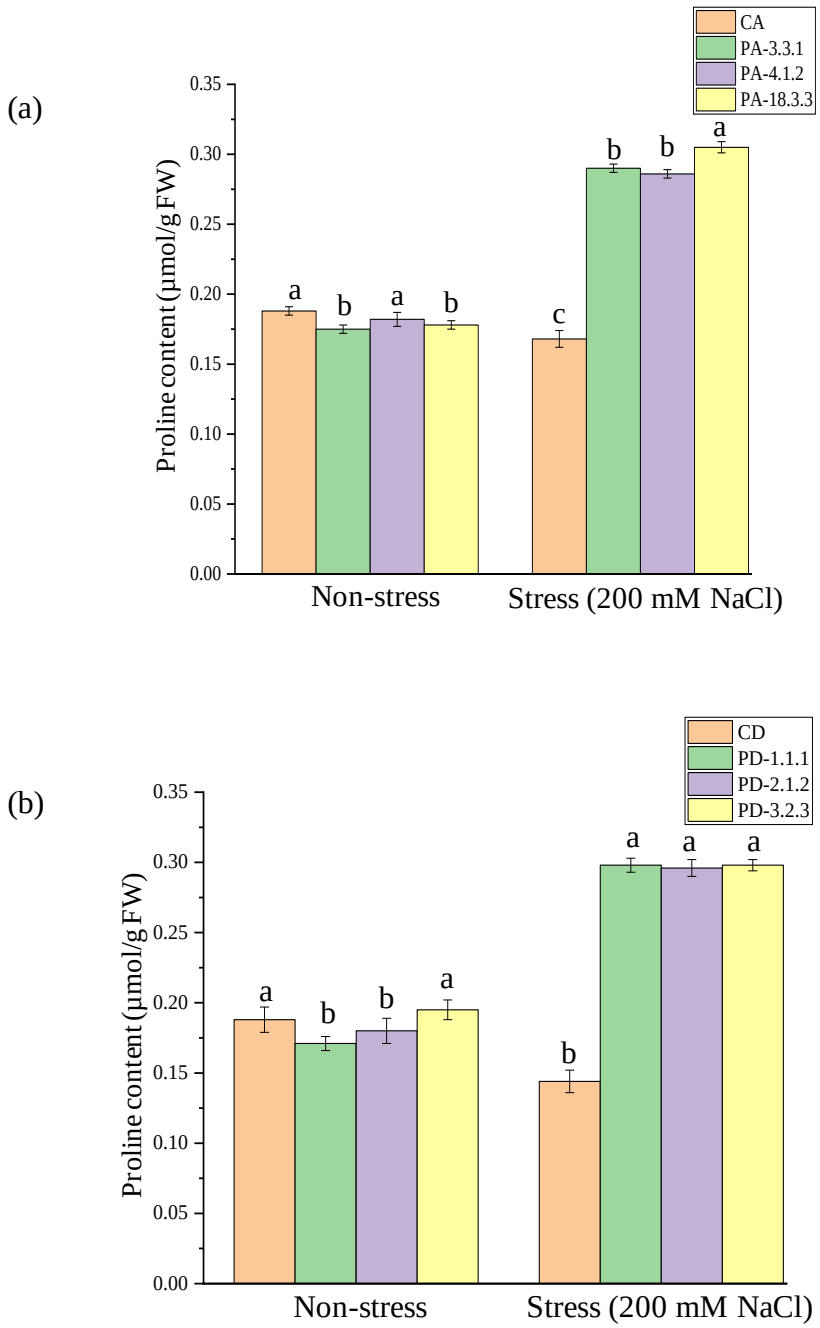

Fig. 40: Proline content under non-stress and 200 mM NaCl salt stress condition in wild type control (CA and CD) and transgenic lines (PA-3.3.1, PA-4.1.2, PA-18.3.3 of Asterix and PD-1.1.1, PD-2.1.2, PD-3.2.1 of Diamant variety. (a) Proline content of Asterix and (b) Proline content of Diamant. Different letters above error bar indicate significant differences at $P < 0.05$ and data were analysed by Duncan's multiple range test. The error bars indicate the mean ± SD.

4.2.6.4 Determination of H_2O_2

In plants, generation of relative oxygen species (ROS) is a common end product of all stress. The quality of ROS produced in plants play a vital role in determining their overall tolerance or sensitivity to stress. The major ROS component, hydrogen per oxide (H_2O_2) was measured in leaves of transgenic lines and wild type control plants in salt stress. To determine the impact of 200 mM NaCl stress on subsequent oxidative stress generation in plants 200 mM NaCl treatment was applied for 15 days. Low H_2O_2 level was detected in leaves of all transgenic lines of Asterix and Diamant. In control condition no significant variability was observed between the transgenic and wild type control plants in terms of H_2O_2. However, significantly increase ROS levels (H_2O_2) were observed in wild type control (CA and CD) in contrast to transgenic lines under salt stress (Fig. 41). Data showed a sharp increase in H_2O_2 level in wild type control (CA and CD) leaves, whereas minor increase of H_2O_2 was observed in transgenic plants confirming reduced damage and higher membrane integrity during stress.

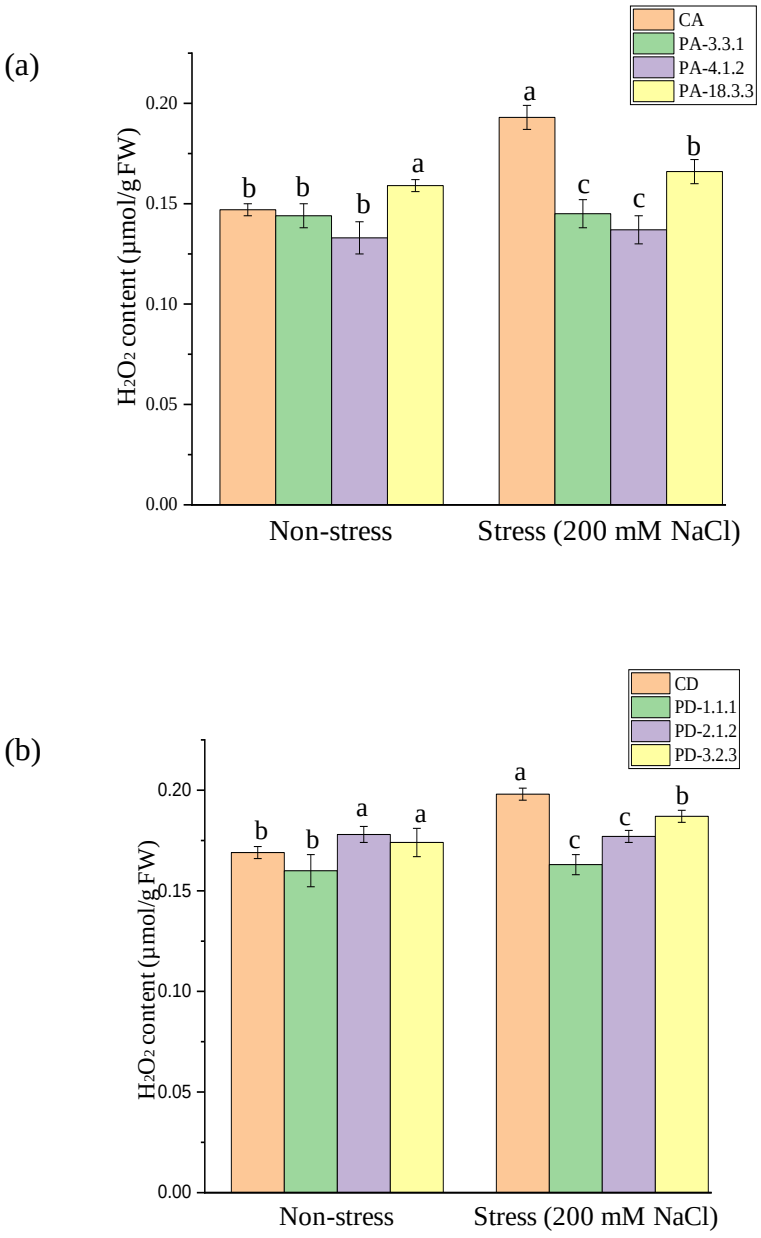

Fig. 41: H_2O_2 content under non-stress and 200 mM NaCl salt stress condition in wild type control (CA and CD) and transgenic lines (PA-3.3.1, PA-4.1.2, PA-18.3.3 of Asterix and PD-1.1.1, PD-2.1.2, PD-3.2.1 of Diamant variety. (a) H_2O_2 content of Asterix and (b) H_2O_2 content of Diamant. Different letters above error bar indicate significant differences at $P < 0.05$ and data were analysed by Duncan's multiple range test. The error bars indicate the mean ± SD.

