CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

# Optimization of Lectin Gene Transformation Protocol in *Potato Cultivar* $GN_2$ by Using GUS as a Reporter Gene

by

Anam Liaqat

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
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*Dedicated to ALLAH Almighty, Hazrat Muhammad (PBUH), my parents, my brother "Awais Liaqat" and my respected teachers for their encouragement, guidance, motivation during my research work and supporting me spiritually throughout my life.*

## CERTIFICATE OF APPROVAL

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# *Abstract*

The developments in transformation technology have enabled the scientists to incorporate gene(s) leading to a particular trait advancing it to a point where only few technical limitations remain. In the present study, a rapid, reproducible and stable *Agrobacterium*-mediated transformation procedure in potato was developed by a combination of different plant growth regulators. Genotype dependency and explant types are important factors affecting transformation efficiency in *S. tuberosum*. *Agrobacterium GV1301* was used for the transformation of lectin to develop resistance in plants of potato cultivar. Internodal parts will be used as explants. During the process, *Kanamycin* and *carboncillin* was used as bacterial selectable marker, Phosphinothricin (PPT) as a plant selectable marker for plant selection, the GusA as a reporter gene and lectin gene. Co- cultivation and infection time periods were also optimized as transformation efficiency was dependent on these factors. Two days of co-cultivation of potato explants with *Agrobacterium GV1301* was best for transformation. Potato cultivar with 08mg/l PPT (phosphinothricin), 0.2mg/l GA3 and 1mg/l BAP showed best transformation efficiency. Furthermore, histochemical assay and PCR were performed to monitor the functionality of desired gene. Overall, the short duration, rapidity and reproducibility make this protocol suitable for wider application of transgenically transformed plants of *S. tuberosum* plants.

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# Abbreviations

**BAP :** Benzyle amino purine (BAP)

**CaMV 35S :** Cauliflower Mosaic virus

**CIM:** Callus Induction Media

**DMF :** Dimethylformamide

**2,4- D :** 2,4-Dichlorophenoxyacetic acid

**($GN_3$) :** Gibberellic acid

**GUS :** $\beta$ - glucuronidase

**GFP :** Green Fluorescent protein

**IAA :** Indole acetic acid.

**LB :** Luria-Bertani

**MS Salts :** Murashige and Skoog

**NAA:** Naphthalene acetic acid

**NptII :** Neomycin phosphotransferase II

**PPT :** Phosphinothricin

**PH :** Potential hydrogen

**SIM :** Shoot Induction media

**T-DNA :** Transfer-DNA

**ZR :** Zeatin riboside

# Chapter 1

# Introduction

Tuber crops like potatoes are widely cultivated and consumed worldwide. Potato Solanum tuberosum) belongs to the family Solanaceae. The world's primary non-grain food commodity, soybean, produced 320 million tons per year. According to popular belief, the crop was first cultivated in Peru (South America), then exported and transported across the globe during military expeditions [1] . Potatoes are used in many products and food types, including as fresh vegetables, raw ingredients in foods and drinks, starch and alcohol, and as animal feed. In comparison to every other essential crop, on much less land the potato plant yields additional nutritional values as well as in harsher climates as much as 85% of the plant is fit for human consumption while the discrimination for cereals is around 50%. Developing countries contributed more than double to food production every year. where potato consumption reached 22 kg per capita per year [2].

Potato is important for supply of food, employment, and earnings in the growing countries where consumption is increasing progressively. The global potato market expanded suddenly in 2015 as prices of food soared unapproachable dozens of low-income countries food security and food balance [3]. Various countries, especially the miserable and most malnourished farm families for nutritional food and other food sources depends on potatoes. The potato is valued by these families because it produces high amounts of nutritional value and solid yields even when other vegetation may fail [4]. For poor farmers in order to be able to earn a living

and maintain their food security, potato yields need to be increased. In order to maximize productivity, new varieties of potato must be developed with higher yield potential and pest and disease resistance, quality seed potatoes must be used, and pest and disease management must improve. Potato is dicotyledonous tuber crop it has significant socioeconomic potential [5]. In today's world, there are more than 5000 varieties of potato most of them are native to South America. Due to its versatility (both raw and processed) and availability to low-income consumers, the crop is popular in Pakistan and elsewhere in the world. A baked potato contains 390 kilojoules per 100 grams [6].

Potatoes grow normally in Pakistan during three different seasons, spring, summer, and autumn. Our plains are cultivated with two kinds of potatoes spring and autumn crops and the hilly areas are cultivated with a third type of potato during the summer months. Potatoes have grown in importance in Pakistan over the years, both for farmers and consumers [7].

After wheat, rice, and maize, potato is the fourth most cultured food crop in the world. In developing countries like Pakistan potatoes are a good source of carbohydrates, vitamins, minerals, proteins, and a lot of trace elements that are needed to meet the energy requirements of the people [8]. There are a number of ways to prepare potatoes, as they are carbohydrate-rich and versatile. Its dry matter content is approximately 20%. When it's freshly picked it contains around 80% water [9]. Starch accounts for about 60-80% of the dry matter. In comparison with other roots or tubers, potato has a very high protein content based on dry weight and is similar to that of cereals. A potato also contains a lot of micronutrients and is low in fat. Almost half of an adult's daily vitamin C requirement is provided by a single medium-sized (150 g) potato if it is eaten with its skin [10]. Its high vitamin C content promotes iron absorption, making potatoes an excellent source of iron. It also contains potassium, phosphorus, and magnesium as well as vitamins B1, B3, and B6. The food contains folate, pantothenic acid, riboflavin, and nutritional antioxidants, all of which reduce the risk of chronic diseases [11]. Potatoes are the best option for food and nutrition security for large sectors of the human population, both in the developed and

developing regions of the world, given their nutritional quality, the wide range of agro-ecosystems in which they can grow and their high dry matter and nutrient output per unit of land area [12].

There are different agroclimatic conditions in Pakistan due to nature. The potato is currently grown on 172.8 thousand hectares in Pakistan resulting in a yield of 3785.9 thousand tons annually, or 18.3 tons per hectare. In 1995-96, there were 78.9 thousand hectares of potato crops, and in 2012-13 there were 172.8 thousand hectares, and 785.9 thousand tons of potatoes were produced in 2018 [13].

More than 99% of Pakistan's seed potatoes are produced locally, and the country is completely self-sufficient in potatoes. Worldwide, there are approximately 1.8 million metric tons of potato produced by countries for consumption. For a country of 132 million people, this is equivalent to 9.3 kilograms per person per year [14].

Potato yields are limited by a number of biotic and abiotic factors. There is insufficient access to high-quality irrigation water, poor soil quality, and inefficient fertilizer application methods. Potato development and productivity are stuck by a number of biological restrictions. These include low-yielding potato varieties, fungi, parasites, bacteria, and viruses [15]. There are a number of foliar diseases in the garden that relate to biotic factors (*Pseudomonas solanacearum*), including Phytophthora infestans (*late blight*), bacterial wilt, and early blight. It is estimated that potato losses due to biotic reactions are around 40% according to estimates on root canker (*Rhizoctonia*), verticillium wilt and fusarium wilt (*Streptomyces scabies*) whereas viral infections have a significant impact on potatoes yield. Abiotic constraints are responsible for 50% of the worldwide decline in agricultural production [16]. Potato production occurs within an environment with poor soil, an inadequate fertilizer supply, severe temperature extremes (heat or cold stress), soil salinity, and drought. Agricultural production throughout the world is most adversely affected by salt and drought [17].

From the pre-harvest to the post-harvest stage, the crop is also plagued by pathogens. Crop productivity is lowered due to different phytophagous insects and pests that may damage crops. The leading sap sucking insect in the world, *Lypaphis erysimi*,

is the most common cause of yield losses in potato crops. In most cases, the sap is sucked away by the larvae and adults. During flowering and seed formation the insects suck the plant sap which prevents nutrients from getting to the seeding pods and therefore affects the health of the plants. Plant aphids can also transmit diseases that cause luteoviruses to crops and cause yield losses [18]. The life cycle of aphids is extremely complicated with over 4400 species found around the world. Crops can be damaged by aphids in many ways and harvests affected in many ways. It is not uncommon for aphids to grow to enormous densities feeding on plants, taking nutrients and sap from them to cause scorching and death. The phytotoxic salivary secretions of aphids can harm vegetation, deform leaves, and cause discomfort. Aphids attacking both peaches and potatoes (*M. persicae* and *M. euphorbiae*) reduce potato production worldwide [19].

Insecticides and pesticides are used extensively to kill these insect pests, causing destruction to the environment and harming consumers and producers alike [20]. Different plant species have been genetically modified to tolerate biotic and abiotic stress using modern technologies. Gene modification is necessary to increase agricultural productivity and nutritional value due to the increasing global demand for such crops. Due to the importance of potato production as well as the demand, both developing and established nations are particularly interested in approaches for improving the potato crop. Plant transformation began in the 1980s, when transgenic potato plants delivered genes through Agrobacterium. Transgenic plants can be created by genetic modification. Genetic transformation is the most popular technique. When a foreign gene is introduced into an organism and expressed the organism undergoes genetic transformation. This occurs when extrachromosomal DNA is introduced into nuclei that has a replicative mechanism, and these genes may persist [21]. As the DNA is diluted by cell division, extra chromosomal expression is usually transient. Foreign DNA can be integrated into somatic chromosomes resulting in expression that persists throughout the lifetime of the organism but which is not inherited.

The incorporation of foreign genes into the genome may also result in their stability. Certain conditions must be met for successful transformation. In addition to

targeting tissues with the ability to reproduce or regenerate, the process should deliver efficient DNA delivery, select for transgenic tissues be reproducible and genotype-independent, and be simple, efficient and cost-effective [22] .

One technique, *Agrobacterium-mediated* transformation, appears to meet these criteria at present Many other crops, including monocots, have benefited from this method because of its advantages. There has been a lot of effort put into developing *Agrobacterium tumefaciens-mediated* transformation protocols for different fruit and vegetable crops. Tissue culture, transformation, and potato regeneration have all improved significantly in the previous two decades [23].

Genetically modified plants are primarily produced using *Agrobacterium-mediated* genetic transformation. In the *Agrobacterium-mediated transformation* method, the natural ability of soil microbes to transform plants is exploited [24] . Plant wounds naturally become infected with *Agrobacterium tumefaciens*, a soil phytopathogen that transmits T-DNA from bacterial cells to host cells through a bacterial type IV secretion system (T4SS). Due to improved understanding of how *Agrobacterium tumefaciens* interacts with host cells, *Agrobacterium tumefaciens* has become the most popular tool for plants to undergo transformation. It is now easier than ever to replace the oncogenes of different types of binary vectors in the T-DNA region to transform plants with *Agrobacterium tumefaciens*. Despite the abundance of genetic resources and genomic resources available to researchers, the most widely studied model plant, Arabidopsis, is readily transformable by *Agrobacterium tumefaciens* in several ecotypes studied, but there are differences in transformation efficiency among varieties. The transformation process involves transferring of T-DNA segment from a bacterium to the host plant cells which can contain a selectable marker and genes of interest, and inserting it into the nucleus. In addition to being easy to use the Agrobacterium system comes with low equipment costs. Additionally, transgenic plants produced through this method often contain copies of the original gene. Due to this, many crops have been adapted to this system, including monocots [25]. *Agrobacterium tumefaciens* was successful in converting tobacco plants to stable forms which led to a major effort in developing. In a review of current literature. Some scientists

found that *tumefaciens-mediated* transformation protocols for tree fruit crops including apples, pears, plums grapes, walnuts and kiwifruits (Ume, Potatoes are also transformed using *Agrobacterium tumefaciens* [26]. Improvements have been made in potato tissue culture, transformation, and regeneration during the last 20 years. However, a simple, cost-effective, and efficient transformation protocol is still lacking [27].

For the purpose of establishing this protocol, a reporter gene system is used. Generating gene fusions is the process of combining two genes (performed in vitro or in vivo) that result in one or both of the genes (reporter or controller) directing the synthesis and translation of the coding sequences of the other gene [28]. Reporters usually encode enzymes. By using reporter enzymes, gene fusion measurement can be greatly enhanced, and many different types of analysis can be achieved. Successful gene fusion systems make use of reporter enzymes that are stable, support amino terminal fusions, have multiple, straightforward, and versatile assays, and have no intrinsic background activity within the organism under study. The ability to detect moderate to low abundance gene expression in individual cells and the ability to distinguish between the enzymatic activity in tissues and organs spatially should be possible in assays. Quantitative assays should be inexpensive and easy to use. The enzyme should be tolerant of all kinds of lab abuse, including changes in pH, ionic environment, and temperature [29].

In plant molecular biology, particularly when using gene fusions, the lack of markers encoding stable, readily assayed reporter enzymes has significantly slowed progress. A number of reporter genes have been used to study gene expression in higher plants, including E. coli. All of these enzymes exist in E. coli including nopaline synthetase (NOS), octopine synthetase (OCS), chloramphenicol acetyl transferase (CAT), firefly luciferase, and bacterial luciferase (lux). The gene fusion analysis is not useful for any of these systems. Plants have a high beta-galactosidase activity, which made testing the beta-galactosidase fusions difficult [30]. Galactosidases are found in virtually all tissues and plants. Since the opianes produced by the enzymes cannot be found in normal plant cells, side effects associated with endogenous reporter enzyme activity were avoided by introducing

the genes from Agrobacterium tumefaciens that encode nopaline synthetase and octopine synthetase tolerant of amino-terminal fusions [31]. Due to the difficulty of measuring and the complexity of using these reporter genes, they are no longer widely used. Most of the time, plant reporter genes that do not normally occur in plant tissues have been used as plant reporter genes, such as chloramphenicol acetyl transferase (CAT) and neomycin phophotransferase (NPTII) [32]. In the study of organelle transport, either NPTII with or without fusion to the amino-terminus can be used [33].

Plant gene expression is likely to advance in the future with the development of new gene fusion systems that are easy to quantify and highly sensitive. Moreover, laboratories should be able to perform qualitative and quantitative analysis without having to invest excessive amounts of money in large, expensive apparatuses. Due to GUS's sensitive fluorometric method of detecting enzyme activity, it has become a preferred choice among many researchers. Additionally, the GUS polypeptide can tolerate changes in its N-terminal structure for the enzyme to function, which enables the construction of fusion genes [34].

In consequence, both enzymes are difficult, tedious, and expensive to assay, as well as showing variable activities in plant cells (typically resulting from enzymes with a more generalized substrate specificity), which limits their sensitivity [35]. Enzyme kinetics is also extremely difficult to quantify CAT or NPTII since endogenous enzymes, such as esterases, phosphatases, transferases, and others, participate in competing reactions. A reasonable method cannot determine where these enzymes are found in the body. Since the 1990s, the focus has shifted to creating light in genetically engineered organisms using luciferase genes. There is little potential for histochemical evaluation or fusion genetics in routine practice when using the firefly luciferase gene [36]. The enzyme is unstable and hard to test accurately [37]. In this study potato was transformed by introducing lectin gene through agrobacterium mediated genetic transformation. Lectins are proteins with high affinity for binding glycans of glycoproteins, glycolipids, or polysaccharides. They can act as recognition molecules within a cell, between cells, or between organisms because of their binding selectivity. Because lectins can be found in a wide variety of

species as well as in a wide variety of organs and tissues, it is considered that they play basic biological roles in plants. The majority of plant lectins that have been thoroughly characterized are secretory proteins, which means that they enter the secretory pathway and then build up in vacuoles, cell walls, or intercellular gaps. Phytohemagglutinin, concanavalin A, soybean agglutinin, pea lectin, and favin, for instance, are all well-known lectins that are present at quite high quantities and build up over time. Both storage proteins and lectins are broken down to release amino acids during germination and seedling growth. In vegetative plant organs such roots, leaves, rhizomes, and stems, lectins are also frequently found in high concentrations. Some of them are vacuolar, whereas others, like the Datura seed lectin that binds chitin, are extracellular. Cereal seeds do include vacuolar lectins, although they are far less common (1 pg/dry grain) and only present in certain cell layers of the embryo [38].

Previous studies revealed that genes of three naturally occurring monocot mannose-binding lectins from snowdrop (*Galanthus nivalis L. agglutinin*, GNA), garlic (*Allium sativum L. leaf agglutinin*, ASAL), and onion (*Allium cepa L. agglutinin*, ACA) and a recombinant fusion lectin between ASAL and ACA genes were expressed in a bacterial system to transform the mustard plants. Ectopic expression of these lectins in mustard plants confirmed their protective capacity on the development of the population of aphids on transgenic plants [39]. Moreover, mannose binding *Allium sativum* leaf agglutinin (ASAL) has been proved to be antifeedant and insecticidal against sap-sucking insects. ASAL coding sequence was expressed in an elite indica rice cv and many fertile transgenic plants were generated [40].

