Optimization of Tissue Culture and Regeneration Conditions for *Spinacia oleracea* and its *Agrobacterium*-mediated Transformation with OmpK Antigen for Developing a Vaccine Against Fish Vibriosis

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Declaration of Originality

I hereby declare that the work presented in this thesis is the result of my own effort and research work, carried out in Plant Biotechnology Lab, Department of Biochemistry, Quaid-i-Azam University, Islamabad, Pakistan.

This thesis is my own composition and no part of it has been presented for any degree previously, nor does it contain, without proper acknowledgement or reference, any material from the published resources to the best of my knowledge.

Iqra Elahi

This thesis is dedicated to
My Loving Parents
For their endless love, affection,
support and encouragement

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LIST OF ABBREVIATIONS

BAP	6-benzylaminopurine
%	Percentage
µl	Microliter
µm	Micrometer
½ MS medium	Half strength Murashige and Skoog medium
BCG	Bacillus Calmette Guerin
bp	Base pair
cm	Centimeter
CTAB	Cetyl Trimethyl Ammonium Bromide
d.H2O	Distilled water
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetra acetic acid
ESAT-6	6 kDa Early Secretory Antigenic Target
GFP	Green fluorescent protein
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HRP	Horseradish peroxidase
i.e	That is
IAA	Indole acetic acid
IFN-γ	IFN-γ
Kb	Kilobase
L	Liter
LA	Luria-bertani agar
LB	Left border
LB	Luria-broth
LFH	Laminar flow hood
M	Molar
MES	(*N*-morpholino)ethanesulfonic acid

mg	Milligram
mg/L	Milligram per liter
ml	Milliliter
MS medium	Murashige and Skoog medium
NAA	Naphthalene acetic acid
NAA	Naphthalene Acetic Acid
NaOH	Sodium hydroxide
nos-P	nopaline synthase promoter
nos-T	nopaline synthase terminator
^{o}C	Degree centigrade
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Power of hydrogen ions
Psi	Pressure per square inch
RB	Right border
TB	Tuberculosis
TBE	Tris-borate-EDTA
TBST	Tris buffered saline with tween
T-DNA	Transfer DNA
TE	Tris-EDTA
Ti	Tumor inducing
Tm	Melting Temperature
TMB	3,3,5,5-tetramethylbenzidine
TSP	Total soluble protein
UV	Ultraviolet
Vir	Virulence
WHO	World Health Organization

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ABSTRACT

Vibriosis, caused by *Vibrio anguillarum*, also called *Listonella anguillarum*, is a deadly haemorrhagic septicaemic disease that affects fish, bivalves, and crustaceans in marine, fresh and brackish water. Vibriosis is one of the most prevalent and harmful infections in marine aquaculture, causing considerable economic losses. To eradicate vibriosis, an innovative, secure, and more stable vaccine must be created. Edible plants can be used as bio-factories for the expression of vaccine antigens. Outer membrane protein K (OmpK) of *Vibrio anguillarum* can be used as vaccine antigen against Vibriosis. The present research work aimed to optimize the tissue-culture conditions for *Spinacia oleracea* and efficient transformation protocol for stable transformation as well as transient transformation of *Spinacia oleracea* with OmpK antigen. Parameters like seed sterilization and seed germination media were optimized before the transformation. Sterilization with 0.2% mercuric chloride and germination on full MS media for spinach seeds provided good results. For tissue culture, nodal explants of *Spinacia oleracea* showed the highest regeneration efficiency on full MS supplemented with BAP 5mg/l and IAA 0.5mg/l. For *Spinacia oleracea*, different concentrations of antibiotic hygromycin were used with the optimal concentration being 10mg/l for nodal explants. Successful transformation with OmpK antigen was carried out using the above-mentioned conditions. Transformed explants were regenerated on selection media supplemented with antibiotic hygromycin. Further, explants with 8 minutes infection time and 3 days co-cultivation time showed 60% regeneration efficiency. The leaves of the spinach plant were infiltrated with *Agrobacterium tumefaciens*, harboring plasmid containing OmpK gene Positive results were shown on all four days of collected leaves. The transformation was confirmed through PCR by using gene-specific primers. Transgene expression in spinach plants were analyzed by quantitative real-time PCR (qRT-PCR) in comparison to the β-actin gene as a control. Further, protein expression was confirmed through Dot-blot analysis, Western blotting, and ELISA. Taken together, the successful expression of the OmpK antigen of *Vibrio anguillarum* in edible plants may facilitate the development of an economical, cost-effective, and subunit edible plant-derived vaccine against vibriosis.