4.2.7 Morphological effect of drought stress in var. Asterix and Diamant

Drought tolerance was also conducted in different lines of two transgenic (TG_2) potato varieties (Asterix and Diamant) and wild type control. *Ex vitro* rooted transgenic and control plants were grown in 8 inch plastic pot for 7 days. Then drought stress was applied by totally with holding water for 13 days. Treated plants were then re-watered for the next 7 days. Transgenic plants had longer shoots and roots than wild type control in case of both the varieties (Figs. 43-46). Approximately 68-80% of transgenic lines survived after watering, whereas recovery from the wild type control plants was very limited (Table 28). Drought stress-recovered transgenic plants were much healthier and greener compared to survived wild type plants.

Fig. 42 (a-f): 13 days after starting drought stress in control and five transgenic TG_2 lines of var. Asterix. (a) Wild type control; (b) PA-3.3.1; ((c) PA-3.4.1; (d) PA 4.1.2; (e) PA-4.5.4 and (f) PA-18.3.3. Scale bar = 4.0 cm.

Fig. 43 (a-f): 13 days after starting drought stress in control and five transgenic TG_2 lines of var. Diamant. (a) Wild type control; (b) PD-1.1.1; (c) PD-1.2.3; (d) PD-2.1.2; (e) PD-2.2.2 and (f) PD- 3.2.3). Scale bar = 4.0 cm.

Fig. 44 (a-f): Recovery after drought stress in control and five transgenic TG_2 lines of var. Asterix. (a) Wild type control; (b) PA-3.3.1; (c) PA-3.4.1; (d) PA-4.1.2; (e) PA-4.5.4 and (f) PA- 18.3.3). Scale bar = 4.0 cm.

Fig. 45 (a-f): Recovery after drought stress in control and five transgenic TG_2 lines of var. Diamant. (a) Wild type control; (b) PD-1.1.1; (c) PD-1.2.3; (d) PD-2.1.2; (e) PD-2.2.2 and (f) PD- 3.2.3). Scale bar = 4.0 cm.

Table 28. Survival rate of wild type control and different transgenic lines of Asterix and Diamant under drought stress

Variety	Control and transgenic lines	Total plants	Number of survivors	Survival rate (%)
Asterix	Wild type Control	25	5	20
	PA-3.3.1	25	18	72
	PA-3.4.1	25	20	80
	PA-4.1.2	25	19	76
	PA-4.5.4	25	17	68
	PA-18.3.3	25	18	72
Diamant	Wild type Control	25	4	16
	PD-1.1.1	25	20	80
	PD-1.2.3	25	18	72
	PD-2.1.2	25	17	68
	PD-2.2.2	25	18	72
	PD-3.2.3	25	20	80

4.2.7 Biochemical and physiological analysis of transgenic lines under drought stress condition in green house

Different experiments were conducted to analyse the biochemical and physiological changes in the transgenic lines under drought stress conditions. Drought stress was mimicked by totally with holding water for 13 days. After 13 days, chlorophyll and carotenoid content, Proline content, H_2O_2 content and electrolyte leakage were determined.

Physiological changes in transgenic potato plants submitted to drought were evaluated by comparing the RWC in control and transgenic lines of both the varieties (Asterix and Diamant). The RWC determines the water status of the plant as a physiological cosequence of cell water deficiency due to drought stress. Under control condition, the relative water content of the control was higher than that of the different transgenic lines (Table 29). However, the drought treatment led to a decrease of RWC in control plants. A lower RWC decrease was obtained in transgenic lines compared to control. Under control condition, the chlorophyll content of the control was higher than that of the different transgenic lines but significance loss in chlorophyll content was observed in control upon drought treated condition. However, most of the transgenic TG_2 lines of Asterix and Diamant showed a lower decrease of chlorophyll content (Fig. 46 and 47)

Total carotenoid content was similar to chlorophyll in control condition. Drought treatment allows a generalized decrease in the concentration of the carotenoid pigment in control plants than transgenic TG_2 lines (Fig. 47 and 48).

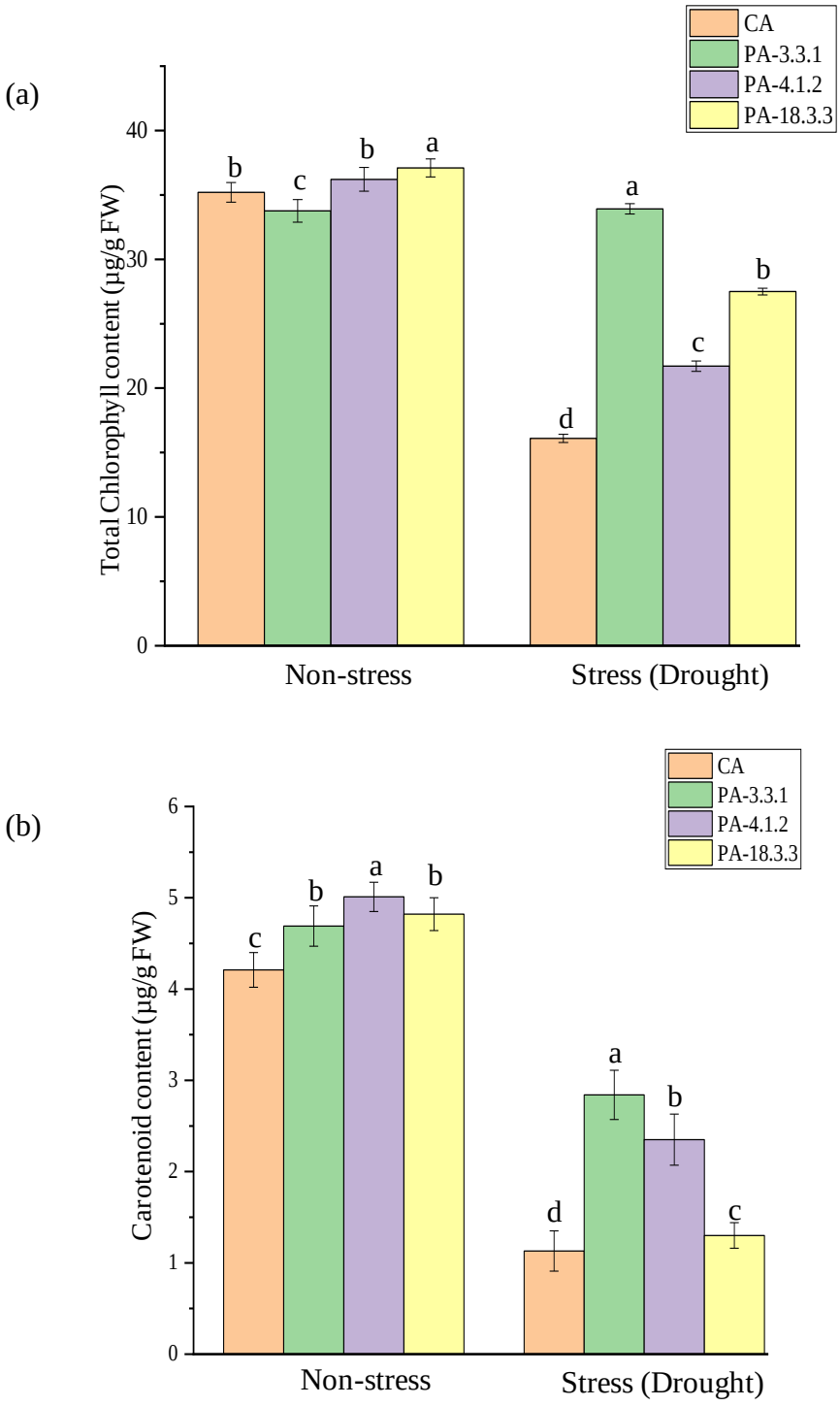

Fig. 46: Total Chlorophyll and Carotenoid contents under non-stress and drought stress condition in wild type control (CA) and transgenic lines (PA-3.3.1, PA-4.1.2 and PA-18.3.3) of Asterix variety. (a) Chlorophyll content and (b) Carotenoid content. Different letters above error bar indicate significant differences at $P < 0.05$ and data were analysed by Duncan's multiple range test. Error bars indicate the mean ± SD.