## 1.1 Problem Statement

Potato is commonly used for food purposes throughout the world. Some of the insects are involved in the damaged crop production i.e., aphids. Aphid as a vector damaged the fruit body of potato which badly reduced the economic return per year of the potato so in this way the production of potato decreases.

## 1.2 Proposed Solution

As a proposed solution gene transformation technology is introduced by the scientists. Lectin gene have resistance against aphid. For this purpose, lectin gene may transfer from garlic to potato with the help of *Agrobacterium mediated* transformation.

## 1.3 Aims and Objectives

The aim was to optimize the conditions required for efficient genetic transformation of lectin gene in potato. The following are the goals of our investigation:

1. To develop the progeny of explant source of potato variety ($GN_2$).
2. To optimize an efficient protocol for genetic transformation of potato through *Agrobacterium* mediated transformation.
3. To enhance regenerate frequency of $GN_2$ variety.
4. Molecular analysis of Lectin gene through guss assay and PCR.

# Chapter 2

# Review of Literature

## 2.1 *Agrobacterium mediated* Genetic Transformation through GUS

This research revealed that in potato, phenomena of genotype specification is genetic transformation, which makes it more difficult to manipulate genes for specific traits and requires protocols optimization. For genetic transformation of potato heat shock treatment was used. By using previously optimized Agrobacterium T-DNA delivery protocols, six different potato cultivars were initially tested for genetic transformation efficiencies. For genetic transformation efficiency, some cultivars were categorized as high and some were low. In addition, one of the objectives of the current study was to learn more about the variation of biochemical basis in genetic transformation efficiency between cultivars. Antioxidant enzymes and phenol levels were significantly different between cultivars with high and low genetic transformation effectiveness. To maximize genotype independence, both cultivars were treated with heat shock before infection immediately. For 15 minutes at 40 °C heat shock treatment significantly increased regeneration in transgenic shoots, transient GUS expression, callogenesis. By using this method, all cultivars could be recovered. Following heat shock treatment, antioxidant enzyme levels and phenol levels were significantly reduced, suggesting that *Agrobacterium*

*tumefaciens-mediated* genetic transformation is more difficult in cultivars with stronger defense systems [41] .

Further research examined the factors that affect the genetic transformation of Kufri Chipsona 1' potato variety. Several experiments have been conducted to determine the maximum transient expression of $\beta$-glucuronidase (GUS) in explants on MS2 medium (10 m silver nitrate, 10 m BAP, 15m GA3) for 2 days, injured by a surgical blade, and co-cultured with *Agrobacterium tumefaciens* strain EHA105 having [O.D600 (0.6)] in the presence of 1 m gold (O.D600 (0.6). By addition of 100 µM acetosyringone in MS2 medium rate of transient GUS expression increased in both the explants. In optimal culture conditions, putative transgenic shoots were regenerated from internodal explants and leaf. Stable integration of T-DNA was achieved by amplifying nptII and uidA genes. It was found that 67.85% of clump shoots expressed GUS uniformly, while 32.15% expressed intermittently indicating chimeric plants. Firstly, stained the tissues with GUS to determine the proportion of non-chimeric transgenic shoots generated by nptII transgene. Transgenic shoot uniformity was directly related to the amount of nptII transgene expressed. The current study suggests that manipulating culture conditions and medium composition may improve the chances of obtaining transgenic shoots. As a result, 'Kufri Chipsona 1' expresses the transgene uniformly throughout all tissues [42].

Transformed the three potato cultivars by using plasmid-based vectors from *Agrobacterium tumefaciens* Ti. Transfer optimization studies were conducted using the $\beta$-glucuronidase reporter gene (GUS) and the Neomycin phosphotransferase II marker. To select transgenics Murashige and Skoog (MS) media containing 100 mg/l kanamycin and 500 mg/l cefotaxime were used. In histochemical assays of GUS activity, X-Gluc was used as a substrate. Southern blots were also used to confirm the transferred genes. Desiree variety of potato achieved the highest transformation frequency (10%) in MS media containing Zeatin riboside and 1.5 mg/l indoleacetic acid. Anaç potato variety showed the lowest frequency (3%) and (2%) among tuber discs and stem explants. This protocol can be used to improve potato through gene manipulation [43]. Two plant pathogenic bacteria that can

transfer their genes through DNA fragments which are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. Plant biotechnologists have adapted this naturally occurring mechanism to make crops that are genetically modified. Over 10% of the world's arable land is used to grow these crops. *Agrobacterium* species were found to be homologous to small interfering RNAs in sweet potato plants. There are two distinct T-DNA regions in the genome of batatas (IbT-DNA1 and IbT-DNA2), and foreign genes are expressed in a variety of tissues. This has been proven through simple quantitative PCR, genome walking, southern blotting and screening and sequencing of bacterial artificial chromosome libraries. The IbT-DNA1 gene have four open reading frames (ORFs). A total of 291 cultigens were examined, none of which contained wild relatives of IbT-DNA1. Several ORFs on IbT-DNA2 are similar to those on rooting loci of *Agrobacterium rhizogenes*. IbT-DNA2 was detected in 45 genotypes, including wild species and cultivated. Sweet potatoes are naturally transgenic, which might have an impact on the consumer distrust of transgenic foods currently [44].

Bangladesh Agricultural Research Institute, Gazipur-1701 was conducted the experiment in Laboratory of Biotechnology, Biotechnology Division. From this experiment it has been demonstrated that an efficient and reproducible transgenic potato plant production protocol can be achieved by inoculating potato *Agrobacterium tumefaciens* strain LBA4404 internode explants with the pBI121 binary vector containing (Gus) as a reporter gene and (nptII) gene a selectable marker which is Kanamycin resistant. Infection time and cocultivation period were optimized by transformation experiments. The majority of explants produced shoots within 21 days of being grown using MS medium treated with kanamycin and zeatin riboside (ZR). Following 21 days of infection, Asterix and Diamant varieties produced 8.27 and 6.42 shoots respectively. Molecular investigation revealed the presence of transgenes. DNA from nptII-positive plants was detected by performing PCR analysis on well-established roots.

The transformation rate for Asterix was 28.97%, whereas Diamant was 24.37 percent. Diamant and Asterix plants produced roots in 12MS medium containing Cefotaxime 50 mg/mg, Kanamycin 50 mg/l, and IBA 0.5 mg/l [45].

Different varieties of potatoes were infected with *Agrobacterium tumefaciens* strain LBA4404 using a pBI121 binary plasmid, which has the nptII and GUS genes. Regeneration was performed directly and with the help of calluses on nodes and internodes. A pair of MS containing different hormones such as (BAP, IAA, GA3) having different concentrations and produced the best results for direct regeneration of shoots in different varieties of potatoes. A large number of shoots were generated from in vitro grown green micro tubers cultivated on MS containing BAP and IAA. For treated explants, this protocol resulted in a transformation rate of 87%. By histochemical analysis GUS gene was detected in the transformed shoots. Transformed shoots were successfully transformed with GUS and nptII genes [46].

The fourth-most significant food crop in the world is potato. In Arab countries, insect and fungal infections are responsible for 20% of potato yield losses. Our transient transformation assay, which is more efficient than conventional transient assays, was developed using implanta agroinfiltratoin in potato seed slides. From DNA extracted from *Bacillus thuringiensis*, PCR was used to amplify a single copy of the Cry1Ca gene (cry1Ca). We injected pRI201-AN-GUS DNA into potato slices for transient assay of the $\beta$-glucuronidase gene (GUS). *Agrobacterium tumefaciens* strain LBA4404, transformed with pRI201-AN-GUS-Cry1Ca, was used for this transformation. Murashige and Skoog media containing kanamycin was incubated genetically modified potato plants with 6-Benzylaminopurine and 1-Naphthalene Acetic Acid. With pRI201-AN-GUS and pRI201-AN-GUS-Cry1Ca, pRI201-AN-GUS-LBA4404 was used to carry out the transient expression assay. It further possesses the Synthetic Cry1Ca gene (cry1Ca) and glucuronidase genes. Agro injection is an efficient transient UIDA expression method for reporter genes and candidate genes. By successfully transforming Cry1Ca potato plants, yields will be increased and food security will be improved.

*Agrobacterium tumefaciens* carries a reporter gene (uidA) that we used to genetically transform potatoes. Histological and molecular analysis proved transient expression of the gene. Success in transforming transgenic potato plants will increase Saudi Arabia's yield and food security in the near future [47]. Further

research examined that three Mexican potato cultivars were regenerated in vitro using leaf, node, and internodal explants before the use of *Agrobacterium-mediated* genetic transformation. In the regeneration protocol, either callus intervention or no callus intervention was considered. To transform *Agrobacterium tumefaciens* strain LBA4404 was used to infect two potato varieties and one was containing GUS and nptII genes and binary plasmid pBI121. Rooted shoots grown on antibiotic-supplemented media without hormones were subjected to a GUS histochemical test and a PCR analysis. PCR and GUS histochemical tests confirmed integration of the nptII gene in transformed shoots [48].

Another research revealed that for the effectiveness in expressing the constitutive E. Coli beta-glucuronidase gene different promoters were evaluated. Promoter fragments were cloned upstream of gusA to develop the plant expression constructs (pRCA-pCAMBIA and pRD29A-pCAMBIA in pCAMBIA-1301 binary vector. To infect Lady Olympia cultivar leaf discs and internodal explants used an Agrobacterium strain GV2260 carrying recombinant plasmids. GUS activity was analyzed histochemically at different stages of transgenic plants. Stress-inducible promoter (pRD29A) was tested with heat, drought, the pRD29A promoter is more active under drought, heat which are showed by a combination of RT-qPCR and GUS flourimetric assays. pRD29A promoter is lower ten folds than GUS expression levels. pRCA promoters in transgenic plants lower gusA transcript levels than 35S promoters in tubers and roots. In flourimetric assays, GUS expression was reduced or absent in tubers. The results suggest that promoters should be used appropriately to drive the expression of foreign genes in potato lines tolerant to stress and to minimize transgenic technology risks [49].

This research reported that the development of novel transgenic plants is possible using tissue-specific promoters for effective expression of transgenes at certain periods or in specific plant tissues. Tuber-specific expression was reported by the laccase promoter independent of developmental stage in transgenic potato, and no GUS reporter expression was seen in leaves stems, or roots. Laccase promoter regulatory components may be dispersed across the promoter, according to a series of 5 deletions. In the leaves and stems of transgenic potato, the laccase promoter

reacted poorly to salt stress, mannitol stress and mechanical injury, but not to cold stress. Weak GUS expression induced by the laccase promoter was found throughout the transgenic tobacco plant, however in the transgenic tomato plant, GUS expression was only found in the roots and seeds. Laccase promoter was shown to be a viable option for promoting high and selective gene expression in potato tubers based on data [50].

Gene expression of GFP and $\beta$ glucuronidase were used in genetic transformation of Dendrobium Sonia 17 potato variety. The time, target distance, gold particle size, and acceleration pressure spent culturing the plasmids before bombardment, and ratio of the plasmids were optimized in this study. A similar response was observed for both reporter systems based on the bombardment parameters. Due to GFP's rapid and non-destructive properties, it could be used as a reporter system since it expressed more than GUS under the same promoter, CaMV 35S. To choose stable putative transformants, genes for antibiotic resistance and GFP were combined [51]. Another research assessed the resistance of the different potato varieties Surprise, Alpha, Bintje, Pimpernel, and Irene to late blight using a transgenic strain of $\beta$-glucuronidase (GUS), which is produced and secreted by Phytophthora infestans. The GUS activity of inoculated leaves was tested four days after inoculation. Cultivars differed significantly. It was possible to distinguish between resistant and susceptible cultivars based solely on GUS activity levels. A positive correlation exists between implanta GUS levels and field resistance measured by Area under Disease Progress Curve (ADPC) [52]. Further research revealed that an *Agrobacterium tumefaciens* that harbors $\beta$-glucuronidase (GUS) was used to enhance potato transformation and regeneration. After transformation and selection stem explants produced more callus and regeneration than leaf explants, resulting in the highest percentage of shoot formation.

There was a genotype-dependent relationship in all cases. Leaf explants exhibited positive expression of GUS ranging from 40 percent (Hermes) to 92.8 percent (Lady Rosetta), and stem explants exhibited highly positive expression ranging from 33 percent (Hermes) to 91.6 percent (Lady Rosetta). The results of RAPD analysis revealed that genotype-dependent soma clonal variations are induced. It

occurs more frequently when leaves are used as explants while stems are genetically stable when used as explants. For each cultivar before and after transformation similarity coefficients were calculated between genotypes and between different stages in tissue culture. A dendrogram was constructed according to the following procedure [53].

Further research examined that to study the 5 flanking region activity of the B subunit gene of ADP-glucose pyro phosphorylase gusA reporter gene was used in transgenic potato (*Solanumtuberosum L.*) and tomato (*Lycopersicon esculentum Mill*) plants. Potato starch sheaths adjacent to leaf veins, stems, and petioles as well as phloem-associated parenchyma are high in $\beta$- glucuronidase (GUS). Neither the mesophyll nor the stomatal guard cells showed any activity of the promoter. There was no expression in the roots. It was found that ovary placental tissues and pollen grains contain $\beta$-glucuronidase activity. Unlike sAGP promoters the potato promoter gusA construct tightly links GUS activity, extractable ADP-glucose pyro phosphorylase, and fruit starch content. The reason for this is that sAGP promoters are transcribed tissues. In contrast to sAGP, sucrose also stimulates agpB1 transcription [54]. Another study showed that cry1Ac gene and a GUS reporter gene were expressed in vegetable crucifers by using a proprietary tCUP gene expression system. T-DNA tagging is used to isolate tCUP gene activation sequences from tobacco for transient and stable expression in cauliflower. Inbred cabbage lines and hybrid cauliflower lines which are using effective transformation protocols cry1Ac insect resistance gene was introduced.

Transgenic cauliflower and cabbage parts with different GUS reporter genes and Cry1Ac insecticidal proteins are studied to describe a regeneration optimization technique. Throughout the plant's development to cauliflower and cabbage leaf tissues show high levels of expression. The tCUP gene expression system mediated a high level of expression of CRY proteins from the cry1Ac gene that was confirmed by ELISA results. After exposure to cabbage loopers, diamond back moths, and cabbage worm larvae, transgenic cabbage and no leaf damage could be seen in cauliflower lines. By using the tCUP gene expression system in cruciferous vegetables like cabbage and cauliflower, BT genes can effectively control

Lepidopteron pests [55]. Further research revealed that Plasmids coding for $\beta$-glucuronidase (GUS) were bombarded on tungsten particles containing a plasmid containing GUS Plus gene, a plasmid coding for $\beta$- glucuronidase (GUS). After 24 hours of bombardment, the number of blue spots resulting from histological GUS assays was compared to assess the effects of different bombardment parameters. GUS expression was examined under several bombardment conditions, including pressure, distance, plasmid content, and fruit maturity stage. It is optimal to bombard the fruit with 1100 psi, 0.83-gram plasmid per shoot, and 6 cm between the stop screen and the fruit to express the GUS Plus gene. As fruits ripen, GUS expression decreases at the fruit shoulder (near the stem end) and increases at the fruit top. Blue spots were most prevalent in the fruit separation zone, which had 2456.91 per cm2. A genetic transformation experiment could be conducted in the future with optimal particle bombardment conditions [56] .

Another study showed that *Solanum tuberosum* in M6 was used to convert *Solanum chacoense* diploid into M6 potato. Using the pBIN19 binary vector, an intronic element was used to interrupt the gusA gene in *Agrobacterium* strains. The 35S CaMV promoter drives GusA. The transformation efficiency of each strain of *Agrobacterium* was determined by histochemical GUS analysis. It was found that leaf and internode explants of Desiree were most effective in transferring gusA genes with *Agrobacterium* strain GV2260, while for S. chacoense M6 AGL1 was used. On cv. In Desiree leaf explants transformed with GV2260, *Agrobacterium* strain GV2260 produced the best callus formation Desiree and *Solanum chacoense* M6.

There was the least number of calluses developing on internode explants from both solanum chacoense M6 and cv. Desiree. Callus induction was observed in cv. CE974 by the AGL1 and LBA4404 strains at around 33% and 23% respectively. Desiree Transgenic potatoes especially diploid varieties will benefit from the research [57]. Further Studies showed that by Using *Agrobacterium-mediated* transformation, an 1107-bp expression promoter for $GLP_2$ in *Oryza sativa* roots was introduced together with a sensor gene for $\beta$-glucuronidase (GUS) in potato plants. *Solani Fusarium* (Mart.) Sacc. and $OsRGLP_2$ promoter was activated

by *Alternaria solani* Sorauer. According to quantitative real-time PCR measurements, promoter activity increased by 4–5 fold every 24 hours after infection. Within 72 hours of treating with *F. salani* Sac. The activity of the OsRGLP2 promoter increased by 15 times, however, *A. solani* Sorauer treatment increased activity 12 times. According to their findings, fungus stress positively affected OsRGLP2 promoter activity. Activation of H2O2-mediated defense genes in potato genomes is strongly correlated with H2O2 hyper accumulation in transgenic plants. As a result of pathogen infection, elicitors appear to activate GT-I and transcription factors (AAAG) in the $OsRGLP_2$ promoter [58].