Keywords: *Spinacia oleracea*, OmpK antigen, Vibriosis, Edible vaccine, *Agrobacterium*-mediated transformation, qRT-PCR, Western blotting, ELISA

1. Introduction

1.1. Fish and its global importance

Oceans provide food, life, and biodiversity. A healthy ocean ensures healthy earth and human societies. 80% of Earth's life is in the water. It is home to biological and carbon pumps and food webs that control our climate and support us all. Ocean health benefits are evident. Fish provides 20% of 3 billion people's animal protein. Ten species produce 30% of marine capture fisheries and 50% of aquaculture productivity. Humans have long relied on fish for protein. Aquaculture produces mostly fish. 174.6 million tons of fish were produced globally in 2020, up from 148.1 million in 2010. (Shahbandeh, 2022). According to FAO, more than 40% of the world's population is within 100 kilometers of the coast. Pakistan's maritime and interior waters are abundant. 204,000 fishermen in Pakistan catch 2,350,000 tons of marine produce annually. The Arabian Sea is biologically fruitful. Carp and trout dominate aquaculture's 150,000 tons of annual fish production (Kaczan & Patil, 2020).

In the face of climate change, stagnant catch fisheries production, rising aquaculture production, and competitive pressure for natural resources, better ocean resource management is essential to guaranteeing global food security and nutritional properties for a growing world population (Guillen *et al*., 2019). Fish are susceptible to microbial disease, and although the spectrum of genuine pathogens seems limited, fish disease is a global concern impacting freshwater and marine fish, feral, cultured, sport, and ornamental fish. Under intensive cultural conditions, vast numbers of fish can quickly develop ill and perish.

1.2. Infectious diseases

Diseases that are spread by organisms like bacteria, viruses, fungi, or parasites are called infectious diseases. Most of the time, the immune system of the host is perfectly operational, which prevents any kind of disease symptoms from showing up. On the other hand, the disease will happen if the host's immune system is weak or if any infectious agent overwhelms its immune system. Aquaculture is still growing so that people can get protein, these protein are nutritious and healthy for them. Fish farming has become less profitable and unsafe due to a variety of factors, including infectious illnesses, a decline in water quality, as well as other environmental pressures. Vibriosis represents one of the most prevalent bacterial infections that kills marine fish, shrimp,

and some freshwater fish. It costs a lot of money to treat and kills a lot of people (Yilmaz *et al*., 2022).

1.3. Vibriosis

An infection known as vibriosis is brought on by a bacterium species of the genus Vibrio, which is common in aquatic habitats. Both cold-water and warm-water fish species are impacted by the sickness. One of the most pervasive and destructive diseases in marine aquacultures, vibriosis results in significant economic losses and mortalities in both freshwater and saltwater fish species, across the globe. Hemorrhages and superficial skin lesions are its main visible signs, and systemic septicemia is usually present. Vibriosis outbreaks have been linked to sudden changes in water temperature, poor water quality, fluctuations in salinity, and inadequate fish diets, especially in the early to mid-summer when the temperature of the water coincides to the ideal growth temperature for Vibrio species (Egidius, 1987).

1.3.1. *Vibrio anguillarum*

Vibriosis is caused by *Vibrio anguillarum*, also called *Listonella anguillarum*, a deadly haemorrhagic septicaemic, bivalves, and crustaceans in marine, fresh and brackish water (Frans *et al*., 2011). *Vibrio anguillarum* is a rod-shaped, Gram-negative bacterium in the family Vibrionaceae. It is polarly flagellated, halophilic, facultatively anaerobic, and non-spore-forming (Madigan *et al*., 2006). At temperatures between 25 and 30°C and on a media with 1.5–2% sodium chloride (NaCl), the bacterium grows quickly and forms colonies that are cream-colored and round (Buller, 2004). *Vibrio anguillarum* infects more than 50 different types of fish and shellfish, which is bad news for the marine fish farming industry (Tan *et al*., 2014).