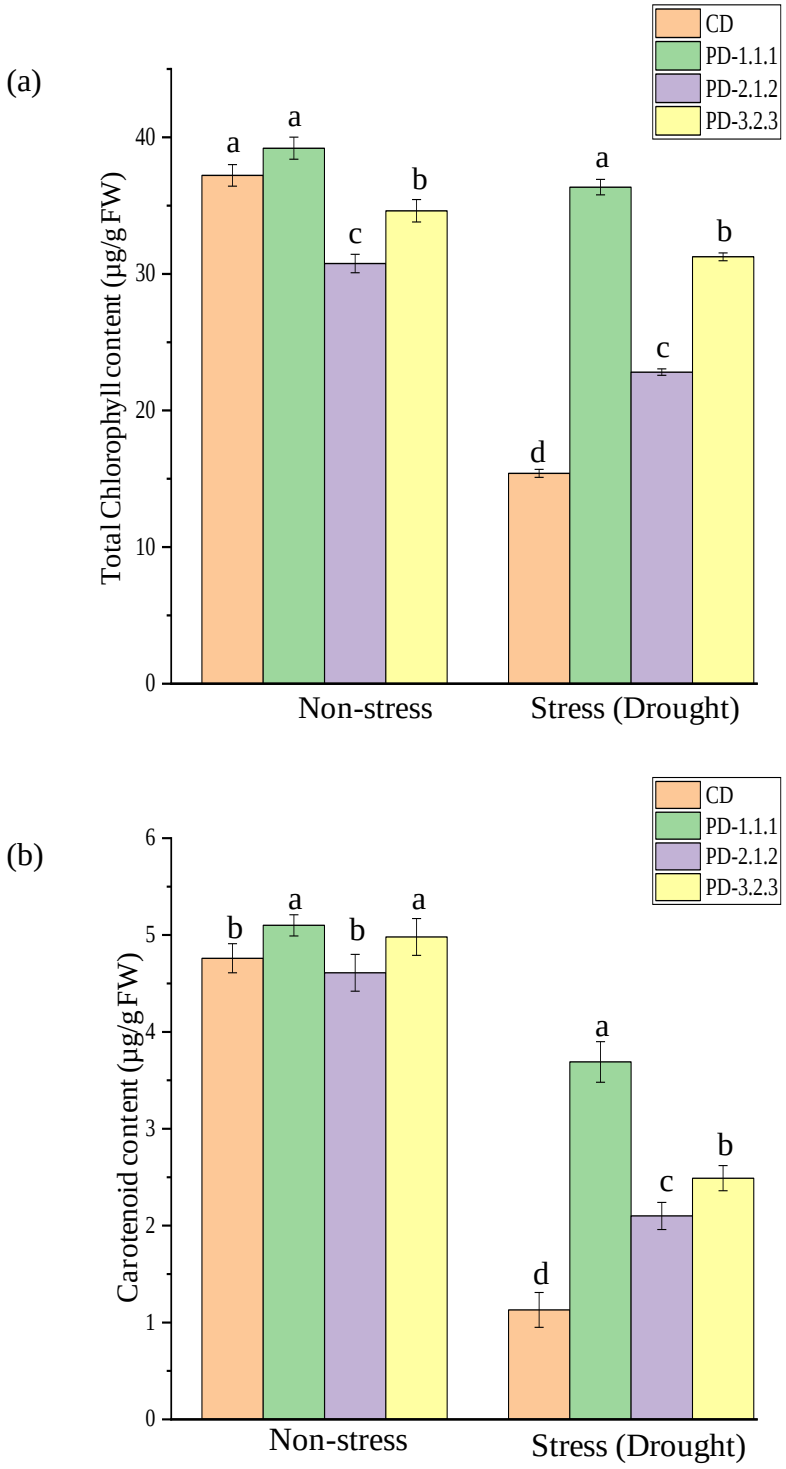

Fig. 47: Total Chlorophyll and Carotenoid contents under non-stress and drought stress condition in wild type control (CD) and transgenic lines (PD-1.1.1, PD-2.1.2 and PD-3.2.1) of Diamant. (a) Chlorophyll content and (b) Carotenoid content. Different letters above error bar indicate significant differences at $P < 0.05$ and data were analysed by Duncan's multiple range test. Error bars indicate the mean ± SD.

In the present study, the increase in proline content was observed in transgenic lines of Asterix and Diamant subjected to drought stress condition (Fig. 48).

To determine the impact of drought stress on subsequent oxidative stress generation in plants drought treatment was applied for 13 days. Low H_2O_2 level was detected in leaves of all transgenic lines of Asterix and Diamant. Only wild type control plants exhibited differences in H_2O_2 in response to drought. None of the transgenic lines exhibited H_2O_2 changes in response to drought (Fig. 49).

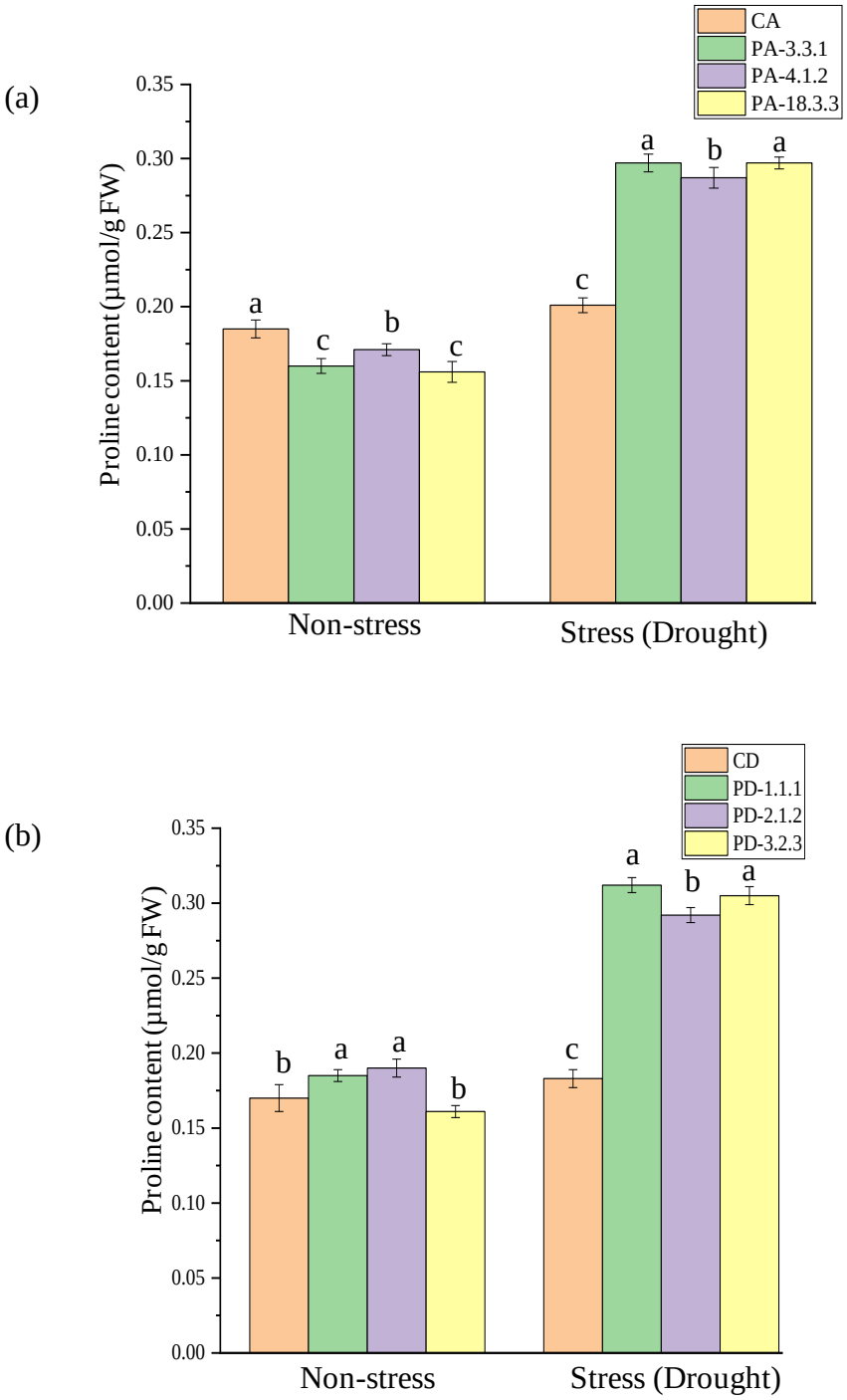

Fig. 48: Proline content under non-stress and drought stress condition in wild type control (CA and CD) and transgenic lines (PA-3.3.1, PA-4.1.2, PA-18.3.3 of Asterix and PD-1.1.1, PD-2.1.2, PD-3.2.1 of Diamant variety). (a) Proline content of Asterix and (b) Proline content of Diamant. Different letters above error bar indicate significant differences at $P < 0.05$ and data were analysed by Duncan's multiple range test. Error bars indicate the mean ± SD.