This research showed that sweet potato has two distinct cDNA and also have amino acids sequence of mature RbcS proteins that are similar to other plants. Green tissue is the only place where IbRbcS1 is expressed, while roots and tubers are the only place where IbRbcS2 is expressed. Green tissue of sweet potatoes showed a higher prevalence of IbRbcS1 than IbRbcS2. $\beta$- glucuronidase (GUS) and the IbRbcS1 promoter were therefore introduced into Arabidopsis. The expression of GUS gene was detected using a reverse transcription-PCR in a semi-quantitative manner. GUS reporter gene levels were similar in CaMV 35S promoter GUS and IbRbcS1 promoter, GUS plants by qPCR. In plants undergoing genetic transformation the IbRbcS1 promoter may prove useful in producing foreign genes [59].

Further research showed that One of the most popular techniques for obtaining transgenic plants involves the transfer and integration of T-DNA from *Agrobacterium* species into the genome of plant. Two methods of genetically modifying potatoes (*Solanum tuberosum*) are presented. In *Agrobacterium tumefaciens* transformation, transformed cells are selected and phytohormones are used to regenerate a new transformed plant after 18 weeks. *Agrobacterium rhizogenes* transformation involves injecting bacteria into stems, and then detecting the newly emerged hairy roots with a red fluorescent marker. Approximately 5-6 weeks after transplantation the plant consists of a wild type shoot and hairy roots. In order transformed hairy roots can self-replicate to increase biomass. To drive the GUS reporter gene, both *Agrobacterium-mediated* transformation techniques were applied. It is possible to identify promoter induction using GUS staining. Both

endodermis and exodermis of transformed potato roots and the lateral roots of *Agrobacterium rhizogenes* roots exhibited GUS activity. These findings indicate that *Agrobacterium rhizogenes* can be used as an alternative tool for studying root genes [60].

Another research revealed that to increase shoot production and the transfer of the Leaf explants from propagating shoots containing the GUS gene without adversely affecting subsequent genetic engineering experiments sucrose concentrations in propagation medium were used in vitro. Adding 29 mM, 44 mM, and 58 mM of sugar did not significantly increase the number of axillary shoots that were longer than 0.5 cm for blue crop. For Duke and Georgiaagem, increasing sucrose concentrations from 15 to 44 mM and 15 to 58 mM increased axillary shoot number. When cocultured with *Agrobacterium tumefaciens* strain EHA105 for four days, shoot explants cultured on 15mM- or 29-mM sucrose expressed GUS most efficiently. The GUS-expressing leaf zones on explants derived from the stalks of cultivars Duke and Georgiagem were significantly lower in 58 mM sucrose-trained shoots compared with 15 mM sucrose-trained shoots, and in 44 mM compared to 15 mM for all three cultivars. Shoot pretreatment conditions can be optimized in blueberry genetic engineering experiments. Blueberry explants should be grown in a proliferation medium containing 15–29 mM sucrose to facilitate genetic transformation [61] .

Further research showed that Transgenic potato plants were produced from some Slovak cultivars (Cvs.) and a breeding line using *Agrobacterium-mediated* transformation. Transgenic plants were grown in vitro and tested for their ability to regenerate using Cv. There are four Albinas, Eta, Malvina, Vila, and line 116/86. Explants and internodes are infected except for those of Cv. Albina regenerated transgenic shoots. Depending on the genotype, transformation efficiency varied. As compared to Desirée the reference cultivar, leaf disc transformation produced more transformants from line 116/86. $\beta$ - glucuronidase transgene activity was tested in transgenic plants. The genotype had no significant impact on the expression levels of the GUS transgene. A high transgene copy number resulted in increased variation of trans-gene expression in plant 116/86 [62].

## 2.2 *Agrobacterium mediated* Transformation through PCR

Another study showed that *Agrobacterium-mediated* transformation of four cultivars of finger millet has been done Using optimized transformation and direct regeneration conditions. In this work, as explants source shoot apical meristems were employed. To improve the transformation conditions, pCAMBIA1301 carrying *Agrobacterium* strain EHA105 was employed. Concentration of Hygromycin, optical density, infection duration, explant survival, co-cultivation duration, concentrations of antimicrobial and acetosyringone and antibiotic concentrations were enhanced to change an increase of frequency. The mean transient GUS expression that was highest (85.1%) was found in a cultivar called CO(Ra)-14. It took 45 days to planting transgenic plantlets in the greenhouse for CO(Ra)-14 to achieve a stable transformation frequency of 11.8%. Southern blots, GUS staining, and PCR were used to confirm gene integration in T0 and T1 generations. Six events from T0 with One transgenic copy revealed a Mendelian pattern of segregation. The original study on high-frequency *Agrobacterium-mediated* finger millet transformation has been published. Following 45 days, the plants were regenerated directly without a callus phase. The proposed protocol may provide a solution to the block for finger millet cultivars [63] .

Further research revealed that, an *Agrobacterium-mediated* transformation method is required to conduct molecular studies on model species a hybrid poplar. An efficient and reproducible callus-based transformation method is presented in this study. CIM1(Callus induction media 1) is the optimal callus induction medium that efficiently induces leaf calli development, whereas SIM1 is the optimized shoot induction medium that induces multiple shoots from calli. An OD600 of 0.6 and an acetosyringone (AS) concentration of 100 m were set as the optimum determinants of the transformation frequency of calli.

Infection time was set at 15 minutes, cocultivation time was 2 days, and preculltivation time was 6 days. It takes approximately 2 months for this method to

produce transgenic plants with over 50% transformation frequency. Histochemical staining for $\beta$-galactosidase (GUS) and PCR-reverse transcription-PCR analysis confirmed the success of the PCR and RT-PCR transformations. After six cycles, the high transformation efficiency of calli cultured from leaves was still retained in subcultured calli. Other poplar species can benefit from this method for developing effective transformation protocols [64] .

This study conducted that protocol for efficient chicory root regeneration in the shoot (*Cichorium intybus var. sativum*) was developed by evaluating different factors, including the concentration of controllers of plant growth in Skoog and Murashige (MS) media, the type of explants, and genotypes. It was found that 1-naphthaleneacetic acid (NAA),6-Benzylaminopurine (6-BAP) on leaf and cotyledon explants promoted callus formation most effectively, and 1-naphthaleneacetic. In MS medium with 6-BAP and IAA multiple adventitious shoots could be produced. Indole-3-butylric acid is effectively present in MS media induces rooting on elongated shoots. Chicory cultivars respond differently to shoot generation in terms of their regeneration abilities. Melci and Hera, a root and Witloof cultivar, regenerated the most shoots. The most effective regeneration technique successfully transformed Melci with the strain C58C1 RifR of *Agrobacterium tumefaciens* (pGV2260) (pTJK136). To determine whether the T-DNA had successfully integrated into the chicory genome, histochemical GUS assays, PCRs, and RT-PCRs, were carried out on putatively transformed plants. Well-developed roots also expressed neomycin phosphotransferase (NPTII) on medium containing 100 mg kanamycin. A simple, efficient, and reproducible protocol may be useful for increasing genetic variation and transferring important genes to root chicory cultivars [65] .

Further research examined that Genetic transformation and regeneration of sweet potatoes in genotype independent systems continue to be of great interest. With the help of an *Agrobacterium-mediated* genetic transformation procedure was created using two cultivars of sweet potato using *Agrobacterium* strain EHA105 that included binary plasmid pBI121 including the GUS and nptII genes Various media and hormone combinations were used with micropropogated plants from 30

days old as explants. Sterility was severe when cytokines were added to different growth regulator concentrations in MS and LS media. However, MS with 2,4D and TDZ showed a low differentiation rate and a good percentage of calluses. Both genotypes had low rooting percentages when exposed to different concentrations of NAA. It was a combination of Gamborgs B5 and NAA that produced the best shoot formation after eight days of growth. An average of 10 weeks for a regenerate and efficiency was 70%. PCR analysis confirmed stable integration of the transgene. Additionally, transcript accumulation was assessed in transgenic lines using PCR analysis and GUS enzymatic assays [66] .

## 2.3 Transformation through Tissue Specific Promoters

Another study showed that there are two methods of genetically modifying *Solanum tuberosum* (potato) plants. Over the course of 18 weeks, phytohormones allowed a whole converted plant to be regenerated from a single transformed cell and an infected leaf. Using a syringe, they injected A. *Rhizogenes* into the stems, and red fluorescent markers were used to identify newly produced hairy roots. It takes 5-6 weeks for a wild type shoot to hybridize with fully grown hairy roots. Both *Agrobacterium-mediated* transformation approaches were used to drive GUS reporter gene expression. By Using the GUS staining method, which is included in the kit, the promoter induction can be located within the cell. It was noted that GUS stained the endodermis and exodermis in both approaches, as well as the lateral roots in the A. rhizogenes transformed roots. Consequently, *A. rhizogenes* may provide a quick and easy way to observe the genes expressed in roots [67].

From further studies about transformation different scientists examined that the development of novel transgenic plants is possible using tissue-specific promoters for effective expression of transgenes at certain periods or in specific plant tissues. Tuber-specific expression was reported by the laccase promoter independent of developmental stage in transgenic potato, and no GUS reporter expression was

seen in leaves stems or roots. Laccase promoter regulatory components may be dispersed across the promoter according to a series of 5 deletions. In the leaves and stems of transgenic potato, the laccase promoter reacted poorly to salt stress, mannitol stress, and mechanical injury, but not to cold stress. Weak GUS expression induced by the laccase promoter was found throughout the transgenic tobacco plant, however in the transgenic tomato plant, GUS expression was only found in the roots and seeds. Laccase promoter was shown to be a viable option for promoting high and selective gene expression in potato tubers based on data [68] .

Another research showed that the beta-glucuronidase (GUS) reporter gene was powerfully induced under drought and salt stress. GUS activity was strongly induced by dehydration in transgenic rice with reverse OCPI1 promoter, suggesting OCPI1 promoter has bidirectional transcriptional activity. OCPI1 was overexpressed in a *japonica cv.* We tested drought resistance in transgenic plants carrying a one transgene copy during the reproductive stage. Positive transgenic plants (overexpressing OCPI1) outperformed wild type plants and the negative transgenic controls (no transgene overexpression) under severe drought stress conditions. Normal growth conditions, however, did not affect transgenic plants' potential yields. Positive transgenic plants' crude proteins showed more potent inhibitory effect than negative transgenic plants' crude proteins. Wild-type plants lost more total proteins when compared transgenic plants with drought stress. Based on these data, OCPI1 may improve rice drought resistance [69].

This study shows that a comparison was made between pRCA and pRD29A promoters in transgenic potatoes for expressing E. coli beta-glucuronidase (gusA) and 35S CaMV promoters. In order to pRCA-pCAMBIA and pRD29A-pCAMBIA promoter segments for developing plant expression constructs were extracted from their original sources and cloned upstream of gusA in pCAMBIA-1301. GV2260 harboring recombinant plasmids was inoculated on Lady Olympia cultivar leaf discs and internodal explants. At various stages of the transgenic plants development GUS activity was analyzed. The stress-inducible promoter (pRD29A) was tested under heat, drought, and both heat and drought stress. under heat,

drought and a combination of some stresses (RT-qPCR and GUS florimetric assays) pRD29A promoter gets more activated. GUS expression increased by pRD29A promoter more than 10 times compared to control promoter. There was a significant reduction in transcript levels of gusA gene in transgenic plants with pRCA promoters compared with those with 35S promoters. According to florimetric assays, GUS expression was also decreased or absent in tubers. Therefore, promoters can be used to potato lines that are resistant to biotic and abiotic stress to maximize the expression of foreign genes transgenic technology risks [70].

## 2.4 Transformation and Phytohormones

Further research examined that Potato genomes contain four promoters. Their plasmids all contained identical gusA gene fusions. All these promoters StUbi, StGBSS, StPat, and StLhca3 were evaluated and shown to be superior to the CaMV 35S constitutive promoter in greenhouse grown plants. Western blot analysis, histochemistry, and flourimetry were used to determine the final quantity of gene product. WN promoters had a greater amount of $\beta$-glucuronidase accumulation than viral CaMV 35S promoters. It was found that StLhca3 promoted activity in green tissue while StGBSS and StPat promoted activity in tubers however, a promoter leakage still occurred. Transgenic lines and tissues differed. According to flourimetric data, a leaf's promoters are ordered as follows: CaMV 35S >StPat >StGBSS >StLhca3 (from highest to lowest). According to the observed variations in expression, transgenic plants expressing the StPat-gusA fusion construct had the highest expression, with CaMV 35S >StLhca3 >StPat >StGBSS >StUbi >CaMV 35S the most effective [71]. From this research it was examined that the *Agrobacterium-mediated* transformation of potato is fast, repeatable, and stable. The *Agrobacterium tumefaciens* strain LBA4404 was introduced into the potato cultivars Lady Olympia, Granola, Agria, Desirée, and Innovator using the pBIN19 expression vector, which encodes the gusA gene under the control of the 35S CaMV promote Screening of primary transformants was performed with Kanamycin (100 mg/L), a plant selectable marker. In terms of efficiency, the internode performed

better than the explant with the explant performing well overall. Based on GUS histochemical tests Lady Olympia, Granola, Agria, Desirée, and Innovator had transformation efficiency scores of 22, 20, 18.6, 15 and 10%, respectively, using internodal explants, and 15, 12, 17, 8 and 6% using leaf disc explants. Regenerated plants also contained the gusA and nptII genes. The molecular analysis of subsequent generations shows that both genes are properly integrated and expressed. Best cultivar for future transformations, according to the results is *Lady Olympia*. Transgenic potato plants benefit from this approach because of its short duration, speed, and repeatability. pRD29A promoters were tested in transgenic potatoes to see how well they expressed E. coli beta-glucuronidase (gusA), compared to the constitutive promoter (pRCA). By cloning the promoter segments from their original sources into the pCAMBIA-1301 binary vector, the PRCA-pCAMBIA and pRD29A-pCAMBIA plant expression plasmids were created. *Lady Olympia* cultivar leaf discs and internodal explants were infected with a recombinant plasmid-carrying *Agrobacterium* strain GV2260. GUS activity was analyzed histochemically in various phases of transgenic plants. The heat, drought, and combination of the two induced stress-inducible promoter (pRD29A) activity was tested on transgenic plants. Real-time (RT-qPCR) and GUS florimetric analyses have shown that drought, heat, and the combination of both can activate the pRD29A promoter. Compared to the control GUS expression was increased 10-fold by the pRD29A promoter. Compared with 35S promoter controlled transgenic plants pRCA promoter controlled transgenic plants had lower levels of gusA transcripts. In tests using GUS florimetric assays, GUS expression in tubers was low. In conclusion, the findings suggest that potato promoters can be effectively used to drive biotic and abiotic stress resistance in potato lines, reducing the risks associated with transgenic technology [72].

Another research showed that bifunctional enzyme, pyrroline-5-carboxylate synthetase, produces proline in plants. Petunia plants have been transformed by *Agrobacterium* to confer water stress resistance (drought, salt, cold) through the p5cs gene. For transformation, the binary vector system used was *Agrobacterium* strain LBA4404 carrying plasmid pBI121. A reporter gene for $\beta$-glucuronidase, a

selectable marker for pyrroline-5-carboxylate synthetase, and a nucleomycin phosphotransferase gene were present in PBI121. In both stress and non-stress conditions, petunia shoot apex was the best explant for transformation, confirming the presence of Gus and p5CS genes. In addition, this is the first report showing that shoot apex transformation is more efficient (73-83%) than leaf disc transformation (0-15%). Plants of the transgenic cultivar 'Super Cascade' accumulated up to 13.2 times more proline under stress compared to their wild counterparts. Transformed plants were more tolerant of stress than wild types. Furthermore, this is the first study to demonstrate how genotype differences affect cold tolerance after transformation with *P. violacea* producing the highest proline content and subsequently the highest cold tolerance [73].

## 2.5 Transformation through Callus Formation

Another study showed that Plant transformation studies can be conducted with fluorescent proteins (FPs) for a variety of purposes, such as evaluating transformation parameters or isolating transgenic cells without selective agents. Several fluorescent proteins have been tested in this study for their applicability to olive transformation, including green (GFP) and red (DsRed). An *Agrobacterium tumefaciens* AGL1 strain carrying cfg, red, or glucuronidase-encoding binary plasmid pXK7FNF2 was used to transform olive embryogenic callus. A transformation rate of 2–8% was achieved for each construct after 3 months of selection in the presence of paromomycin. Epifluorescence and confocal laser scanning microscopy were used to examine the expression of FPs during olive plant regeneration. Olive somatic embryos (SE) transformed with pXK7SNF2 plasmid displayed a strong signal of GFP during proliferation, while SE transformed with pXK7FNF2 showed a weak signal of GFP. GFP could neither be detected in leaves nor roots of plants transformed with both vectors after embryo conversion. A confocal laser microscope and epi-fluorescence zoom microscope were used to visualize DsRed expression in regenerated leaves and roots, respectively. DsRed expression in SE transformed from a different olive embryogenic line was also detected using pXK7RNR2.