1.3.2. Susceptible fish species

Numerous species are economically significant to the larviculture and aquaculture industries, including rainbow trout (*Oncorhynchus mykiss*) (Holt, 1970), salmon (*Salmo salar L.*), cod, eel, turbot (*Psetta maxima L.*), ayu (*Plecoglossus altivelis*), sea bream (*Sparus aurata L.*) (Buller, 2004; Toranzo *et al*., 2005), bivalve mollusks, and crustaceans are affected by vibriosis (Paillard *et al*., 2004).

1.3.3. Clinical signs of the disease

Red spots, weight loss and lethargy on the ventral and lateral parts of the fish, swollen. Black skin lesions ulcerate and bleed, and exophthalmia are typical external clinical

indications of vibriosis. Splenomegaly is seen internally, along with enlargements of the kidney and intestines that may be filled with a clear, sticky liquid. The kidney tubules' epithelial cells are vacuolated, and there are haemorrhages in the renal parenchyma. Iron deposits are seen to accumulate in the kidney's melanomacrophage centres. But in severe epizootics, the infection spreads so rapidly that most infected fish die without displaying any clinical symptoms (Angelidis, 2014; Austin & Austin, 2016).

1.3.4. Mode of transmission of *Vibrio Anguillarum*

According to O'Toole *et al*., (1996), Vibriosis in fish can be caused by cutaneous infection as well as oral exposure to the bacteria through polluted food or water. In most cases, the infection begins as a result of bacterial skin penetration through regions where the mucus has been removed. This microbe is highly resistant to the mucus' high antibacterial activity. The mucous layer is constantly replenished to prevent germs from attaching to epithelial cells, although *Vibrio anguillarum* typically enters through wounds or a disrupted mucous layer (Weber *et al*., 2010). Vibriosis can also be brought on by ingesting *Vibrio anguillarum* through food or water, especially in larval fish since their gastrointestinal systems are not acidic enough to kill the bacterium (Engelsen *et al*., 2008; Larsen, 1984). A vital source of nutrients for adhesion, colonization, and proliferation in the gut after passing through the stomach is intestinal mucus. By using endocytosis, *Vibrio anguillarum* moves across the epithelia after being released from the lamina propria. The bacteria then enter the bloodstream, which can result in blood poisoning, septicemia, or an infection of a number of internal organs, including the kidney, and liver (Grisez *et al*., 1996).

1.4. Vaccine against vibriosis

1.4.1. History

It is not advisable to use antibiotics for preventive measures in aquaculture because they leave antibiotic residues in the end product, promote antibiotic resistance, and spread antibiotic resistance to other organisms. As a result, disease prevention by vaccination is the main focus. Generally speaking, fish vaccination involves giving fish a certain pathogen's antigen in order to elicit specific immunoprotective reactions (Wali, 2016). David C. B. Duff is known as the "Father of Fish Immunization" since he published the first paper on fish vaccination (Khati *et al*., 2021). The first vaccines designed specifically for aquaculture were those against vibriosis, and they often offer very high

levels of protection (Colquhoun & Lillehaug, 2014). The vaccine should produce a high level of protection against various pathogenic strains, be efficient throughout the whole production process, and be affordable (Gudding *et al*., 1999; Toranzo *et al*., 2009).

1.4.2. Several types of fish vaccines

The creation of vaccinations against Vibrio species that infect fish has advanced significantly. Figure 1.1 illustrates the numerous fish vaccines developed, such as traditional live attenuated vaccines, inactivated bacterins, DNA vaccines, monovalent and polyvalent vaccinations, subunit vaccines, edible plant-based vaccines and nanoparticle-based vaccines (Bedekar *et al*., 2022; Ji *et al*., 2020; Mondal & Thomas, 2022; Mutoloki *et al*., 2015; Toranzo *et al*., 2009). Vibriosis in aquaculture can be decreased with the use of vaccines against Vibrio species since they can generate powerful immunological reactions in fish who just get them (Hstein *et al*., 2005; Toranzo *et al*., 1997).