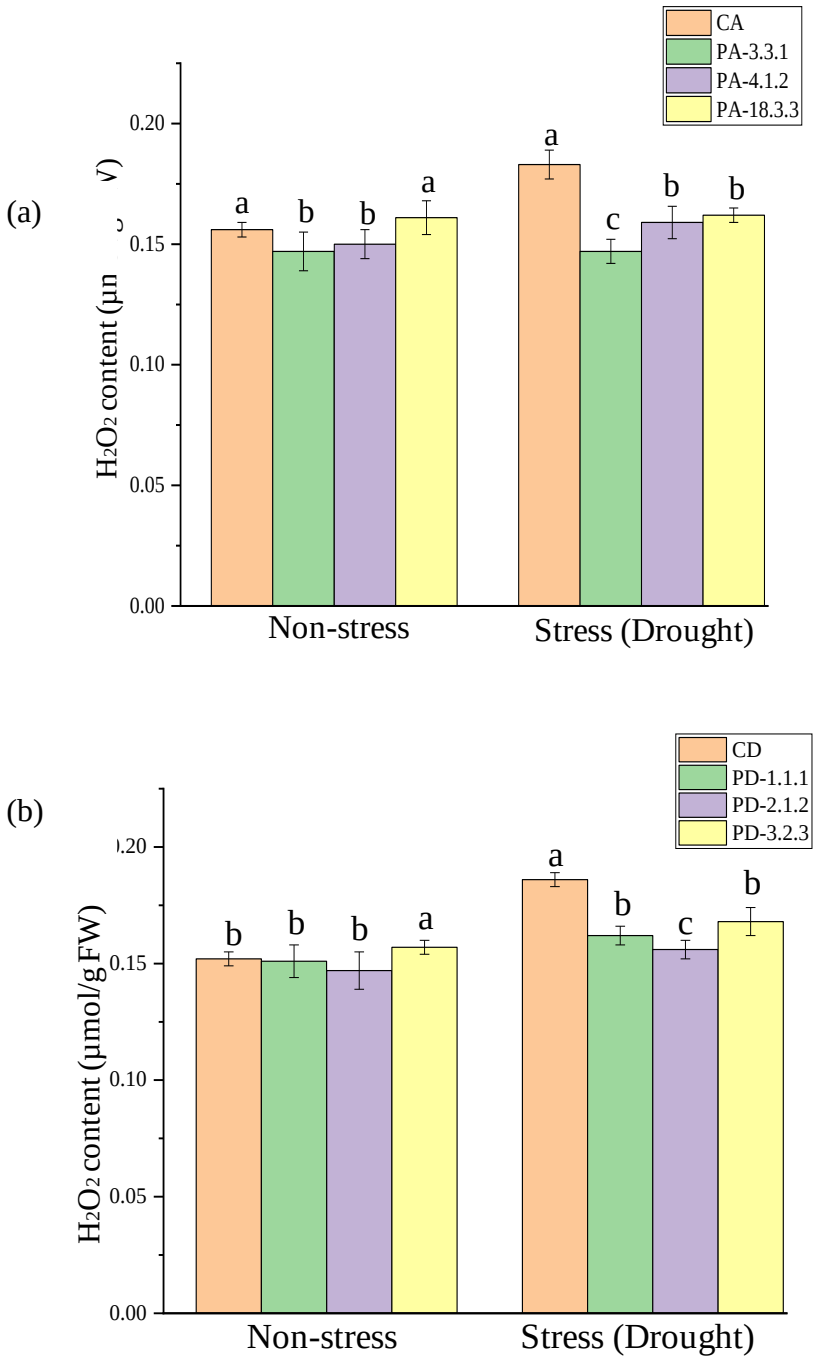

Fig. 49: H_2O_2 content under non-stress and drought stress condition in wild type control (CA and CD) and transgenic lines (PA-3.3.1, PA-4.1.2, PA-18.3.3 of Asterix and PD-1.1.1, PD-2.1.2, PD-3.2.1 of Diamant variety). (a) H_2O_2 content of Asterix and (b) H_2O_2 content of Diamant. Different letters above error bar indicate significant differences at $P < 0.05$ and data were analysed by Duncan's multiple range test. Error bars indicate the mean ± SD.

Table 29. Measurement of Relative Water Content (RWC) of leaf after 13 days of applying drought stress in green house (var. Asterix)

Variety	Transgenic TG_1 lines	Fresh weight (mg) FW (±SD)	Turgid weight(mg) TW (±SD)	Dry weight (mg) DW (±SD)	Relative Water Content (±SD)
Asterix	Wild type control	464.661±1.67^{h}	660.661±4.01^{g}	27.64±1.70^{c}	66.17±1.07^{c}
	PA-3.3.1	1424.331±5.02^{a}	1683.33±15.56^{a}	176.66±5.85^{a}	74.13±1.82^{a}
	PA-3.4.1	1303.331±0.40^{c}	1641.0±11.53^{b}	175.66±9.29^{a}	68.73±1.01^{b}
	PA-4.1.2	1259.03±0.60^{e}	1552.0±15.23^{cd}	155.0±6.55^{b}	71.15±1.32^{ab}
	PA-4.5.4	1127.66±2.51^{g}	1316.33±14.84^{f}	134.66±5.68^{cd}	75.45±0.38^{a}
	PA-18.3.3	1136.01±3.52^{g}	1346.66±12.58^{e}	145.66±10.01^{bc}	73.55±0.55^{ab}
Diamant	Wild type control	429.66±4.50^{i}	609.0±7.93^{g}	26.0±10.92^{e}	66.30±0.40^{c}
	PD-1.1.1	1378.331±6.62^{b}	1635.0±15.0^{b}	174.0±8.18^{a}	73.67±1.18^{a}
	PD-1.2.3	1283.331±0.40^{c}	1574.01±0.81^{c}	145.0±7.0^{bc}	72.33±0.23^{ab}
	PD-2.1.2	1163.661±4.84^{f}	1356.66±10.40^{e}	128.66±6.11^{d}	76.30±0.49^{a}
	PD-2.2.2	1147.01±7.52^{fg}	1334.33±9.29^{ef}	136.66±9.07^{cd}	75.73±1.46^{a}
	PD-3.2.3	1267.33±11.59^{d}	1537.66±7.50^{d}	146.33±5.50^{bc}	72.90±0.93^{ab}

Mean±SD followed by similar letters are non-significant according to Duncan's Multiple Range Test (DMRT) at $p<0.05$.

4.2.8 Leaf discs assay and chlorophyll content measurement in TG_3 lines of Asterix and Diamant

Leave discs were excised from healthy and fully expanded non-transgenic and transgenic (TG_2) potato leaves of similar age. Discs were floated in a 20 ml solution containing 0, 100 and 200 mM NaCl for 72 h. After treatment these leaf discs were blotted with Whatman no. 1 filter paper and weighed before measuring chlorophyll. Higher chlorophyll content was observed in leaf discs of transgenic plants at 0, 100 and 200 mM salt stress condition than wild type control plant (Figs. 50 and 51). Total chlorophyll and

carotenoid content were also measured from TG_3 lines of Asterix variety. Lower decrease in chlorophyll and carotenoid was observed compare to wild type control (Fig. 52).

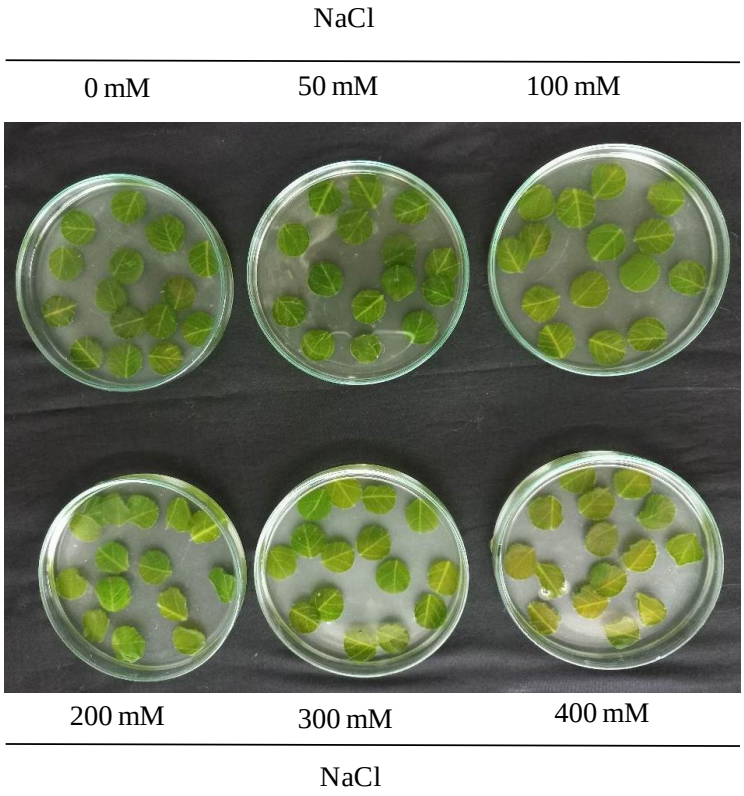

Fig. 50 : Leaf discs assay of Control in different concentrations of salt stress 0, 50, 100, 200, 300 and 400 mM NaCl in case of var. Asterix.