According to these results, FPs can be used to genetically transform olive embryogenic cells with DsRed being more effective than GFP [74].

This research showed that throughout the world and in Vietnam, sweet potatoes, or *Ipomoea batatas (L.)* are important food crops. Genotype-dependent in vitro responses make it a recalcitrant crop for gene transformation and tissue culture. This study investigated the transformation of cry8Db from *Bacillus thuringiensis* into KB1 sweet potato variety via *Agrobacterium*. In a plant transformation experiment, the C58cv strain carried a pBI121 backbone containing the cry8Db delta-endotoxin gene that is regulated by the 35S CaMV promoter, as well as the neomycin phosphotransferase gene (nptII) that is used to select new plants. Plants were inoculated and cocultured with bacteria from shoot tips and leaves explants, along with *A. tumefaciens*. During callus production and plant regeneration selection occurred. The production of 201 transgenic putative plant lines resulted in 21 plants being confirmed as transgenic by PCR and Southern blot. The cry8Db gene was confirmed in four putative transgenic lines transferred into greenhouse soil pots. According to the biological activity evaluation conducted under controlled conditions, untransformed plants had higher levels of infestation by sweet potato weevils (*Cylas formicarius*) than transgenic plants [75].

Further research showed that *Agrobacterium tumefaciens-mediated* transformation was used to transform several varieties are prestigious sweet potato cultivars. Embryogenic suspension cultures were established in LCP media using induction medium containing 2 mg/l 2,4-D. The hpt gene was selected as a selectable marker along with an intron-interrupted uidA gene as a visible marker. Co-cultivated the suspension cultures with *Agrobacterium tumefaciens* strain LBA4404 carrying pCAMBIA1301, carrying the hpt gene. Antibiotics suppress the growth of *Agrobacterium*, and hygromycin can be used to select the best dose for transformation. Using cefotaxime and hygromycin at 10 mg/l, 485 putative transgenic plant lines were produced. Based on PCR, GUS, and Southern blotting, 92% of the regenerated plants were transgenic. There are usually one to three insertions of T-DNA in transgenic plants. A total of 308 transgenic plants survived in the greenhouse and in the field. The majority of them produced storage roots after

three months of cultivation in greenhouses or fields. A robust transgenesis method that can be applied to a wide range of sweet potato genotypes allows us to verify the function of sweet potato endogenous genes [76]. This study showed that to study molecular aspects in order to effectively transform hybrid poplar 84K an *Agrobacterium-mediated* transformation technique is needed. The study describes a reproducible and efficient method for callus-based transformation. A high efficiency callus induction medium (CIM1) was used to induce calli from leaves, and a shoot induction medium (SIM1) was used to induce multiple shoots from calli. *Agrobacterium* concentration was optimized at 0.6 OD600, *Agrobacterium* infective suspension was optimized at 100 M acetosyringone, infection time was 15 minutes, cocultivation was 2 days, and precultivation was 6 days to optimize transformation frequency. With a transformation frequency greater than 50%, transgenic plants can be obtained in approximately 2 months. Reverse transcription-PCR and polymerase chain reaction (PCR) analyses the production of stable transformants was successfully verified by $\beta$-glucuronidase (GUS) staining. The high transformation efficiency was also evident after six cycles of subculture using calli from leaves as explants. By using this method as a reference, other poplar species can also be transformed [77].

## 2.6 Effect of Abiotic Stresses on Plants

Another study showed that Abiotic stresses negatively affect the growth of sugarcane. Several genes that respond to abiotic stress have been genetically engineered into sugarcane to confer tolerance to abiotic stress. Under drought and salt stress, we examined the regulation of OsC3H52 by transforming the CP77-400 sugarcane variety with the promoter of the OsC3H52 gene. A CIM was used to induce calli. *Agrobacterium* and biolistic transformation were used to transform the zinc finger protein gene promoter region of OsC3H52 into sugarcane calli using pBI221 and pGreenII0129. CIM3 (5 mg/ L-1 2, 4-D + 10% coconut water) showed the highest callus induction rate (93%) according to a study using various induction media. By using 8.5g/l recombinant pGreenII0129 in biolistic transformation, efficiency

was increased by 28%. The efficiency of agrobacterium-mediated transformation was 13% when using recombinant pBI221 plasmid. The efficiency of 100 M acetosyringone in *Agrobacterium-mediated* transformation was higher (13%). After a 15-day selection with 25mg/l Hygromycin and 300 mg/l cefotaxime, the calli were transformed by biolistic and *Agrobacterium-mediated* mechanisms. Successful transformation of the calli was confirmed by PCR and GUS expression. A further evaluation of the OsC3H52 promoter was conducted under drought and salt stress. The GUS reporter gene was highly expressed under drought and salt stress conditions [78].

## 2.7 Transformation of Lectin

From this study it was revealed that Plant lectins have insecticidal activity. The lectin gene has been isolated and introduced into *Brassica juncea cv.* Varuna uses *Agrobacterium-mediated* genetic transformation to combat aphid resistance. Direct cloning of the PCR amplified lectin gene into pCAMBIA1300 binary vector was achieved using restriction sites KpnI and XbaI. *Agrobacterium tumefaciens* (GV3101) allowed tissue-specific expression of lectin genes in *Brassica juncea* using LL + rolC construct in pCAMBIA1300. Cotyledon explants showed an average transformation efficiency of 30-40% for the LL and CPPI genes. PCR and RT-PCR were used to confirm that the transgenes were present and expressed in the transgenic *B. juncea* plants [79]. Further research shows that Using Orysata, a rice lectin with mannose specificity belonging to the jacalin-related lectin family, investigated the insecticidal activity of the lectin. The beet armyworm, green peach aphid, and pea aphid are three significant pest insects in agriculture thus, research was done to see how successful Orysata was against them. On transgenic tobacco lines expressing Orysata, bioassays were conducted using beet armyworms, green peach aphids, and pea aphids. Based on the expression level of 0.6-1.1%, the expression level ranged between 38 and 71 g/g FW. A significant reduction in larval weight gain and mortality was observed in the larval stages of beet armyworm. Aphid mortality was greatly reduced when they fed on leaves expressing

Orysata. Pea aphid mortality was high at relatively low lectin concentrations when fed a man-made diet supplemented with Orysata. Additionally, the results indicated that Orysata, a jacalin-related lectin exhibited strong insecticidal activity, suggesting that it could be used as a valuable impact agent against both biting-chewing pest insects as well as piercing-sucking pest insects [80].

Another study shows that it remains a challenge even today, to impose resistance in plants through genetic manipulation and breeding. The purpose of this study was to isolate two major defense genes of plant origin, lectins from lentils and protease inhibitors from chickpeas which are insecticidal against phytophagous pests. Using extension PCR, two genes are stacked into one ORF and a fusion gene construct is prepared. The fusion gene was expressed using a phloem specific promoter rolC, so the construct was transfected into *Brassica juncea cv.* Using hygromycin selection, the transformed putative plants were tested for regeneration. PCR and Southern hybridization show 17% overall transformation efficiency for transformed plants after fusion gene integration. Transgenic plants showed reduced leaf damage as compared to non-transformed control plants during the aphid resistance test [81].

From this research it was examined that the effect of lectin on wheat aphids. A gene encoding mannose binding lectin from *Pinellia pedatisecta* was cloned during this study. In nucleotide composition, it was similar to *Pinellia ternata agglutinin* (pta) by 92.69% and in aminoalkanoic acid composition by 94%. By particle bombardment transformation, the ppa gene driven by the ribulose bisphosphate carboxylase small subunit gene (rbcs) promoter, was inserted into wheat cultivar Baofeng104.

A total of 54 transgenic plants were generated. PCR and RT-PCR analysis confirmed ppa gene inheritance expression, and 7 homozygous transgenic lines were obtained. Compared with BF104. In seven ppa transgenic wheat lines, aphid growth rates were lower, and inhibition rates were better. The number of aphids per tiller of transgenic lines was significantly lower than that of wild type BF104 after two years of aphid bioassays in separate fields. As a result, ppa might be a promising biotechnological candidate for providing aphid-resistant wheat [82].

By Using an in vitro assay plant derived lectins were evaluated for their anti-PVY (potato virus Y) activity. Crocus vernus corm tetramer lectins were isolated, precipitated, dialyzed, filtered on the PD 10 column, and chromatographed on SP Sepharose gels to isolate subfractions. Based on in-silico studies, it was estimated that Crocus vernus lectin (CVL) and selected PVY proteins interact stable IC50 values for CVL were determined by cytotoxicity tests on HepG2 cells. There was no cytotoxic effect of the CVL extract based on the IC50 values. CVL hemagglutinated rabbit erythrocytes at 100 g/ml based on its hemagglutination activity. Through a quantitative real-time PCR assay, CVL lectin anti-PVY activity was evaluated. According to the study, CVL was effective against the PVY targeted gene at 30 g/ml, 60 g/ml, and 90 g/ml concentrations and up to 100% inhibition of PVY mRNA was achieved at all three concentrations. The CVL gene appears to be an efficient tool for controlling the potato virus Y in the future [83].

# Chapter 3

# Research Methodology

## 3.1 Optimization of an *Agrobacterium tumefacien* Mediated Gene Transformation through GUS

### 3.1.1 Explant Source

Explants of potato cultivar "$GN_2$" that were free of disease were used as explant source. Cultivars of $GN_2$ were collected from tissue culture lab of NIGAB (National Institute for Genomics and Advanced Biotechnology) potato research program.

### 3.1.2 Surface Sterilization

Washing and rinsing potato tubers with detergent and distilled water were done for 2 to 3 minutes. After that, the tubers were dried well and placed in a dark environment for two to three weeks until sprouting began. Healthy and disease-free tubers of the $GN_2$ were treated with a fungicide (0.05% bavisting) and then dried for 10 -15 minutes. The tubers were treated with 50mg/L $GA_3$ for 30 minutes to break the dormancy. The Sproutings were cut from potato tubers and placed on

moist sand. For in vitro culture initiation, sprouted tubers were cut into parts and their epical meristems were used. A mixture of 50%, 60%, and 70% concentrations of Colorox was applied for 10 minutes to potato explants after that washing three times with autoclaved distilled water.

### 3.1.3 Multiplication

In order to optimize the most suitable media for multiplying $GN_2$, MS medium with sucrose and different concentrations of $GA_3$ with pH 5.8 was used for establishing potato cultures. Sterilized sprouts were cultured on the multiplication media. A full-length plantlet with 6 to 8 nodes was formed after 3 to 4 weeks from buds. Plantlets of potato sprouts are fully grown after 2 to 3 weeks, which are then used in experiments. Composition of multiplication media is given in Appendix (3.1)

### 3.1.4 Regeneration

By cutting flourishing explants into small pieces and transferring them to regeneration media allowed the cells to thrive. Different concentrations of BAP (1mg/L, 2mg/L, 3mg/L) and $GA_3$ (0.1mg/L, 0.2mg/L, 0.3mg/L) used in regeneration media. The next step was to test different combinations of BAP and $GA_3$ concentrations. A final optimum concentration was determined using the following formula, regeneration frequency was calculated and the most responsive treatment was selected. Composition of regeneration media is given in Appendix (4.2).

### 3.1.5 Preparation of Bacterial Culture

A developed *Agrobacterium tumefaciens* strain GV3101 harboring PCAMBIA 1301 plant expression vector was used to transform $GN_2$. The vector contains a kanamycin resistance gene and a GUS reporter gene for analyzing gene function in a simple sensitive manner. Stocks of glycerol were strick down onto Luria– Bertani

(LB) agar plates containing 50mg/L of kanamycin and 25mg/L of rifampicin. After 48 hours of incubation, the plates were removed from the oven. It was stored at 4C when colonies of Agrobacterium were observed. From pure culture plates, a single rounded colony was selected and cultured in autoclaved falcon tube having LB with kanamycin (20µl) and Rifampicin (40µl). After that, it was shaken for 24 to 48 hours.

### 3.1.6 Infection

Explants with the desired GUS gene were infected. For this purpose, bacterial cultures with optimized density (0.5-0.6) were centrifuged for 10 to 15 minutes at 5000 rpm. After discarding the supernatant, the pellet was resuspended in MS media. In a laminar air flow cabinet, the explant was infected for 5-7 minutes with this inoculum.

### 3.1.7 Co-Cultivation

During the co-cultivation period, *Agrobacterium mediated* transformation occurs because the T-DNA is transferred into the genome of the host plant. A cocultivation media consisting of MS salt, vitamins, 2-4D, proline and gellen gum. In order to optimize the co-cultivation media different time durations (24,48,72 hours) and concentrations of proline (0.8g/L ,0.6g/L,0.11g/L) were observed. The pH of media was established at 5.8. Composition of co-cultivation media is given in Appendix (5.3).

### 3.1.8 GUS Histochemical Assay

GUS histochemical assays was conducted at various stages of transformation to assess gusA gene functionality. A blue precipitate was visualized at the site of enzyme activity detected in the histochemical assay when X-Gluc was hydrolyzed by

GUS. It was determined that 0.6 mg/l X-Gluc, 10mM EDTA, 100mM NaPO4(PH-7), 0.1% Triton X-100, and 20% methanol would be required to make the GUS buffer which has a pH of 8 due to the presence of sodium phosphate. The plantlets were incubated in this buffer overnight at 37°C and de-stain the samples with 70% ethanol. Explants infected with GUS were counted out of total explants infected to determine transformation efficiency. The following protocol was followed to prepare 50ml of fresh GUS staining solution.

### 3.1.9 GUS Staining Buffer

Table 3.1: GUS staining buffer components

| **Components** | **Stock Concentration** | **Working Concentration** | **For 1ml** | **For 50ml** |
|---|---|---|---|---|
| $NaPO_4(PH)_7$ | IM | 100Mm | 100 µl | 5ml |
| $K_3Fe(CN)_6$ | 0.05M | 0.5mM | 10 µl | 0.5ml |
| $K_4Fe(CN)_6.2H_2O$ | 0.05M | 0.5mM | 10 µl | 0.5ml |
| EDTA .2Na (PH 8) | 0.5M | 10Mm | 20 µl | 1ml |
| Triton X-100 (V/V) | 10% | 0.1% | 10 µl | 0.5ml |
| $d2H_2O$ | | | 638 µl | 31.9ml |

### 3.1.10 Add to GUS Staining Buffer

Table 3.2: The addition to GUS staining buffer

| **X-Gluc** | 50mg /ml | 0.6mg /ml | 12 µl | 0.6 ml |
|---|---|---|---|---|
| **Methanol** | 100% | 20% | 200 µl | 10 ml |
| | | | 1 ml | 50 ml |

### 3.1.11 Stock Solution Preparation

1 X-Gluc is dissolved in 1ml of Dimethylformamide (DMF) to make a stock of 50 mg/L. X-Gluc solution should always be prepared fresh. For X-Gluc preparation, we can also use methanol. The following steps were involved:-

1. Make 1M $Na_2HPO_4$. $2H_2O$ by dissolving 4.45 g in 25 ml of distilled water.
2. Prepare 1M $Na_2HPO_4$ by dissolving 3.45 grams of NaH2PO4 in 25 ml of distilled water .
3. By adding 13.68 ml $Na_2HPO_4$ (1M) and 6.32 ml $NaH_2PO_4$ (1M) to 0.1M sodium phosphate buffer $NaPO_4$ (pH 7) at 25°C, prepare 0.1M sodium phosphate buffer $NaPO_4$(pH 7).
4. $K_3Fe(CN)_6$ (potassium ferric hexacyanoferrate) is prepared by dissolving 0.16463g in 10 ml of distilled water to obtain 0.05M $K_3Fe(CN)_6$ . To store for a long period of time, store at -20C. If it darkens, discard it.
5. Make 0.05M $K_4Fe(CN)_6.2H_2O$ (potassium ferrocyanide) by dissolving 0.2119 g in 10 ml of distilled water.
6. To make 0.5M EDTA.2Na, dissolve 2.8 g of EDTA.2Na in 15 ml of distilled water. And to make a 10% stock solution, dissolve 2.5 ml of Triton X-100 in 22.5 ml of distilled water.

## 3.2 Optimization of an *Agrobacterium Tumefacien* Mediated Lectin Gene Transformation Protocol

### 3.2.1 Explant Source

This experiment was conducted using healthy and disease-free explants of potato (*Solanum tuberosum*) cultivar $GN_2$. Cultivars of $GN_2$ were collected from tissue culture lab of NIGAB potato research program.