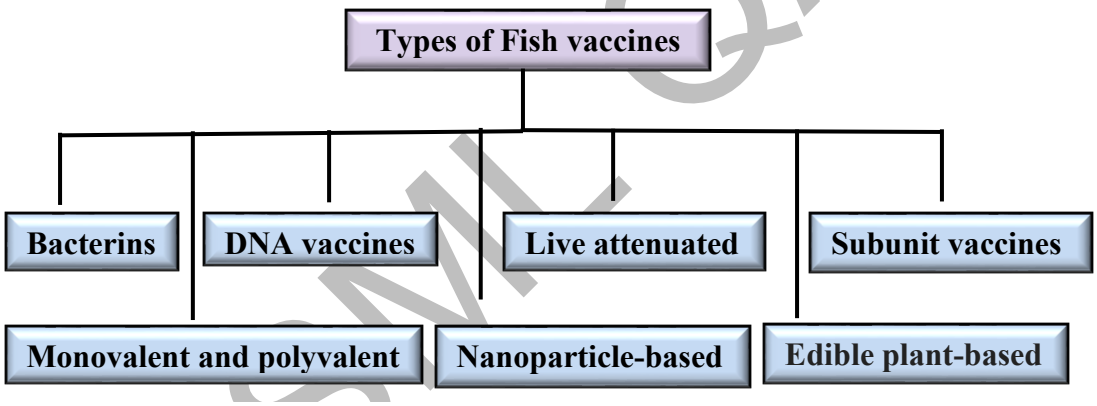


Figure 1.1: Types of fish vaccine.

1.5. Outer membrane protein K (OmpK)

Outer membrane protein K (an immunogenic protein) is a potential candidate for a subunit vaccine against *Vibrio anguillarum*. It is well recognized that outer membrane proteins, crucial components of Gram-negative bacteria, are essential for bacterial adaptive responses like antimicrobial, serum and bile resistance, uptake of ion and solute, acquiring iron and bacterial colonization. OmpK in particular has been proven in multiple studies to be highly potent immunogenic due to its exposed epitopes just on cell surface, and to have the potential to serve as a candidate for a fish vaccine against *V. anguillarum* (Frans *et al*., 2011; Hong *et al*., 2009; Khushiramani *et al*., 2012; Wang *et al*., 2003). The antigenic variety of bacterial strains, including those from the same species, has made it difficult to develop vaccines. However, serotype-conserved OmpK

could be used as prospective vaccine candidates, and numerous studies have shown their efficiency and viability (Maiti *et al*., 2020).

1.6. Plant biopharming

Plant biopharming, commonly referred to as molecular farming, is the cultivation of transgenic plants that have undergone genetic modification in order to create "humanised" pharmaceutical substances for use in humans. Instead of growing crops for food, they are being used as a bioreactor to produce therapeutic recombinant proteins thanks to recent developments in molecular biology and plant biotechnology (Bergougnoux, 2014). Because they can produce recombinant proteins in endless quantities safely and affordably, plants are prospective biopharming factories. The most frequently studied plants for biopharmaceutical development at the moment are corn, soybeans, rice, tobacco, spinach, tomato, broccoli, lettuce, banana, and potatoes. These plants can be genetically altered to produce the desired substance, typically a protein, in their fruit, leaves, seeds, or tubers (Morris *et al*., 2009).

In the past 20 years, molecular farming and transient expression systems have been employed in plants to produce pharmaceuticals, medications, vaccines, monoclonal antibodies, and immunomodulatory proteins in large quantities. These plants are referred to as bioreactors or factories (Yemets *et al*., 2014). A successful plant production system for recombinant biologicals requires the appropriate heterologous plant expression system, the best arrangement of regulatory elements that control gene expression, regulation of post-translational processing of recombinant products, and efficient purification methods for product recovery. These biological products are economical, readily available, stable, safe, and effective (Dhama *et al*., 2020; Ko & Koprowski, 2005).

The market does indeed have vaccines for some diseases, but the majority of them are highly expensive. Developing nations cannot afford to control disease with such expensive vaccines. Instead, efforts are being made to create inexpensive edible vaccinations that have numerous advantages over commercial vaccines (Ahmad *et al*., 2012; Khushiramani *et al*., 2012; Leblanc *et al*., 2020).

1.7. Plant-based molecular pharming of edible vaccines

Plant-based edible vaccines, as defined by Lössl and Waheed (2011) and Naik (2022), are recombinant protein vaccines in which selected antigens are produced by certain

plant species and administered orally in the form of an edible vaccine in order to elicit protective immunity against particular infections. Figure 1.2, which demonstrates how edible vaccines are made, shows that this process begins with the identification of the gene encoding the antigenic protein and its introduction into the plant that would generate the food (edible vaccines), which could then be distributed globally. B cells and T helper cells operate as the main mediators of the immune response after ingestion of an edible vaccine and subsequent passage of the antigenic protein through M cells, which are skilled at delivering antigens to dendritic cells (Concha *et al*., 2017; Mondal & Thomas, 2022).