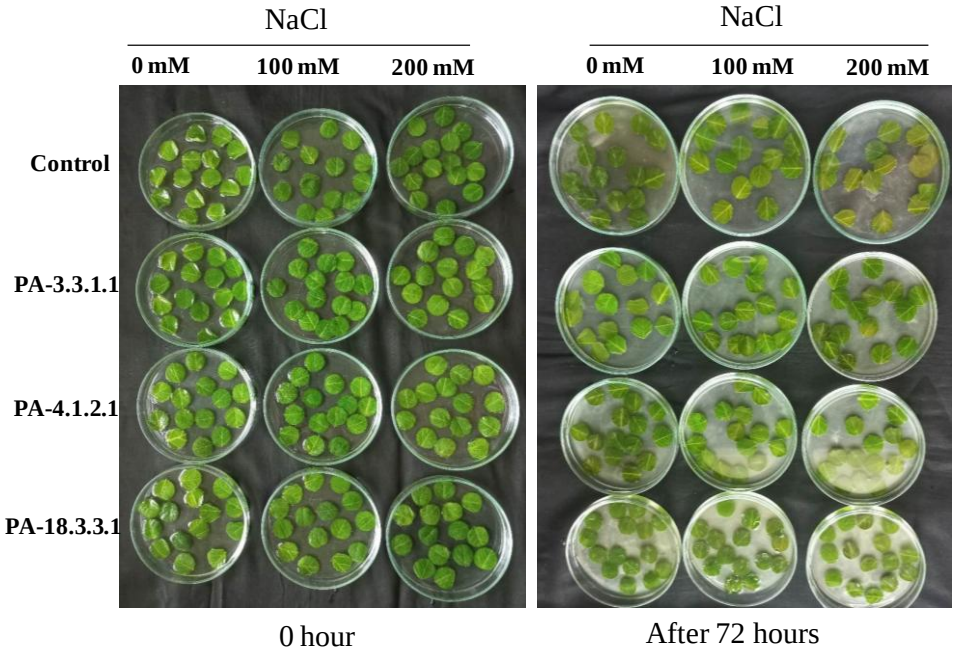

Fig. 51 : Leaf discs assay of control and three transgenic TG_3 lines (PA-3.3.1.1, PA-4.1.2.1. and PA-18.3.3.1) of var. Asterix.

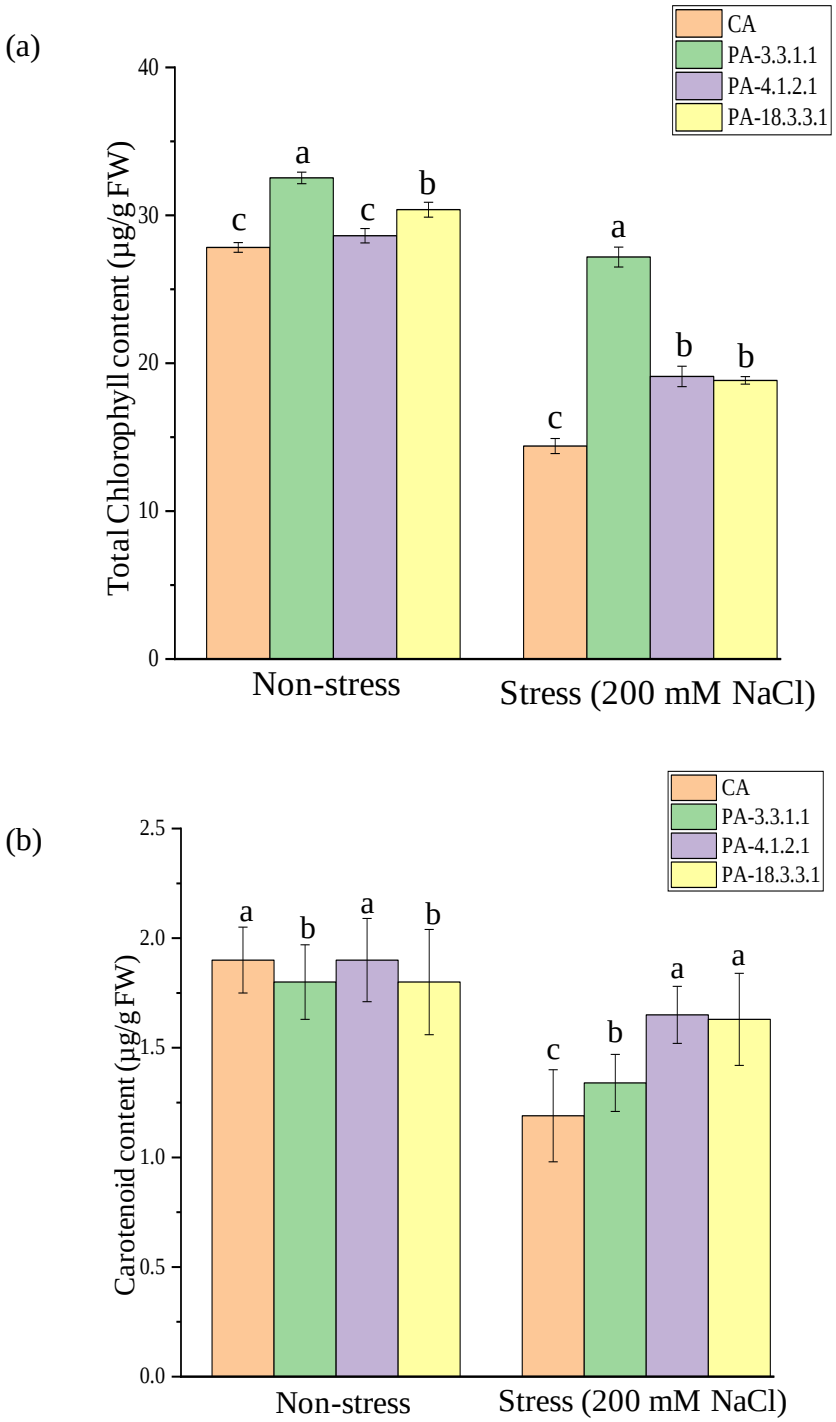

Fig. 52 : Total Chlorophyll and Carotenoid contents under non-stress and 200 mM NaCl salt stress condition in wild type control (CA) and transgenic lines (PA-3.3.1, PA-4.1.2 and PA-18.3.3) of Asterix variety from leaf discs assay. (a) Chlorophyll content and (b) Carotenoid content. Different letters above error bar indicate significant differences at $P < 0.05$ and data were analysed by Duncan's multiple range test. Error bars indicate the mean ± SD.

5. DISCUSSION

5. DISCUSSION

The fourth most significant vegetable crop in the world is the cultivated potato (*Solanum tuberosum* L.). However, abiotic factors like salinity and drought significantly reduce potato output. Numerous factors, including water stress, ion toxicity, nutrient imbalances, oxidative stress, metabolic process change, membrane disruption, reduced cell division and expansion, etc., are impacted by high salinity on plants (Hasegawa *et al.* 2000). The advancement of contemporary agriculture has been greatly aided by the use of genetic engineering, which has improved crop quality, productivity and disease resistance (Klümper *et al.* 2014). Therefore, genetic engineering can be very important in creating potato varieties that can withstand salinity and drought.