### 3.2.2 Surface Sterilization

potato tubers were washed and rinsed with distilled water and detergent for 2 to 3 minutes. After that, the tubers were dried well and placed in a dark environment for two to three weeks until sprouting began. Healthy and disease-free tubers of the $GN_2$ were treated with a fungicide (0.05% bavisting) and then dried for 10 -15 minutes. The tubers were treated with 50mg/L $GA_3$ for 30 minutes to break the dormancy. The Sproutings were cut from potato tubers and placed on moist sand.

### 3.2.3 Multiplication

Multiplication media as optimized in GUS was used for lectin gene transformation. Appendix (5.1)

### 3.2.4 Preparation of Bacterial Culture

In order to transform $GN_2$, a developed *Agrobacterium tumefaciens* strain GV3101 harboring PCAMBIA 1301 plant expression vector was used. For bacterial selection, the vector contains a kanamycin resistance gene and a lectin gene. Firstly, glycerol stocks were used to revive the bacterial culture. In order to achieve this

goal, 1µl of culture was spread on solid LB plates containing the selectable marker Phosphinothricin (PPT). After 48 hours, the plate was incubated at 28°C. Colonies of Agrobacterium were stored at 4°C when they were observed. In an autoclaved falcon, a single rounded colony was picked from pure culture plates and cultured with 20ml liquid LB containing Carbanciline (20µl) and Rifampicin (40µl). After that, it was shaken for 24 to 48 hours.

### 3.2.5 Infection

A lectin gene was introduced into the internodal parts of the explants. For this purpose, the optimized density (0.2-0.3) of bacterial culture was centrifuged at 5000 rpm for 10 to 15 minutes. A pellet was resuspended in MS media after the supernatant was discarded. In a laminar air flow cabinet, this inoculum was used to infect the explant for 10 to 15 minutes.

### 3.2.6 Co-Cultivation

Previously optimized protocol of GUS was used for lectin gene transformation. Appendix (5.3)

### 3.2.7 Lethal Dose

For the conversion experiment, various concentrations of PPT (phosphinothricin) (250,350,500µL/L) were tested on culturing explants. As a lethal dose, the concentration at which only transformed explants survived was used.

### 3.2.8 Selection

The selection media contain PPT, MS salt, BAP, $GA_3$ and vitamins. pH of the media was adjusted to 5.8. After that washing of co-cultivated explants was done

3-4 times for 5 minutes with liquid MS containing 500mg/L cefotaxime. After drying on sterile blotting paper, the explants were shifted to selection media. the callus forms within 4-5 weeks. The transformed plants were then placed in regeneration media. Composition of selection media is given in Appendix (5.4).

### 3.2.9 Regeneration

The previous media used in GUS also used in lectin gene transformation. Appendix (5.2)

### 3.2.10 DNA Extraction through CTAB Method

CTAB was used to isolate DNA from transformed plants. Chemicals and apparatus used in this process were prepared and autoclaved before extraction began. A solution of 0.5M EDTA, 5M NACl and 1M Tris HCl was prepared for the preparation of 2X CTAB. Room temperature was used to store the stocks. Preparation of 2% CTAB was followed by preparation of the stocks.

For DNA extraction, 0.5g leaf samples were grinded in liquid nitrogen in pestle mortar until they were fine powders. Powder was added to 700µL of preheated CTAB in an Eppendorf tube. The reaction was catalyzed by preheated CTAB. A period of 30 minutes was then spent incubating the sample at 65C. Then 700µL of chloroform: isoamyl alcohol was added 24:1 followed by 700µl of chloroform: isoamyl alcohol. Three layers were formed after centrifuging the solution at 10000 rpm for 15 minutes. A solution of 0.5M EDTA, 5M NACl and 1M Tris HCl was prepared for the preparation of 2X CTAB. Room temperature was used to store the stocks. Preparation of 2% CTAB was followed by preparation of the stocks. Another Eppendorf tube was filled with chilled isopropanol (700µL) and only the uppermost layer (700µL) was removed. To obtain pellets, it was centrifuged once again at the same speed and time. A 70% ethanol solution was used to wash and dry this pellet. Finally, the DNA was dissolved in 50µl of ddH2O and stored at 20°C [84].

### 3.2.11 PCR Analysis

Primer3 software will be used to design internal gene primers for lectin gene. 20µl of the reaction were carried out with Taq polymerase from Fermentas.

Table 3.3: Primers used for amplification of lectin

| **OLIGO** | **Length** |
|---|---|
| ATGGGTCCTACTACTTCATCTCCT | 24bp |
| TCAAGCAGCACCGGTGCCAACCTT | 24bp |

The PCR profile set as:

1. DNA denaturation at 94°C for 3 minutes
2. Amplification of product size 546bp took 35 cycles and was made as:
3. DNA is denatured at 94°C for 30 seconds, primers are annealed at 52°C for 30 seconds, then extended at 72C for 20 minutes.

### 3.2.12 Preparation of Agarose gel

For the preparation of gel firstly need of horizontal electrophoresis apparatus. A DC power source, pipte, agarose powder and sample for the experiment. Dilute the concentrated buffer with distilled water, mix the agarose powder with diluted buffer solution in flask. Dissolve the agarose powder by boiling the solution for one minute then remove and mix it well. Cool the gel at 60°C when gel cool seal the ends of gel casting tray with rubber and caps. Place the well template into appropriate notch.

### 3.2.13 Gel Electrophoresis

After PCR product extraction, transgenic plants were conformed on a 1% agarose gel with a 1kb ladder.

# Chapter 4

# Results and Discussions

## 4.1 Optimization of an *Agrobacterium tumefacien mediated* Gene Transformation through GUS

### 4.1.1 Explant Source

Explants of potato cultivar "$GN_2$" that were free of disease were used as explant source. The cultivar of this variety ($GN_2$) was collected from Tissue Culture Lab of National Institute of Genomics and Advanced Biotechnology (NIGAB) Islamabad. Shown in (Fig4.1)

FIGURE 4.1: Explants source

### 4.1.2 Surface Sterilization

Before the surface sterilization pretreatment of potato explants with combination of fungicide and antibiotic agents was effective. potato explants sprouted within 10 days. After 21 days culture showed full elongation of the nodes, reaching a length of 3-5 cm. Plant growth hormones supplemented nutrient medium significantly affected the efficiency of in vitro propagation. Chlorox concentration results during surface sterilization are shown in figure (4.2). Along X-axis graph contains three different treatments of Chlorox at different concentrations and along y-axis graph contains the survival percentage of explants. T1 having 70% Chlorox concentration had the lowest contamination rate. The surface sterilization treatment of T2 60% (Chlorox) was better than the surface sterilization treatment of T3 50% (Chlorox). The highest survival rate was also reported in T1 treatment, followed by T2 and T3. Figure (4.3) showing the best effect of Chlorox on potato ($GN_2$) at 70% Chlorox concentration.

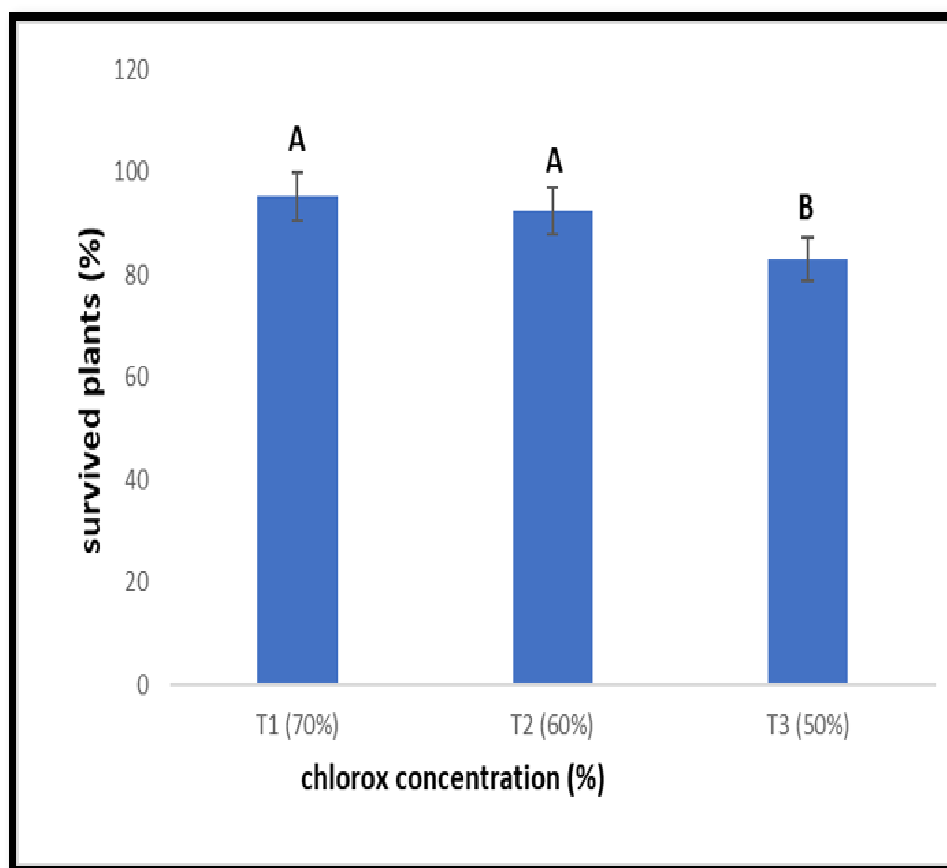


FIGURE 4.2: Effect of different Chlorox concentrations on Explant

TABLE 4.1: ANOVA table for the Effect of Chlorox on invitro micropropagation of potato (GN2)

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Replication | 2 | 10.706 | 5.353 | | |
| Treatment | 2 | 250.802 | 125.401 | 9.45 | 0.0305 |
| Error | 4 | 53.059 | 13.265 | | |
| Total | 8 | 314.567 | | | |

MS = Mean square, F=Significance probability, p=probability value, Grand Mean =90.140, CV (Coefficient of variation) = 4.04 LSD= 8.2564

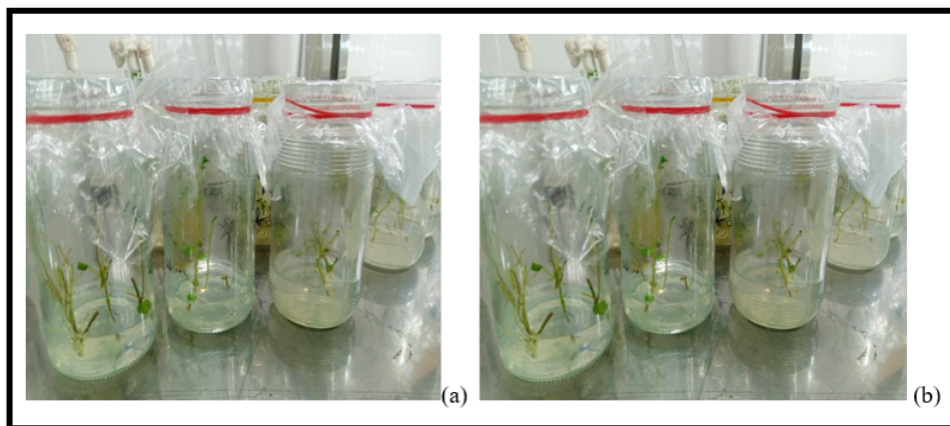

FIGURE 4.3: (a) & (b) showing best growth at 70% Chlorox concentration

### 4.1.3 Multiplication

Gibberellic acid ($GA_3$) plays an important role in plant growth and development. Additionally, it stimulates the development of xylem, hypocotyl, and internode, as well as germination of seeds. $GA_3$ is necessary for stem elongation. To determine the optimal concentration of $GA_3$ different concentrations of the substance were tested for growth of potato explants ($GN_2$). Based on statistical analysis, GA3

concentration significantly influenced the frequency of explant multiplication. In the graph along x-axis contains three different treatments of $GA_3$ hormone at many concentrations(0.4mg/L,0.5mg/L,0.6mg/L) and along y-axis showing survival percentage of explants. $GA_3$ concentrations of 0.5mg/L had the highest growth rate, with a mean value of 41.667, followed by 0.4mg/Land 0.6mg/L with increasing $GA_3$ GA3 concentration, the survival rate of explants decreases. Figure 4.4 shows the best growth of plants at 0.5mg/L concentration of $GA_3$ hormone. Previous researchers observed that during the shoot initiation stage in tea it was demonstrated that 0.5 mg/L $GA_3$ resulted in the greatest number of shoots and the greatest shoot elongation rate. A 2mg/L $GA_3$ supplemented MS medium significantly promoted shoot elongation in *Cerasus campanulate* [85] . Current results showed that 0.5mg/L GA3 concentration is best for shoot elongation rate. From Previous research it was observed that sweet potato shoots could grow significantly taller when combined with 10 mg/L $GA_3$. From this study it was indicated that adding 1.0 mg/L $GA_3$ to multiplication media increased shoot elongation by 33.5% in Jack Purple [86] . According to previous research it was observed that the effect of $GA_3$ on shoot growth, growing shoots in a medium supplemented with GA3 for about 4 weeks. The concentrations of $GA_3$ (Serva) were 0, 1, 2, and 4 mg/L. Each $GA_3$ concentration was repeated in triplicate. The best growth was observed at 2 mg/L after 3 weeks of culture [87]. The present data is not in accordance with supported data.

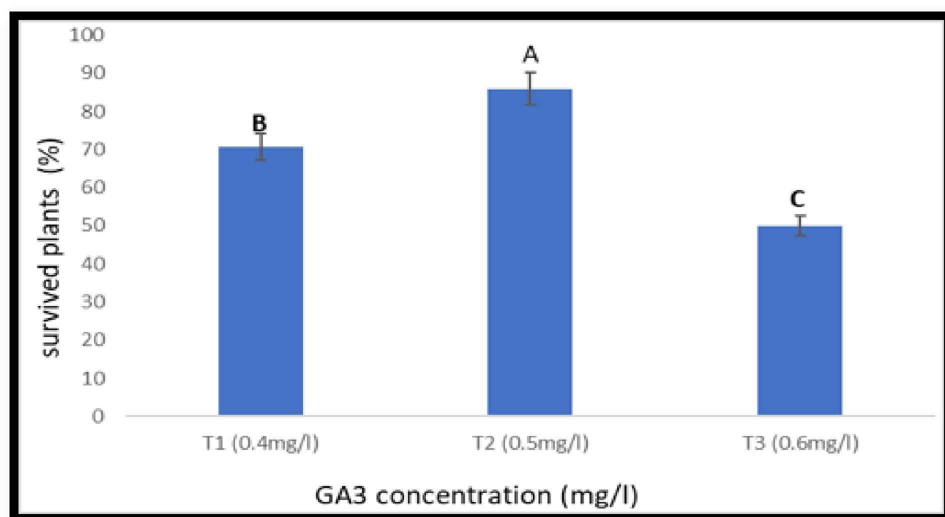


FIGURE 4.4: Effect of $GA_3$ on the growth of Explants

TABLE 4.2: ANOVA Effect of $GA_3$ concentration on multiplication of ex plants ($GN_2$).