The objective of the current study was to create an effective method for genetic transformation mediated by *Agrobacterium* that would allow for the creation of potato (*Solanum tuberosum* L.) lines that were drought and salinity tolerant and could be grown in Bangladesh. For this investigation, plant materials included four potato varieties: Asterix, Diamant, Granola, and Lady Rosseta. To develop potato lines that are resistant to salinity and drought, genetically modified *Agrobacterium* strain EHA105 with binary plasmid pCAMBIA1301-PDH45 was employed. To assess the capacity of diverse explants from various potato varieties for transformation, the *Agrobacterium* strain LBA4404/pBI121 was utilized as a marker strain. For four different potato varieties, an effective *in vitro* regeneration methodology was developed in the initial phase of the inquiry. This technique is essential to develop an effective *Agrobacterium*-mediated genetic transformation system for any crop. For two chosen potato varieties, the second step intended to create an effective *Agrobacterium*-mediated genetic transformation system. To determine the effectiveness of their transformation, four different explants, including nodal segments, microtuber discs, intermodal segments and leaf explants were

investigated. Using a genetically modified *Agrobacterium* strain, steps were also taken to improve a number of transformation-related parameters. The transformants produced in this work were identified by PCR analysis in order to analyze the introgression of desired foreign genes.

In this study, development of *in vitro* axenic culture of potato was established. Surface sterilization procedure of potato sprouts were optimized for this purpose. For obtaining maximum number of contamination free explants surface sterilization of potato sprouts was done using 70% alcohol and 0.1% $HgCl_2$. It was found that most efficient sterilization includes treatment with 0.1% $HgCl_2$ for 8.0 min followed by 70% alcohol for one min. This treatment resulted contamination free culture and 100% explants were regenerated. It was observed that regeneration frequency was low with increasing sterilization time. Explants washed for longer times with 70% alcohol and $HgCl_2$ turned brown and did not response towards *in vitro* regeneration. On the other hand, contamination rate was high with lower sterilization period. Few other studies suggested that 15 min of sterilization time was optimum for different potato varieties before regeneration (Khan and Rabbani 1999; Sarker and Mustafa 2002).

Borna *et al*. (2010) used sterilization period of 8.0 min with 0.1% $HgCl_2$. Khatun *et al.* (2012) used a sterilization protocol with 70% (v/v) ethanol for few seconds then 0.1% $HgCI_2$ containing two drops of Tween-20 per 100 ml solution for 30 seconds. Zaman *et al.* (2015) reported potato sprout sterilization with 70% ethanol for one minute and then 10% chlorox for 10 minutes. This differential response towards surface sterilization may be variable due to genotype and physiological status of the mother stocks.

In this study, potato sprouts were used as initial explants to obtain *in vitro* plants. After surface sterilization, 1.0-2.0 cm long sprout segments including one or two nodes were cut and inoculated into regeneration medium. Akhter *et al.* (2006) used pieces of sprout as

starting material for *in vitro* regeneration. Four types of explants, namely, nodal segments, microtuber discs, intermodal segments and leaf were used in this study for all four varieties. Among the four explants, nodal segments showed higher regeneration frequency. Previously, Hussain *et al.* (2005) reported that nodal explants have high regeneration potential which was followed by shoot apices. Recently, Borna *et al.* (2019) reported efficient shoot regeneration from microtuber discs. Sarker and Mustafa (2002) used leaf, nodal segments and inter-nodes of two local potato varieties for regeneration. They reported highest regeneration from leaf explants. Philip and Hampson (1995) showed high regeneration frequency from internode and leaf tissue explants of potato.

An efficient and reproducible *in vitro* regeneration system is required to develop a transformation protocol (Gardner 1993). Plant regeneration system in potato was either followed direct organogenesis using single media for all phases or indirect organogenesis (Philip and Hampson 1995; Hansen *et al.* 1997) where callus was initiated on one medium containing auxin and shoot formation on another medium containing cytokinins. For potato *in vitro* regeneration, MS (Murashige and Skoog 1962) medium was generally used as culture medium. Some researchers performed hormone free MS medium for *in vitro* plant production. On the other hand, studies are done on the effect of different concentrations and combinations of growth hormones for *in vitro* potato reproduction (Badoni and Chauhan 2009). Several researchers have reported about adventitious regeneration in potato using nodal, inter nodal, leaf disc, petiole, tuber disc explants, via direct and indirect organogenesis (Biswas *et al.* 2010; Onamu *et al.* 2012 and Ghosh *et al.* 2014). During the present study, regeneration experiments were conducted using nodal segments, microtuber discs, intermodal segment and leaf explants via direct and indirect organogenesis.

For better *Agrobacterium* infection and regeneration from pre-existing meristem above mentioned explants with physical injury or cut surface on explants were used in the present study. It is reported that use of nodal segments as explants, infected cells of wounded areas of the explants can reconstitute the meristematic area without callus formation (Onamu *et al.* 2012).

In the current investigation, various concentrations of BAP and IAA were utilized separately or in combination on MS medium to assess their impact on the regeneration of numerous shoots. At 5.0 mg/l BAP, the greatest number of shoots per explant was discovered. When BAP concentration exceeded 5.0 mg/l, there was a tendency for shoot regeneration to decline. Borna *et al*. (2010) and Sarker and Mustafa (2002) both noted a similar BAP effect on potatoes. The current study's findings indicate that Lady Rosseta, Asterix, Diamant, and Granola respond best to MS media that has been supplemented with 1.0 mg/l IAA and 4.0 mg/l BAP in the case of nodal segments and microtuber disc explants.

BAP alone did not respond as well to *in vitro* regeneration as BAP plus IAA did. Utilizing microtuber explants, MS media supplemented with 4.0 mg/l BAP and 1.0 mg/l IAA revealed the largest number of multiple shoots in all four potato species. Borna *et al.* (2010) also reported high shoot regeneration from nodal explants and microtuber discs (Borna *et al*. 2019).

When intermodal segments and leaf explants were grown on MS media supplemented with various BAP and IAA concentrations, callus was also developed. With rising BAP and IAA concentrations, callus development frequency rose as well. Within 55 to 60 days on MS with 4.0 mg/l BAP, 1.0 mg/l IAA, and 0.5 mg/l GA_3, it was observed that shoot buds started to emerge from those calli. But in the present study, multiple shoot formation

through callus was less in number than direct shoot regeneration from nodal segments and microtuber explants. Direct shoot regeneration from explants without introducing a callus phase showed much higher regeneration efficiency (Visser *et al.* 1989). Therefore, in the present study emphasis was given on direct organogenesis.

Khatun *et al.* (2012) reported that leaf and internode showed best response on medium with NAA and BAP. This variation may be due to different genotypes used in the study. It is generally known that genotype among potato varieties had a significant impact on both shoot regeneration and transformation efficiency (Sheerman and Bevan 1988; Wenzler *et al.* 1989; Phillip and Hampson 1995).

It was observed in the present investigation that leaf explants has poor response towards shoot regeneration compared to nodal segments, microtuber discs and intermodal segments. For further confirmation, different concentrations of GA_3 were also used in combination with BAP and IAA and observed their combined effect on regeneration and multiple shoot proliferation from leaf explants of four potato varieties. Among the hormones, GA_3 is well known to enhance shoot regeneration and elongation.

Two local potato varieties' leaf, nodal segments, and inter-nodes were used by Sarker and Mustafa (2002) for regeneration. They reported highest regeneration from leaf explants. Philip and Hampson (1995), showed high regeneration frequency from internode and leaf tissue explants of potato. Khatun *et al.* (2012) reported that leaf and internode showed best response on MS medium with NAA and BAP. Parveen *et al.* (2014) found best shoot regeneration from internode using 0.2 mg/l GA_3+0.5 mg/l IAA + 1.0 mg/l BAP.

Healthy root induction at the base of *in vitro* regenerated shoots is a crucial step in the development of plantlets. Throughout the current study, it was observed that a number of roots spontaneously formed from the *in vitro* grown shoots. However, it was found that spontaneous roots were less successful at transferring *in vitro* developed plantlets to the

soil. To move plants into soil, separate root induction was required. In order to induce roots, 0.2-0.5 mg/l IAA and 0.2-0.5 mg/l IBA were introduced individually to half and full concentrations of MS media. Although roots were induced largely in all auxin-combination treatments in the current investigation, hormone-free MS was discovered to be the most successful treatment for root induction in regenerated shoots in all four varieties of potato.

Plantlets that had developed strong roots enough to be transplanted to soil did well in the field. However, Sarker and Mustafa (2002) found best response for root formation on half strength of MS medium supplemented with 0.1 mg/1 IAA. Borna *et al.* (2010) reported that MS medium containing 0.2 mg/1 IBA showed best response in developing roots of Diamant, Cardinal and Granola.

Overall results of present study indicated that potato varieties used in present investigation were very responsive towards regeneration. Therefore, these materials may also be suitable for the incorporation of desired genes through *Agrobacterium*-mediated genetic transformation to improve their agronomic or qualitative properties.