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Replication | 2 | 384.22 | 192.111 | | |
| Treatment | 2 | 1986.89 | 993.444 | 82.03 | 0.0006 |
| Error | 4 | 48.44 | 12.111 | | |
| Total | 8 | 2419.56 | | | |

MS = Mean square, F=Significance probability, p=probability value, Grand Mean= 68.778, CV =5.06, LSD= 7. 8892

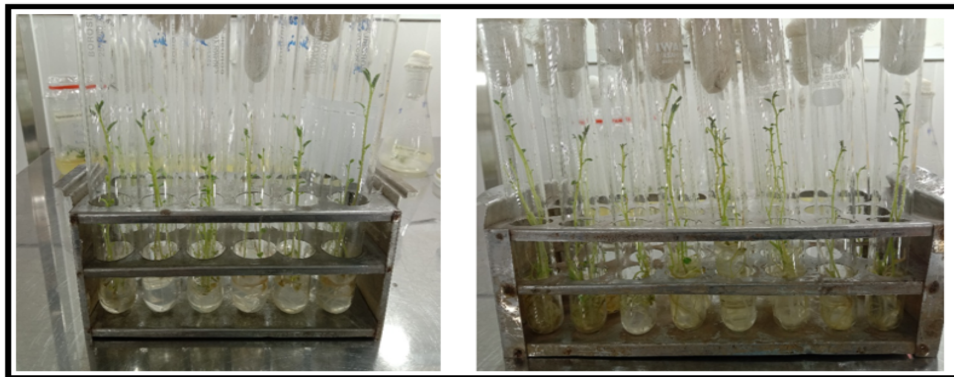

FIGURE 4.5: $GA_3$ Concentration on multiplication of explants

### 4.1.4 Regeneration

#### 4.1.4.1 Different $GA_3$ Concentrations During Regeneration

To optimize the regeneration media for the transformation of potato (GN2) different concentrations of GA3 (0.1, 0.2, and 0.3mg/L) were tested. Statistical results shown in graph along x- axis showing the three different treatments of $GA_3$ at regeneration media and along y-axis showing the survival percentage. According to

the statistical analysis, the difference between treatments is highly significant. A concentration of 0.2mg/L, with a mean value of 59.778, was found to be the most effective in generating maximum transformations shown in fig (4.6) When $GA_3$ concentrations were increased from 0.1 to 0.2 and 0.3mg/L, transformation efficiency decreased. Best growth results at 0.2mg/L shown in fig (4.7) The principal challenge in potato tissue culture is to optimize its regeneration protocol considering the fact that a new special optimized condition for regeneration. Previous literature suggested that wheat tissue culture is highly dependent on genotype Hence, it is essential to understand and optimize the factors influencing potato regeneration. In this study potato regeneration media $GA_3$ was found to be equally effective. $GA_3$ was used at different concentrations (0.1, 0.2, and 0.3mg/L) and growth parameters were observed. Shoot induction was highest with MS medium containing $GA_3$ at 0.1 mg/ L with increased internodal distances. Adding $GA_3$ to treated germplasm significantly increased plant height and root length, but it inhibited growth of nodes and roots. 0.1 mg of $GA_3$ per liter significantly increased the number of leaves on plants [88] . The results obtained are in accordance with previous studies which showed highest regeneration frequency in the presence of $GA_3$. However, the concentration used in this study was 0.5 mg/l instead of 0.1 mg/l [89] . Another study contradicted the above finding in which the best regeneration of wheat cultivar (Zhoumai 18) can be obtained by adding $GA_3$ at the concentration of 4 mg/L in MS medium. After 82.65% regeneration efficacy was reported [90] . These results do not support the idea of already reported findings.

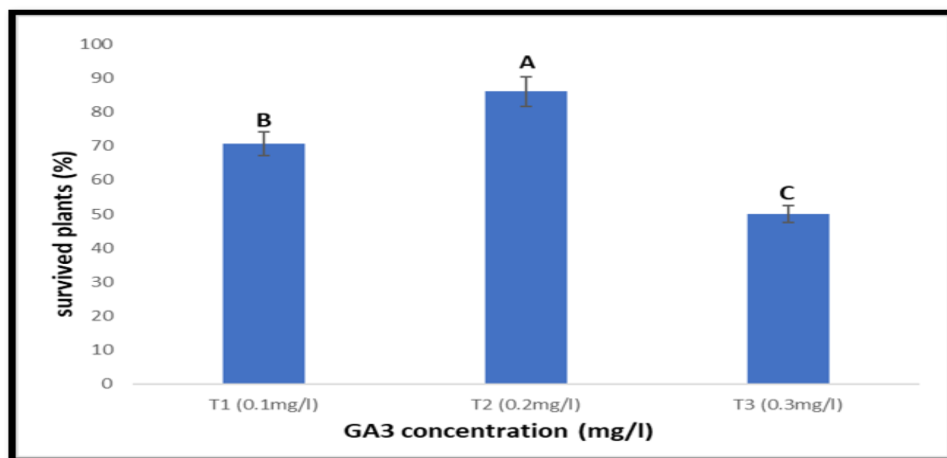


Figure 4.6: Effect of $GA_3$ hormone on Explants at Regeneration media.

TABLE 4.3: ANOVA Effect of different concentrations of $GA_3$ on regeneration of potato ($GN_2$)

| **Source** | **DF** | **SS** | **MS** | **F** | **P** |
|---|---|---|---|---|---|
| Replication | 2 | 314 .22 | 179 .222 | | |
| Treatment | 2 | 1786.82 | 857. 99 | 79. 01 | 0. 0004 |
| Error | 4 | 39 .11 | 9.121 | | |
| Total | 8 | 2140 .15 | | | |

MS = Mean square, F=Significance probability, p=probability value, Grand Mean= 59.778, CV= 4.66, LSD=6.8191

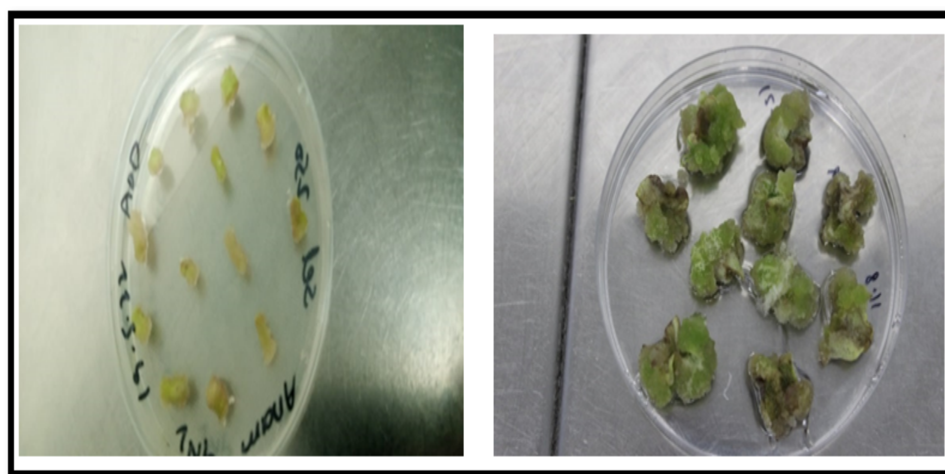

FIGURE 4.7: Different concentrations of $GA_3$ on regeneration

#### 4.1.4.2 Different Concentrations of BAP (mg/L) with Optimized $GA_3$ Concentration on Regeneration

In addition, we studied the effect of different concentrations of BAP (1 mg/L, 2 mg/L, and 3 mg/L) along with the optimal concentration of $GA_3$, which is 1 mg/L. The BAP concentration along with $GA_3$ on x- axis and survival percentage of plants on y-axis are shown via graph. Best results were observed at 1mg/L with a mean value of 37.972, followed by 2 and 3mg/L. At 2 and 3 mg/L, the mean values

were 30.000 and 24.000, respectively. There was a significant difference between the treatments. Shown in fig (4.8) different scientists studied the effect of $GA_3$ along with BAP. Shoot elongation was observed when BAP was added to the media despite the simple effect of $GA_3$ not showing any difference. Regeneration requires BAP since medium without BAP failed to generate multiplication other than single shoot growth. When BAP concentration was increased a maximum number of shoots were produced. However, when BAP concentration was increased further (5 to 7 mg/L), fewer shoots were produced, necrosis occurred, and fasciation occurred [91] . Current results with comparison of previous results showing that BAP along with $GA_3$ shows good results in plants shoot elongation when both hormones added in the regeneration media figure(4.9). This study shows that the best regeneration obtained at 10 mg/L BAP along-with 2 mg/L $GA_3$ in the medium [92] . These results do not match with the present study. The difference is due to different explants sources as well as different genotypes. In this study different scientists used twelve different types of media for optimizing in-vitro regeneration in four potato varieties and found the highest regeneration on MS medium containing 12 mg/L BAP and 0.5 mg/L $GA_3$ [93] . These obtained results are not in-line with the findings of this research study. The underlying reason of difference in achieved results is potentially due to difference in genotypes.

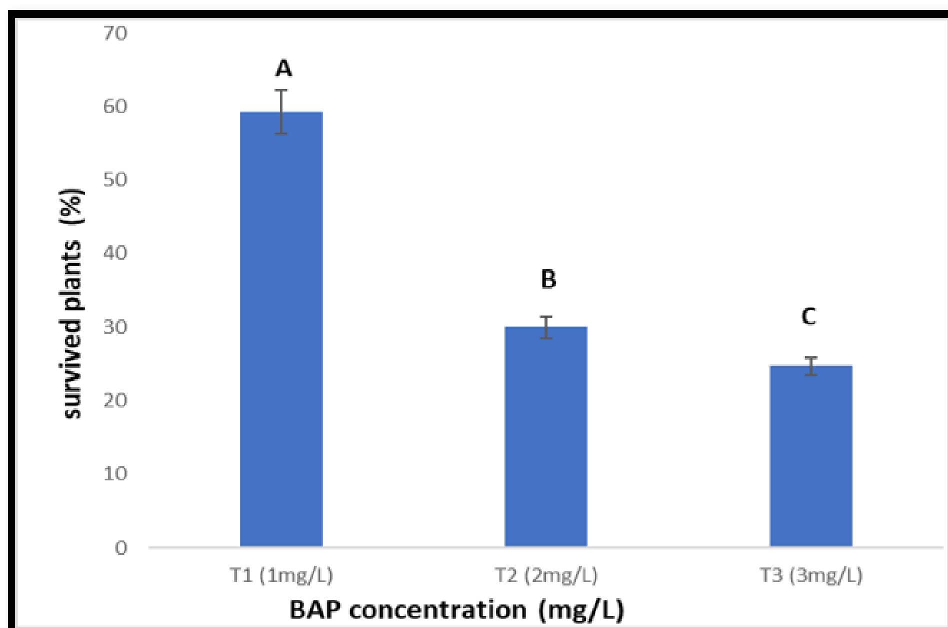


FIGURE 4.8: Effect of BAP along with $GA_3$ on Explants on regenerative media

TABLE 4.4: ANOVA Effect of different concentrations of BAP (mg/l) with optimized $GA_3$ concentration on regeneration

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Replication | 2 | 60.18 | 30.09 | | |
| Treatment | 2 | 2080.01 | 1040.01 | 124.70 | 0.0002 |
| Error | 4 | 33.36 | 8.34 | | |
| Total | 8 | 2173.56 | | | |

MS = Mean square, F=Significance probability, p=probability value, Grand Mean =37.972, CV= 7.61, LSD= 6.5469

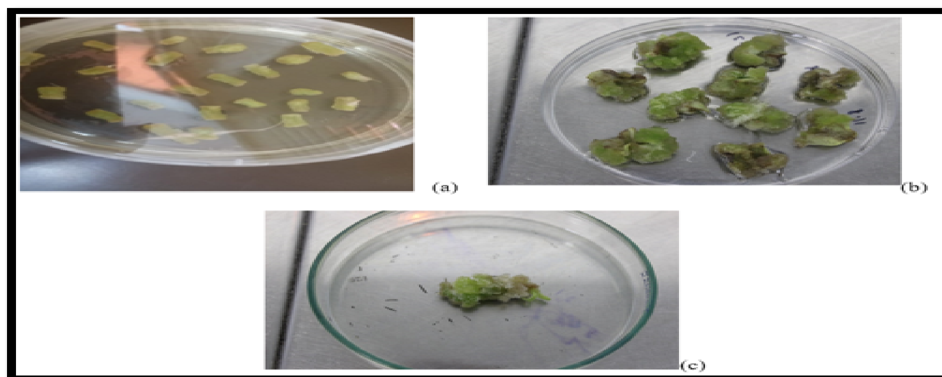


FIGURE 4.9: (a) showing the survival (%) of BAP at 1mg/l (b) showing the survival (%) at 2mg/l and (c) showing the survival (%) at 3mg/l at regeneration media.

### 4.1.5 Infection

Further optimization of bacterial density was achieved using multiplied plants. On ($GN_2$) cultivar of potato three different optical densities were tested. The results of the statistical analysis shown in fig (4.10) indicated that the treatments as well as their interaction were highly significant. With a mean value of 62.333, 0.6 was

recorded as the maximum transformation efficiency, followed by 0.5 and 0.55 respectively. Steps of infection are shown in fig (4.11) Optical density of inoculum greatly affects the transformation efficiency. So, it is necessary to optimize the optical density of inoculum for successful transformation. The bacterial culture of different optical densities (O. Ds) 0.0, 0.25, 0.5, 0.75 and 1.0 at 600nm were utilized to investigate their impact on the competence of different wheat cultivars. In our experiments, the highest transformation efficiency (12.5%) was observed when O.D.600nm = 0.5 [94] . The findings contrasted sharply with those of our study. Highest modification efficiency while using strain EHA105 shows the maximum transformation efficiency. when the optical density was O.D.600nm = 0.75. from another study maximum transformation efficiency was obtained) when the O.D.600nm = 1.0 was used. To transformed immature wheat and barley embryos with Agrobacterium having an O.D.600nm = 1.5. used bacterial culture density of O.D.600nm = 2.0 to transform wheat inflorescence tissues [95] . These results do not support the idea of already reported findings. Previous research reported that the transformation of cotyledonary leaves, plantlet leaves, and hypocotyls was achieved with and without preculture treatment using Bacterial culture (*Agrobacterium tumefaciens*) with optical densities ranging from 0.3 to 0.6 at 600 nm. In the case of calli transformation, bacterial cultures with an optical density of 600 nm of 0.2-0.8 were used, however none of the calli were positive in Gus histochemical staining.

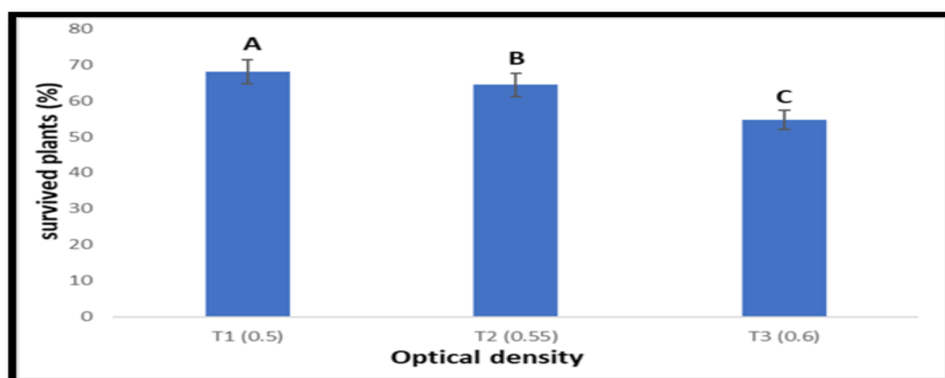


FIGURE 4.10: Optimum optical density for better growth of bacteria

The maximum transformation efficiency (100%) was observed in precultured explants with O.D.600nm=0.3 regeneration. The maximum efficiency was 66.67%

when the O.D.600nm was 0.5 regeneration in the case of non-precultured explants [96]. This study revealed that the GUS assay showed at OD600=0.6, maximum transformation which was achieved by strain EHA105 (40%) over LBA 4404 (36%). The transformation efficiency decreased when the OD value increased or decreased. The organism could not be transformed if the OD value exceeded 0.8, and extensive tissue damage was caused by the overgrowth of bacteria when the OD value exceeded 1.0 [97] . Our results do not support the above data.

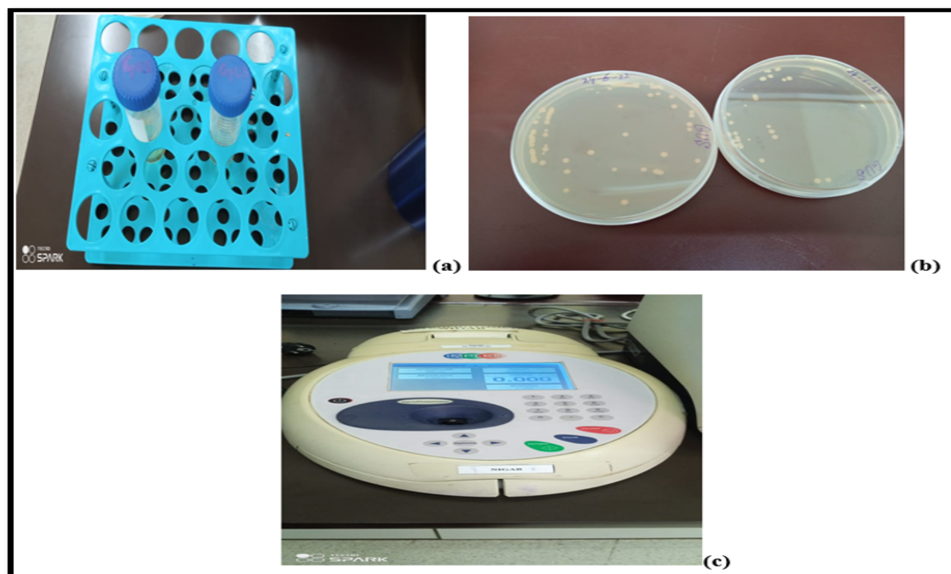


FIGURE 4.11: (a) Showing the bacterial cultre of GUS (b) Showing the glycerol stock and (c) Showing the spectrophotometer to check the optical density.