Second phase of present investigation dealt with the establishment of genetic transformation methodology for the selected potato varieties cultivated in Bangladesh. A few procedures for transformation of potato have been described by several workers (Ishida *et al.* 1989; Newell *et al.* 1991; Conner *et al.* 1991; Visser *et al.* 1989; Sheerman and Bevan 1988; Stiekema *et al.* 1988; Tavazza *et al.* 1988; Wenzler *et al.* 1989; Mitten *et al.* 1990; Sarker and Mustafa 2002; Borna *et al.* 2010; Shin *et al.* 2011; Shimazaki *et al*. 2016).

The DEAD-box helicase family member pea DNA helicase 45 (*PDH45*) actively promotes salinity stress tolerance. In response to salinity stress, the over-expressed *PDH45* transgenic plants can sustain cell viability (Sanan-Mishra *et al.* 2005).

Additionally, they noted that salt, dehydration, wounding, and low temperature all increase *PDH45* expression, indicating that it plays a widespread role in abiotic stress adaption (Sanan-Mishra *et al.* 2005). Increased abiotic stress tolerance was seen when *PDH45* was overexpressed, which is consistent with this (Amin *et al.* 2011; Sahoo *et al.* 2012; Manjulatha *et al.* 2014; Augustine *et al.* 2015; Nath *et al.* 2015).

In the current investigation, *PDH45* was overexpressed by transformation experiments utilizing pCAMBIA1301-PDH45. Nodal segment explants have produced transformed callus. Potato has also undergone chloroplast transformation (Sidorovet *et al*. 1999), which has a number of benefits, such as no gene silencing; nevertheless, the tissue culture needed can be challenging (Chakraverty *et al*. 2007). It is also possible to create transgenic potato lines devoid of markers without the use of a selection process. According to Ahmad *et al*. (2008) the desired gene of interest was present in about 2% of the regenerated putatively altered shoots created without selection. It is thought that a number of variables, including the optical density (O.D.) of the *Agrobacterium* suspension culture, the incubation time for explants in the bacterial suspension, the co-cultivation time, and others, affect the process of genetic transformation mediated by this organism (Mansur *et al.* 1993). During the present investigation, factors that influence successful transformation such as *Agrobacterium* strain, explant type, incubation period of explants, density of bacterial suspension were optimized. These studies were conducted using genetically engineered *Agrobacterium tumefaciens* strain, namely, LBA4404/pBI121 (construct I) and EHA105/pCAMBIA 1301-PDH45 (construct II). Construct I contain *gus* and kanamycin resistant *nptII* gene. Construct II contains salinity and drought tolerant gene *PDH45* and *hptII* gene resistant to hygromycin. Transformation experiments were carried out with Asterix and Diamant varieties of potato. Rate of

multiple shoot regeneration from leaf explants was high but the transformation efficiency was very low when leaf were used as explants.

In this investigation, it was found that *Agrobacterium* culture on solid YEP was more efficient for transformation. It was also observed that higher transformation response obtained from culture supplemented with acetosyringone during incubation (200 µmol) and co-cultivation (100 µmol). The vir functions are improved by acetosyringone during transformation (Stachel *et al*.1985). The ability of an *Agrobacterium* strain to change into several other plant species is also known to enhance with the presence of a moderately virulent vir region (Atkinson and Gardner 1991; James *et al*. 1993; Janssen and Gardner 1993). Droste *et al.* (2000), found transformed plants using 100 µM acetosyringone.

The T-DNA of pCAMBIA1301-PDH45 contains the *hpt* gene, which grants transformed cells hygromycin resistance. So, 20 mg/l of hygromycin was used to select transformants. Only transformed cells can survive because hygromycin severely inhibits explant development. For this reason, immediately after co-cultivation, selection pressure employed. After 4 months of culture well-developed shoots were obtained from infected nodal explants. Naturally occurring callus formation led to the expansion of explants and the production of many shoots from the callus. The development of the shoot and callus were selected for antibiotic use. All control shoots failed to survive at 20 mg/1 hygromycin during the current experiment and perished after 20 days. In the past, it has been mentioned that a pre-culture phase and/or a delayed selection with kanamycin were utilized to generate regeneration from explants with high transformation frequency in flax, alfalfa, peanut, and chickpea (Pezzotti *et al.* 1991; McHughen 1989; Cardi *et al.* 1993).

In this study transformation efficiency of explants towards *Agrobacterium* strain LBA4404/pBI121, it was seen that when the bacterial density (OD at 600) was gradually

increased (0.5, 0.6, 0.8 and 1.0), percentage of GUS positive explants was also increased in case of nodal segments. Here, in case of Asterix variety of potato 0.6 was found optimum OD which showed 90 % GUS positive expression. It was discovered that the incubation period has a direct impact on transformation effectiveness. It was found that the incubation time of explants in the bacterial suspension boosts transformation efficiency. However, prolonged contact to a bacterial suspension led to weakening of cells and cracks when handling explants, which decreased the potential of plants to grow further. Taking into account all of these details, the current investigation determined that a 30 min incubation period of the explants with an *Agrobacterium* strain LBA4404/pBI121 solution was appropriate. In order to achieve effective transformation, a 30 min incubation time followed by 3 days of co-cultivation was maintained. The findings of Borna *et al*. 2010 and Lecardonnel *et al*. 1999 were both supported by this outcome. For *Agrobacterium* strain EHA105/pCAMBIA1301-PDH45, the optical density of the bacterial suspension was 0.5 at 600 nm, needed to achieve the maximal transformation efficiency. The goal of effective transformation was maintained by a 20 min incubation period and 2 days of co-cultivation.

Agrobacterium strain LBA4404/pBI121 contained *nptll* gene and *Agrobacterium* strain EHA105/pCAMBIA1301-PDH45 contained *hptII* gene. This *nptII* gene gives kanamycin resistance to transformed cells and *hptII* gene for hygromycin resistance. Therefore, using various concentrations of kanamycin and hygromycin, selection of transformants was carried out. One of the key elements determining the effectiveness of genetic transformation and tissue regeneration is a delicate balance between effective negative selection that eliminates non-transformed cells and promoting the capacity of transformed cells to regenerate (Nguyen *et al*. 2016). This is the reason that instead of encouraging

regeneration from the co-cultivated explants immediately following the application of kanamycin/hygromycin for selection.

Well developed shoots were obtained from transformed nodal segment explants after 90-95 days of culture on MS with 4.0 mg/l BAP, 1.0 mg/l IAA, 0.5 mg/l $GA_{3,}$ 300 mg/l Carbenicillin and 20 mg/l hygromycin. Higher kanamycin doses were used in the current work to efficiently screen transformed shoots for *Agrobacterium* strain LBA4404/pBI121. During this experiment, it was discovered that none of the control shoots survived at 200 mg/1 kanamycin after 15 days. Shoots that survived on a medium containing 200 mg/1 kanamycin were therefore regarded to have undergone transformation. Following such a selection process, transformed shoots were recovered, and these shoots were then made to establish roots while being exposed to selectable agents. To detect expression of *GUS* gene in transformed explants and plantlets histochemical GUS assay (Jefferson 1987) was carried out. Such histochemical assay of selected shoots demonstrated the presence of blue colored cells which indicates characteristic expression of *GUS* gene. Plant parts (shoot tip, leaves and stem) of transformed plantlets showed presence of GUS positive blue color in their respective tissues expressing the stable integration of *GUS* gene. A part of macerated tissue of explants was also observed under fluorescent microscope and tranformed cells showed blue colour.

Initially several regenerated shoot buds infected with *Agrobacterium* strain LBA4404/pCAMBIA1301-PDH45 were directly transferred to MS + 4.0 mg/l BAP and 1.0 mg/l IAA + 20 mg/1 hygromycin. In this study, all non transformed plants died in presence of 20 mg/1 hygromycin within about 20 days.

During this study, leaf explants were also used to investigate the possibility of transformation with *Agrobacterium* strain LBA4404/pBI121 and

EHA105/pCAMBIA1301-PDH45. But transformation efficiency was very low in comparison to nodal explants.

In the present study, all shoots obtained through selection were subjected to rooting on half MS medium with 200 mg/l carbenicillin. After sufficient development of roots from the selected shoots, putatively transformed T_0 plantlets of Asterix and Diamant variety were successfully transplanted to soil. All transplanted plants survived in soil and T_0 plantlets showed better acclimatization capacity compared to controls. High survival rate of transformed plantlets in soil is most likely due to well root development or the proper acclimatization of the plantlets. Well developed matured minitubers were collected from T_0 plants of var. Asterix and Diamant and from those minitubers TG_1 plants were developed. TG_2 plants were developed from TG_1 minitubers.