TABLE 4.5: ANOVA Assessment of various optical density on transformation efficiency in potato ($GN_2$)

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Replication | 2 | 216. 000 | 108. 000 | | |
| Treatment | 2 | 284.667 | 142. 333 | 106.75 | 0. 0003 |
| Error | 4 | 5. 333 | 1. 333 | | |
| Total | 8 | 506. 000 | | | |

MS = Mean square, F=Significance probability, p=probability value, Grand Mean =37.972, CV= 7.61, LSD= 6.5469

### 4.1.6 Co-Cultivation

#### 4.1.6.1 Proline Concentration

During co-cultivation explants were treated with proline concentrations of 0.05g/L, 0.08g/L and 0.11g/L. Statistically significant interactions were observed between treatment and explant selection. Figure 4.12 given below shows an analysis of statistical data which showed that the maximum selection value was reached when explants were treated with 0.11g/L resulting in a mean value of 49.667. Following that, 0.05g/L and 0.08g/L had mean values of 30.667 and 54.000, respectively. Table (4.6) also showing the results of proline which are varying the level of proline on transformation efficiency in potato ($GN_2$). Different researchers investigated that the effect of proline on cultures of mature rice seeds going through in vitro callus formation. In the medium without proline, the average callus formation was reduced to 70.4%, whereas in the medium with proline, 85.3% callusing was observed. A similar increase in fresh callus weight was observed when proline was added [98] . Corn multi-shoot cultures were studied to determine how proline affected GUS expression. By adding 0.7g/l of proline to the medium, the GUS gene was more likely to be transferred into corn tissue.

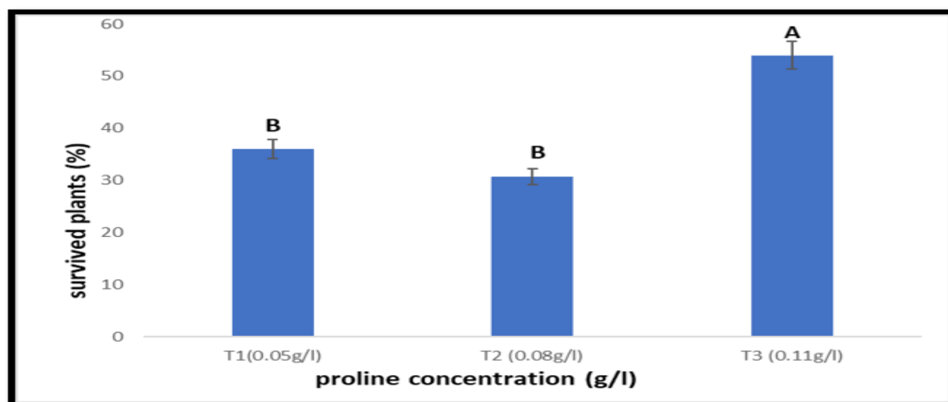


FIGURE 4.12: Effect of different proline concentrations on infected Explants

However, a high level of proline (3 g/L) promotes the growth of *Agrobacterium* in corn tissues [99] . The present results are not in accordance with previous ones. Another finding contradicted the effects of proline supplement with concentrations from 10 g/L up to 32 g/L on switchgrass transformation. Based on the results,

supplement of 20 g/L proline was chosen for further transformation experiments [100]. According to this study our results are different because we have observed the best survival of plants at 0.11mg/l proline concentration.

A successful transformation depends on several factors. Optimum co cultivation time is the most important factor among these (A large number of researchers stated that co- cultivation time significantly effects the regeneration of transformants and hence transformation efficiency of several plant species. The success or failure of transformation can also depend on co- cultivation. Therefore, it is predictable to determine optimum co-cultivation time to have maximum transformation efficiency [101] .

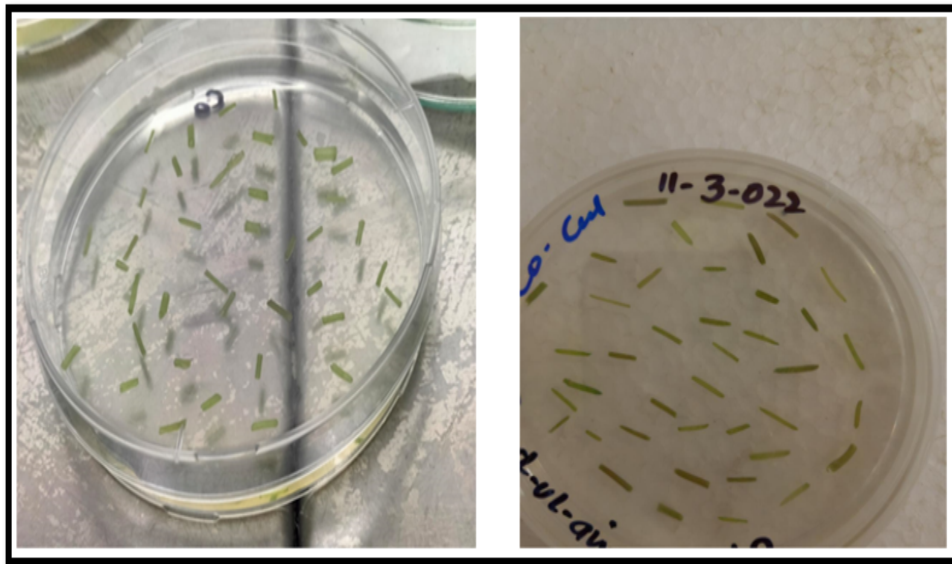


FIGURE 4.13: Proline concentration during co cultivation of potato ($GN_2$)

TABLE 4.6: ANOVA Effect of varying level of Proline on transformation efficiency in potato ($GN_2$)

| **Source** | **DF** | **SS** | MS | **F** | **P** |
|---|---|---|---|---|---|
| Replication | 2 | 82.667 | 41.333 | | |
| Treatment | 2 | 738.667 | 369.333 | 40.29 | 0.0022 |
| Error | 4 | 36.667 | 9.167 | | |

TABLE 4.6: ANOVA Effect of varying level of Proline on transformation efficiency in potato ($GN_2$)

| **Source** | **DF** | **SS** | MS | **F** | **P** |
|---|---|---|---|---|---|
| Total | 8 | 858.<br>000 | | | |

MS = Mean square, F=Significance probability, p=probability value, Grand Mean 62.333, CV= 1.85, LSD= 2.6177

### 4.1.7 Time Duration of Co-Cultivation

In experiments on potato ($GN_2$) explants, cocultivation time for 24, 48, and 72 hours led to maximum selection. In graph along x-axis showing that three different treatments were applied to check the time duration of explants on co-cultivation media and along y-axis showing the survival percentage of explants along with time duration. The highest selection value was observed when cocultivation time was 24 hours with the mean value of 42.333, followed by 48 hours and 72 hours with the means of 45.000 and 41.600 respectively. At 24 hours, the maximum selection was recorded. As shown in figure (4.14) and best survival of explants at 24 hours shown in figure (4.15). There are several factors that contribute to a successful transformation. One of the most important factors of co-cultivation is the optimal co-cultivation time. According to many researchers, co-cultivation time has a significant impact on the regeneration of transformants and transformation efficiency. Transformation can also be affected by co-cultivation. For maximum transformation frequency, determining optimum co-cultivation times for each cultivar is essential. A maximum transformation frequency was achieved by comparing two co-cultivation times on each cultivar (24 and 48 hours). The co-cultivation time of 24 hours was found to be the best and generated the most transformants in all wheat lines under study. Four wheat cultivars showed decreased efficiency when their co-cultivation time was increased from 24 to 48 hours [102]. Another finding contradicted that in monocots, most common co-cultivation time is 24 hrs.

Its maximum range is upto 48 hrs. The transformation frequency decreased for higher or lower co-cultivation times [103] . Another finding shows that 3 days co cultivation time is optimum for Narcissus tazzeta transformation [104] . Another study also documented 3 days co cultivation time for three different plant species. The 3 days duration for optimum co cultivation time is best while transforming barley calli with bar gene and uidA gene [105] . The optimized co-cultivation conditions for successful transformation of rice and found that 3 days co cultivation produced higher number of transformants. This study stated that 36 hours is the optimum co cultivation time since it produced high transformation frequency in tomato. another study revealed that the 4 days co cultivation duration in rice to be optimum duration for maximum transformation efficiency [106] . The results obtained are in accordance with previous studies.

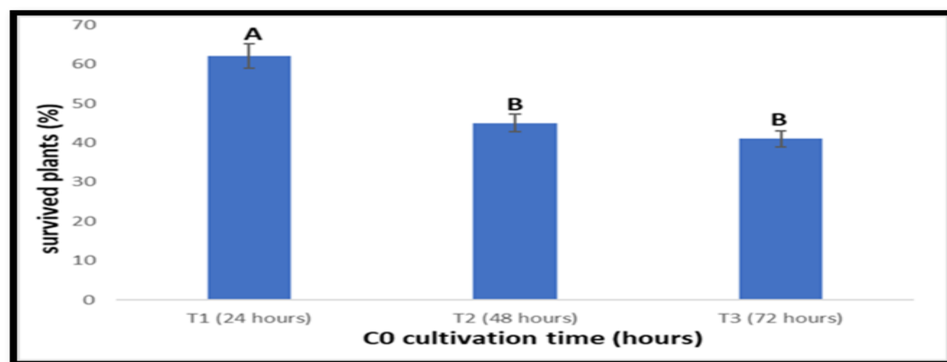


FIGURE 4.14: Effect of different time durations on infected Explants

TABLE 4.7: ANOVA table of Assessment of Co-cultivation time periods on transformation efficiency in potato ($GN_2$)

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Replication | 2 | 38.000 | 19.000 | | |
| Treatment | 2 | 612.667 | 306.333 | 20.65 | 0.0078 |
| Error | 4 | 59.333 | 14.833 | | |
| Total | 8 | 710.000 | | | |

MS = Mean square, F=Significance probability, p=probability value, Grand Mean= 42.333, CV =9.10, LSD 8.7310

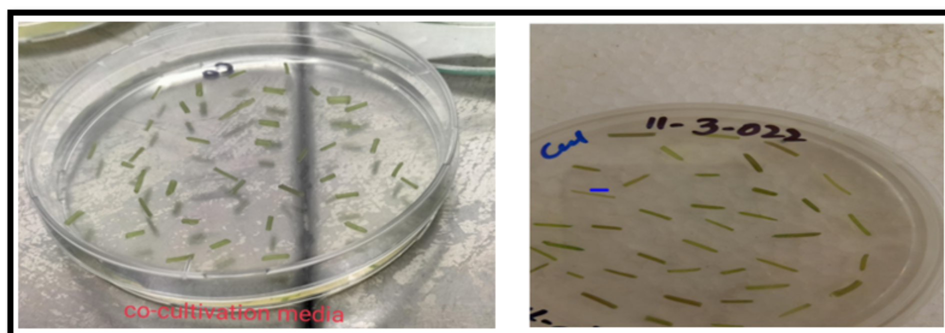


FIGURE 4.15: Co cultivation time period of potato ($GN_2$)

### 4.1.8 GUS Assay

Transient GUS transformation efficiency of GV1301 strain was observed in 60 explants of $GN_2$ Varity. GUS expression was observed in 10 explants with transformation efficiency of 16.66%. as shown in table (4.8).

TABLE 4.8: Effect of Transient GUS transformation efficiency of Gv3101 strain.

| **Variety** | **No of explants** | **GUS expression** | **Transformation efficiency** |
|---|---|---|---|
| $GN_2$ | 60 | 10 | 16. 66% |

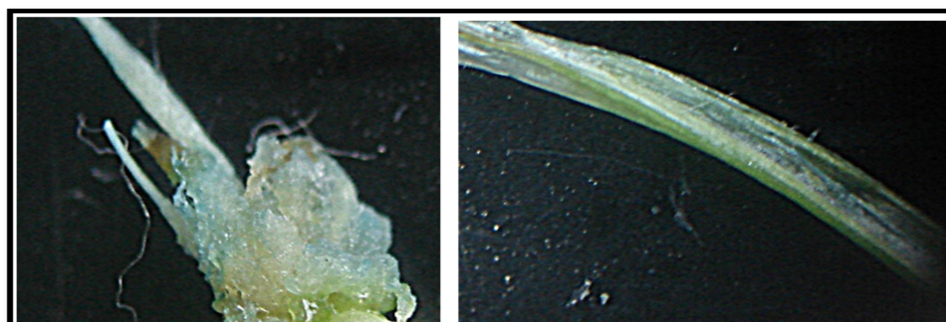

FIGURE 4.16: Co cultivation time period of potato ($GN_2$)

## 4.2 Optimization of an *Agrobacterium tumefacien* Mediated Gene Transformation for Lectin Gene

### 4.2.1 Explants Source

Explants of potato cultivar "$GN_2$" that were free of diseases were used as explant source. The cultivar of this variety was collected from the National Institute of Genomics and Advanced Biotechnology Islamabad.

FIGURE 4.17: Explants source

### 4.2.2 Surface Sterilization

As Previously optimized protocol (GUS) was used for lectin gene transformation.

### 4.2.3 Multiplication

Also Previously optimized protocol (GUS) was used for lectin gene transformation during multiplication. Appendix (5.1)

### 4.2.4 Infection

Further optimization of bacterial density was achieved by using multiplied plants. On potato ($GN_2$), three different optical densities were tested. Results are shown

in figure 4.18 indicated the treatments as well as their interaction were highly significant. With a mean value of 36.222, 0.3 was recorded as the maximum transformation efficiency, followed by 0.25 and 0.2 respectively. In error bar graph along x-axis showing the optical density with three different treatments and along y-axis showing the survival of explants at different concentrations of optical density. Previous findings revealed that the Bacterial density significantly influences the delivery and integration of Transfer DNA into the host genome. This in turn affects the transformation efficiency. Higher bacterial density damages the explants, reduces cell recovery and stable transformation frequency. Therefore, it is crucial to optimize the bacterial density to maximize the transformation efficiency. Four bacterial densities (0.25, 0.50, 0.75 and 1.00) were experimented at OD600nm. The maximum transformation efficiency was 28.59% that was obtained in Lasani-08 at OD600nm 0.50. Rest of the three cultivars also performed best at this bacterial density [107] . These results are different from current results the difference is due to different explants source as well as genotype.

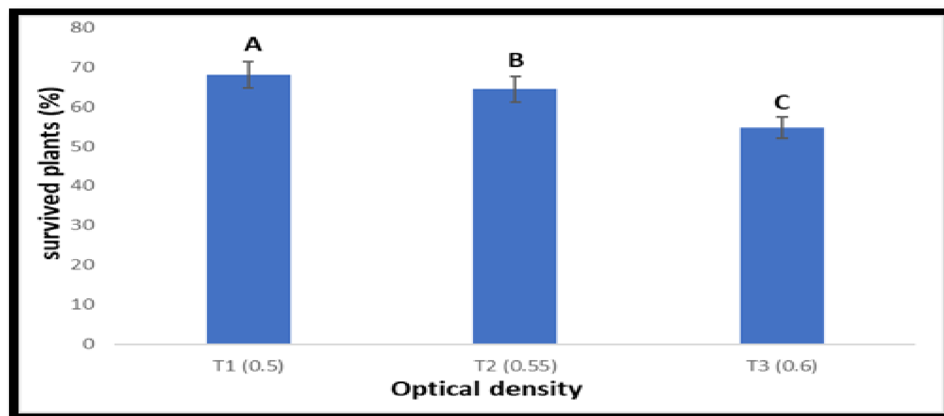


FIGURE 4.18: Explants source

Another scientist achieved results that were opposing with the work of previous scientist who was observed the transformation frequency more at OD600nm = 0.2 in rice. By increasing OD, they reported a decline in transformation efficiency [108] . some scientists improved the transformation frequency of rice at OD600nm = 0.02 in the presence of 76 µM acetosyringone during plant bacteria interaction. In another experiment, they used 100mg/L cysteine along with 76 µM acetosyringone and got highest transformation frequency at OD600nm =0.04 in the same

rice cultivar. From more studies it was reported that at OD600nm = 0.8 transformation of rice cultivar, ADT 43 would be maximized if co-cultivated for 2 days on LS30-AsPC medium. Further increase or decrease in OD, the transformation efficiency got disturbed. some scientists infected tobacco and Arabidopsis explants with Agrobacterium culture having OD = 0.4 and got highest transformation frequency [109] . All of these findings do not collaborate with our results. This may be due to difference in plant species. Our results have significant resemblance with the findings of previous ones who used 0.50 OD600nm to infect the rice calli and got maximum transformation efficiency as compared to other OD.