In present study, genomic DNA was extracted from leaves of *in vitro* putative transformed plantlets. The protocol followed for DNA extraction was Chloroform-Isoamyl Alcohol DNA Extraction Protocol or CTAB method (Doyle and Doyle 1987)**.** Presence of *GUS, nptII*, *PDH45* and *hptII* genes in transformed plantlets was confirmed by PCR analysis using gene-specific primers. A total of 218 putative transgenic shoots out of approximately 700 infected explants in case of Asterix and 204 out of 698 in case of Diamant were recovered in final selection with EHA105 strain of *Agrobacterium* containing *PDH45* and *hptII* genes. Therefore, the frequency of recovery of putative transformed shoots was about 31.30 % in case of Asterix and 29.50 in case of Diamant. By using 1% (w/v) gel electrophoresis, amplified DNA was analyzed. The presence of *PDH45* and *hptII* genes in the T_0 and TG_1 (transformed first tuber generation) plants was also confirmed by PCR analysis.

Crop varieties that can withstand stress will be created in order to address the looming issue of food security (Mahajan *et al*. 2005 and Tuteja *et al*. 2007). The main factors that reduce crop productivity are salinity and drought.

In abiotic stress tolerance of plants *PDH45* gene plays crucial roles. In the present study, effect of *PDH45* gene in transgenic potato var. Asterix and Diamant and their response to NaCl salt and drought stress was evaluated. Half strength of Hoagland solution was used for the preparation of hydroponics condition to evaluate the salt stress. Relative water content total chlorophyll, carotenoid, proline and H_2O_2 content were measured both in salt and drought stress condition. The primary biological pigment in plants, chlorophyll, helps to show growth, nutrient status, and crop productivity. In comparison to wild type controls, transgenic lines showed a lessened drop in chlorophyll content. Relative Water Content (RWC) in wild type control plants decreased significantly in response to NaCl salt treatment, whereas RWC in transgenic plants decreased substantially less. Relative oxygen species (ROS) are generated mainly as by product of various cellular metabolic pathways such as photosynthesis, respiration and electron transport chain. Stress causes the quantity of ROS to multiply, which shuts down all cellularly significant pathways. H_2O_2 is regarded as another oxidative stress biomarker (Baby and Jini 2011). Proline serve as osmoprotectant to protect macromolecules from denaturation (Mosaddek *et al*. 2013, Surekha *et al*. 2014). Higher proline accumulation in response to cadmium exposure has also been commonly observed (Aghababaei and Raiesi 2015 and Dinakar *et al*. 2008). According to earlier findings, overexpression of StDREB1 and StDREB2 in potatoes increased the amount of free proline (Bouaziz *et al*. 2013). Similar to that, the present work amply demonstrated that after NaCl treatment, proline concentration in transgenic lines dramatically rose. This increase is much higher than that measured in wild type control plants. It confirmed involvement of *PDH45* gene in activation of proline

synthesis pathway under NaCl salt stress condition. Performance of transgenic potato lines during salt stress was evaluated by leaf discs assay in presence of salt (200 mM). Retention of chlorophyll in leaf discs of wild type control plant is very low or negligible in comparison to those of transgenic.

In a greenhouse, the ability of transgenic lines and non-transgenic potato plants to produce tubers under salinity stress was assessed. During a 90-day period, five plants from each line were cultivated under non-saline and salinity stress conditions (100 and 200 mM NaCl). The number and weight of the harvested tubers were counted. When grown in an environment free of salt, the non-transgenic potato produced more tubers than the transgenic lines. Between transgenic and non-transgenic potatoes, there were no appreciable differences in minituber. All lines produced less tuber under the 100 mM NaCl environment than they did under the non-saline condition. Transgenic lines survived under severe salt stress conditions (200 mM) for 80 days, but non-transgenic plants wilted after 45 days. While both transgenic lines produced an average of 4.0-5.0 minitubers, non-transgenic potatoes only produced one or two tubers.

Plants have evolved a number of adaptations to maintain growth in water-limited environments (Mahajan and Tuteja 2005). Plants undergo drought stress when they don't get enough water compared to their actual needs. Potato plants employ a number of physiological, biochemical and molecular techniques to battle drought stress (Dahal *et al*. 2019).

From the explanation above, it can be inferred that the selection method created for this study has been successful in retrieving transformed plantlets. This protocol can be used to develop transgenic potato plants from transformed tissue following proper selection. Moreover, protocol developed here for the *in vitro* regeneration of plantlets from the co-cultured explants requires very simple hormonal supplement. So, this transformation

compatible regeneration system can also be used in the future transformation experiments in other varieties of potato or any other related plant species. Optimization of various factors influencing transformation will help to obtain transgenic potato plants having stable integration of *PDH45* or salinity and drought tolerant gene. Here, protocol was primarily developed using *PDH45* gene, screenable marker gene like *GUS* and selectable marker gene like *nptII*. Transformation techniques developed in the present study were efficient, reliable and can be utilized for the development of abiotic stress tolerant potato varieties.

6. REFERENCES

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7. APPENDIX

7. APPENDIX – A

ABBREVIATIONS

The following abbreviations have been used throughout the text:

A.	:	*Agrobacterium*
BAP	:	6-benzylaminopurine
BARI	:	Bangladesh Agriculture Research Institute
BBS	:	Bangladesh Bureau of Statistics
bp	:	base pair
C	:	Centigrade / Celsius
CaCl2	:	Calcium chloride
CaMV	:	Cauliflower Mosaic Virus
cm	:	Centimeter (s)
CTAB	:	Cyle tetramethyl ammonium bromide
dNTP	:	Deoxy nucleoside tri-phosphate
e. g.	:	Example gratia, for example
et. al.	:	et alil and others
etc	:	et cetra, and the rest
FAO	:	Food and Agriculture Organization
Fig/s	:	Figure / Figures
FW	:	Fresh weight
GM	:	Genetically modified
g	:	gram (s)
GUS	:	ß-glucoronidase
Ha (s)	:	Hectare
Hr (s)	:	Hour (s)
HCL	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
IAA	:	Indole- 3 – acetic acid
IBA	:	Indole- 3 – butyric acid
i. e.	:	id est = which to say in other words
Kan	:	Kanamycin
Kb	:	Kilo base pair
Kcal	:	Kilocalorie
KNO_3	:	Potassium nitrate
l	:	Litre
LB	:	Liquid Broth
m	:	Meter (s)
M	:	Molar
mM	:	Millimolar
mm	:	Milimeter
mg	:	Milligram
mg / l	:	Milligram per liter
min (s)	:	Minute (s)
ml (s)	:	Milliliter (s)
MS	:	Murashige and Skoog Medium 1962
MT	:	Metric tonne

MW	:	Molecular weight
NaOH	:	Sodium hydroxide
Na_2 – EDTA	:	Sodium salt or ferric ethylene diamine tetra acetate
NH_4NO_3	:	Ammonium nitrate
No.	:	Number
NOS	:	Nopaline synthase
nm	:	Nanometer
*npt*II	:	Neomycine phosphotransferasae II
OD	:	Optical density
PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of Hydrogen
rpm	:	Rotation per minute.
sec.	:	Second
Sp. / Spp.	:	Species
t	:	Ton
T_0, TG_1, TG_2	:	Transgenic lines (First and second tuber generation)
T- DNA	:	Transfer DNA
US	:	United States
USDA	:	United States Department of Agriculture.
US$	:	United States dollar
UV	:	Ultraviolet Wavelength
Var. (s)	:	Variety (s)
Vir	:	Virulence region
Viz	:	Namely
v / v	:	Volume by volume
WHO	:	World Health Organization
Wt.	:	Weight
w / v	:	Weight by volume
X – gluc	:	5-bromo-4-chloro-3-indolyl glucoronide
YEP	:	Yeast Extract Peptone
YMB	:	yeast extract Mannitol Broth
µ	:	Micron
µM	:	Micromole
µl	:	Micro liter
µg	:	Microgram
1 N	:	1 Normal
%	:	Percentage
+ve	:	Positive

APPENDIX - B

Murashige and Skoog (MS) Medium 1962

Components	Concentration (mg/l)
Macronutrients	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
Micronutrients	
$FeSO_4.7H_2O$	27.80
Na_2-FeEDTA	37.30
$MgSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Inositol	100.00
Sucrose	30,000.00

pH adjusted to 5.8 before autoclaving.