Table 4.9: Assessment of various optical density on transformation efficiency in potato ($GN_2$).

| **Source** | **DF** | **SS** | MS | **F** | **P** |
|---|---|---|---|---|---|
| Replication | 2 | 38. 222 | 19. 111 | | |
| Treatment | 2 | 384. 889 | 192. 444 | 21. 12 | 0. 0075 |
| Error | 4 | 36. 444 | 9. 111 | | |
| Total | 8 | 459. 556 | | | |

MS = Mean square, F=Significance probability, p=probability value, Grand Mean= 36.222, CV= 8.33, LSD= 6.8427

### 4.2.5 Co-Cultivation

As Previously optimized protocol of (GUS) was used for lectin gene transformation during co cultivation. Appendix (5.2)

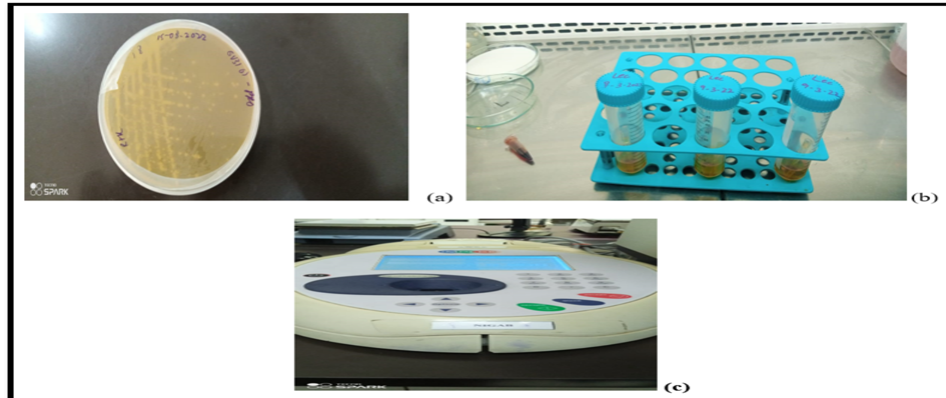


FIGURE 4.19: (a) showing the glycerol stock of lectin gene (b)showing the bacterial culture and spectrophotometer which is used to check the optical density.

### 4.2.6 Selection

#### 4.2.6.1 Different PPT Concentrations

For the evaluation of lethal doses of phosphinothricin (ppt) for transformation experiments, various concentrations of PPT (6mg, 08mg, and 10mg) were used on explants. According to the statistical analysis, there is a highly significant difference between treatments. There was a significant interaction between variety and treatment. According to the data, PPT concentration inversely relates to the survival rate of transformed explants. Figure (4.20) shows that at 8 mg/l, the highest survival rate was recorded with a mean value of 49.222, followed by 6mg/l and 10 mg/l.

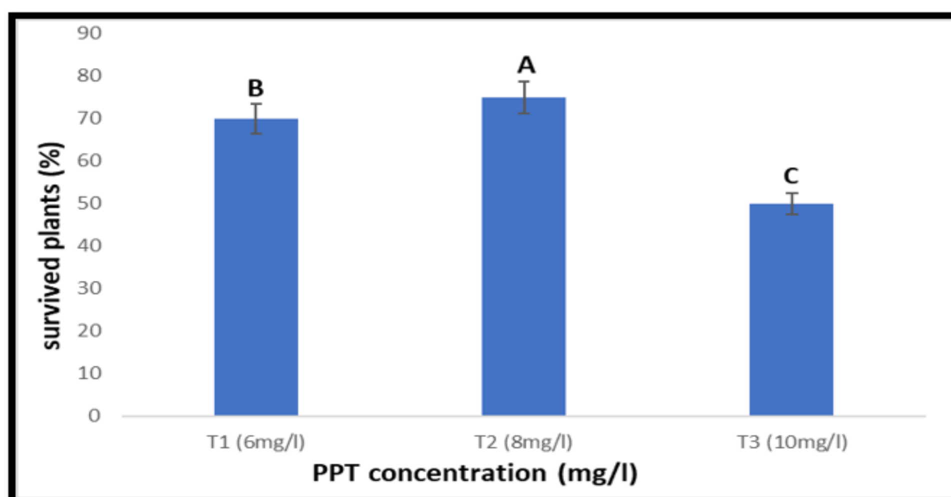


FIGURE 4.20: Effect of different PPT concentrations on Explants

As a result, potato ($GN_2$) died at a lethal dose of 8mg/l.as shown in fig (4.21) Some scientists studied the effect of PPT on embryo axes. Optimal selection pressure and plant tissue to PPT responses were determined by culturing damaged embryo axes on shoot regeneration medium having different concentrations of PPT (2, 5, 10 or 20 mg/L). Inhibition from mature embryo axes Inhibition of shoot proliferation was observed on medium containing 10 mg/L PPT [110] . Our results have significant resemblance with the findings of previous ones.

Another study also contradicted the effect of PPT on the transformation efficiency of Bottle gourd. Various concentrations of PPT (0, 0.5, 1, 2, 5 and 10 mg/L) were incorporated into the shoot induction medium to determine the optimal concentration for selecting transgenic shoots. At 2.0 mg/L, the best results were obtained [111] .

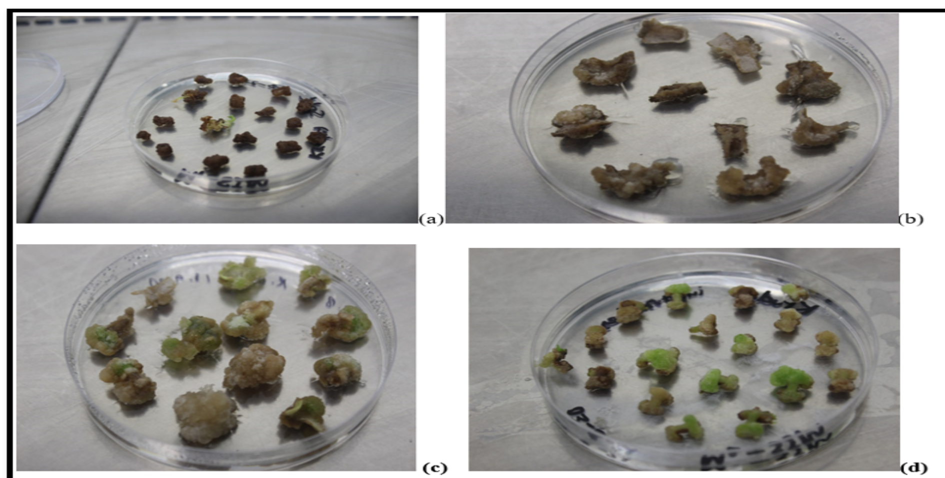


FIGURE 4.21: a, b, c and d showing the Assessment of optimum level of potato at different concentrations for transformation experiment in potato ($GN_2$).

TABLE 4.10: ANOVA table of Assessment of optimum level of ppt (phosphinothricin) for transformation experiment in potato ($GN_2$).

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Replication | 2 | 26.<br>89 | 13.<br>44 | | |
| Treatment | 2 | 3230<br>.89 | 1615<br>.44 | 36.<br>35 | 0.<br>0027 |

TABLE 4.10: ANOVA table of Assessment of optimum level of ppt (phosphinothricin) for transformation experiment in potato ($GN_2$).

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Error | 4 | 177<br>.78 | 44.<br>44 | | |
| Total | 8 | 3435<br>.56 | | | |

MS = Mean square, F=Significance probability, p=probability value, Grand Mean= 49.222, CV= 13.54, LSD =15.113

Further research assessed that in vitro development of in vitro grown shoots and embryonic tips exposed to increasing concentrations of PPT. Therefore, the PPT concentrations increased gradually during selection as 0.5, 0.75, and 1.0 mg/L. The regeneration rate of embryonic tips was improved when the dose was raised to 1.0mg/L and a greater number of resistant tips were produced. PPT was added to shoot initiation medium in five different concentrations (0.0, 0.25, 5, 7.5, 10mg/L).

### 4.2.7 Regeneration

As previously optimized protocol of (GUS) was used in regeneration media during lectin gene transformation Appendix (5.2)

### 4.2.8 Development of Transgenic Plant and PCR Conformation

#### 4.2.8.1 Transformation of Potato ($GN_2$) Cultivar by Using Lectin Gene

Lectin gene was transformed by using Agrobacterium mediated- transformation protocol against aphid resistance in potato ($GN_2$). For the transformation of

potato ($GN_2$), PCAMBIA 1301 vector which containing lectin gene with 35S promoter and PPT (phosphinothricin) selectable marker was used.

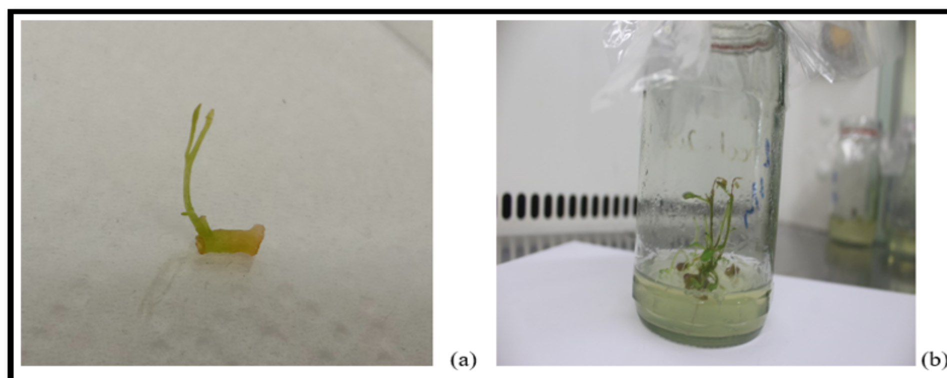


FIGURE 4.22: a and b showing the transgenic plants of potato ($GN_2$)

#### 4.2.8.2 PCR analysis

1970 calli of potato ($GN_2$) were infected with *Agrobacterium* having Lectin gene. Only five plants were regenerated. Among these regenerated plants, 2 were PCR positive transgenic plants.

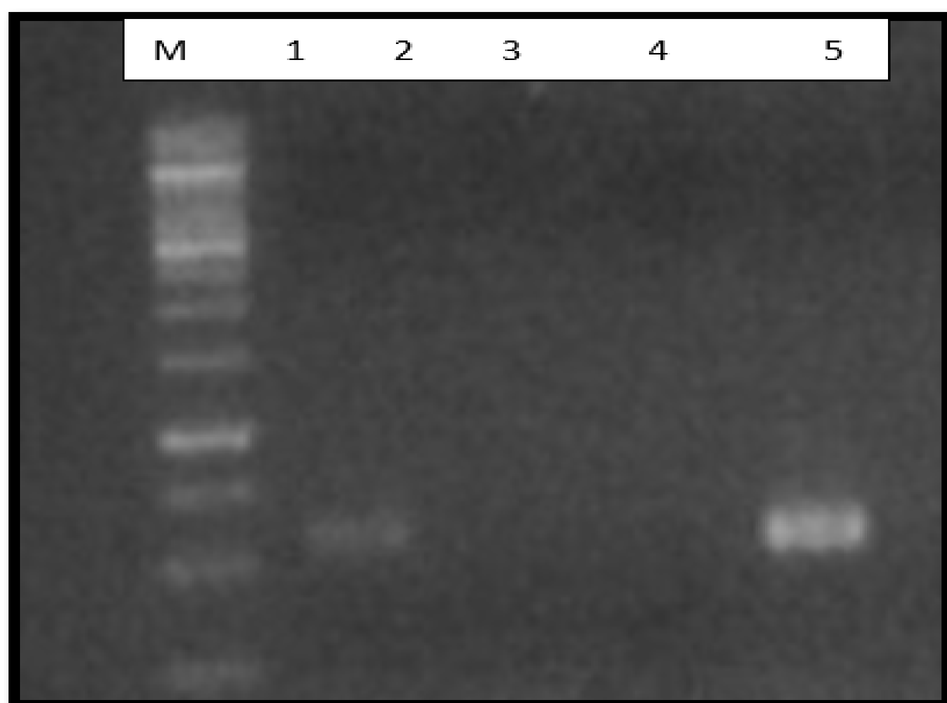


FIGURE 4.23: Conformation of transgenic plants through PCR analysis. M, 1kb DNA ladder 1-5 amplification of 546bp fragments of transgenic plants.

TABLE 4.11: Transformation efficiency of lectin gene in potato ($GN_2$)

| **Variety** | **Number of Uncontaminated Selected calli** | **No of Regenerated Calli** | **No of Transformed Plants Produced** | **Transformation Efficiency** |
|---|---|---|---|---|
| $GN_2$ | 1970 | 430 | 5 | 2 |

**Summary**

Potato (*L. solanum tuberosum*) belongs to the family Solanaceae. Potato is a more valuable and essential crop due to its high nutritional values with sufficient number of macronutrients and use as a food source. After wheat, rice, and maize, potato is the fourth most cultured food crop in the world. Many biotic and abiotic factors restrict the yield of potatoes. A number of determined abiotic issues to reduced yields in Pakistan. These include issues with soil quality, inefficient fertilizer application methods, and insufficient access to high-quality irrigation water. 50% of the global decrease in agricultural production is due to abiotic constraints. There are several biological restrictions that affect the development and productivity of potatoes, including low-yielding potato varieties, fungi, parasites, bacteria, and viruses. potato losses due to biotic reactions to be around 40%. Aphids can develop up to great densities in feeding plants, taking nutrients and sap from plants to induce scorn and death of plants. Green peach and potato aphids (*M. persicae and M. euphorbiae*) are major potato-infesting insect pests that harm global potato output. Currently in Pakistan, potato is grown on 172.8 thousand hectares with a yield of 18.3 tons per hectare and an annual production of 3785.9 thousand tons. More than 99% of Pakistan's seed potatoes are produced locally, and the country is completely self-sufficient in potatoes. For the generation of genetically modified transgenic plants, *Agrobacterium*-mediated genetic transformation is the most common method. The *Agrobacterium-mediated transformation* approach takes use of the soil microbe Agrobacterium's inherent capacity to change plants. It is possible to construct the T-DNA vector to include a selectable gene of interest, which are then transported from the bacterium to

the host plant cells during transformation. GUS gene used as a reporter gene to check the transformation efficiency of gene. Due to GUS's sensitive fluorometric method of detecting enzyme activity, it has become a preferred choice among many researchers. Additionally, the GUS polypeptide can tolerate changes in its N-terminal structure for the enzyme to function, which enables the construction of fusion genes. Transformation of lectin gene into selected potato ($GN_2$) to develop resistance against fungal and bacterial diseases because lectin gene recognition at the cellular and molecular level. Sterilized the explants with 70% of Chlorox and cultivated on multiplication media for 10-15 days. Cut the internodes of ($GN_2$) potato and inoculated on the bacterial culture and then put on the co- cultivation media for 2 -3 day. After two to three days washed explants with autoclaved distilled water and cefotaxime for 3 times and inoculated on selection media for regeneration. After regeneration of plants extracted the DNA and performed the PCR by using specific primers to confirmed gene of interest is present or not.

# Chapter 5

# Conclusions and Recommendations

The conclusion and recommendation of this research titled "**Optimization of lectin gene Transformation protocol in Potato Cultivar ($GN_2$) by Using GUS as a Reporter Gene**" are given below.

**Conclusion**

This study presents suitable hormonal composition of the regeneration media, type of explants and most suitable protocoal for *Agrobacterium-mediated* potato transformation. According to the results, the use of internode explants is recommended as a generally better procedure taking all aspects of transformation and regeneration into account. Additionally, the other factors affecting transformation efficiency were also described. The samples having lectin showed better resistance to insect and other pathogens than those samples that did not get infected with lectin. This will certainly help in future to overcome challenges of yield losses by reducing pathogenic attack.

**Recommendation**

*Agrobacterium mediated* transformation can be used for the different other crops to protect them from bacterial and fungal diseases and improved crop production.

To produce more resistant varieties in future its best to transfer resistant genes into non- resistant genes. To reduce bacterial and fungal diseases in potato crops this technology will certainly help to overcome the challenges of yield loses.

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# An Appendix

TABLE 5.1: Composition of multiplication media

| Nutrients | Quantity |
|---|---|
| MS Salt(g/l) | 4.43 |
| GA3 | 50µl |
| Sucrose(g/l) | 30 |
| Gellen gum(g/l) | 2.8 |

TABLE 5.2: Composition of Regeneration Media.

| Nutrients | Quantity |
|---|---|
| MS salt(g/l) | 4.43 |
| Sucrose(g/l) | 30 |
| Vitamin 500X | 3000µl/l |
| BAP | 1000µl/l |
| GA3 | 100µl/l |
| Gellen gum | 2.8g/l |
| PH | 5.8 |

TABLE 5.3: Composition of Co Cultivation Media

| Nutrients | Quantity |
|---|---|
| MS salts(g/l) | 4.43 |
| Sucrose(g/l) | 60 |
| BAP (mg/ml) | 1 |

TABLE 5.3: Composition of Co Cultivation Media

| **Nutrients** | **Quantity** |
|---|---|
| Cefotaxime | 250µl |

TABLE 5.4: Composition of Selection Media

| **Nutrients** | **Quantity** |
|---|---|
| MS salt | 2.165g/l, |
| Sucrose | 15g/l |
| Benzyl amino purine | 500µl/l |
| Naphthalene acetic acid | 50µl/l |
| Gibberellic acid | 50µl/l |
| Vitamins | 1000µl/l |
| Ph | 5.8 |
| PPT | 8mg/l |