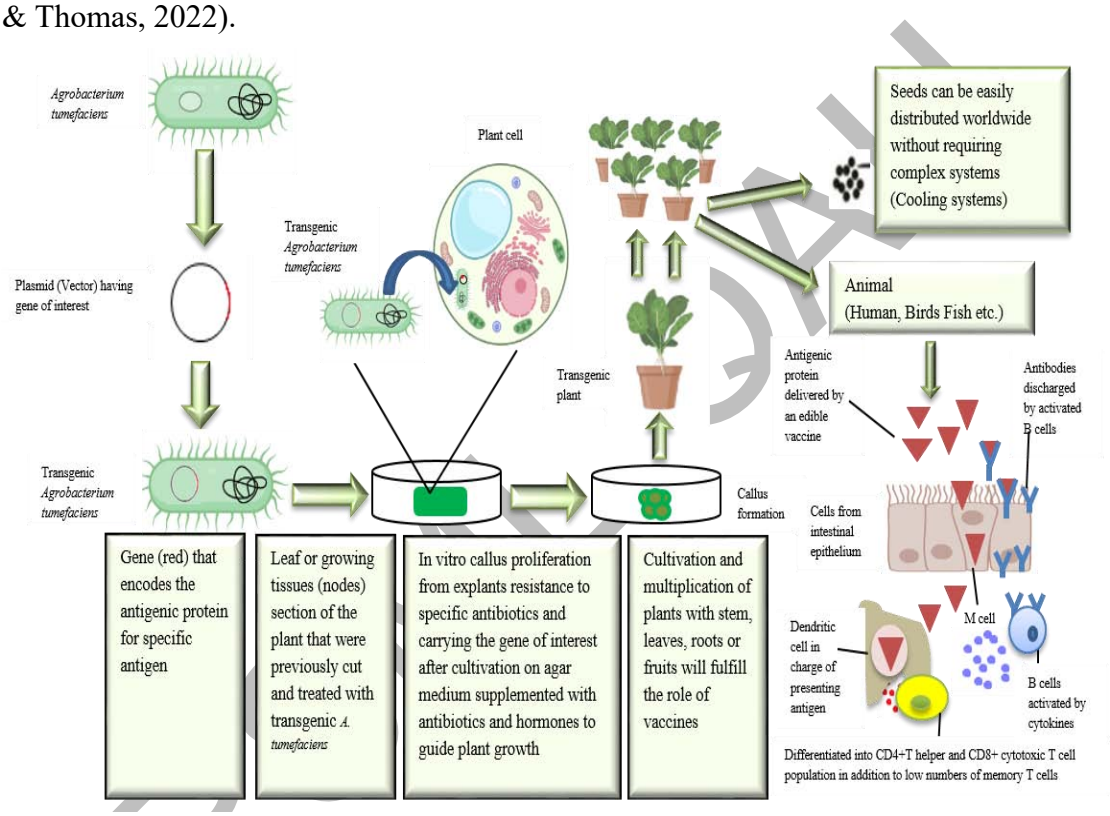


Figure 1.2: Procedures involved in obtaining an edible vaccine and an immune response.

1.7.1. Immunological mode of action of plant based edible vaccines

The genetically engineered plant or plant component that is eaten orally and expresses the potential vaccine antigen passes through the mastication process. The majority of plant cell breakdown is brought on by the action of bacterial or digestive enzymes in the colon, which releases the vaccine antigens (Gunasekaran & Gothandam, 2020; Mondal & Thomas, 2022c). Because Peyer's patches (PP) are a rich source of secretory immunoglobulin (IgA) producing plasma cells, they have the capacity to fill mucosal tissue and function as mucosal immune effector sites (Buonaguro & Butler-Ransohoff, 2010; Streatfield, 2006; Takahashi *et al*., 2010). The disintegration of the edible vaccine

close to PP, which is made up of lymphoid nodules on the gut's outer membrane and contains follicles that grow the germinal center in response to antigenic stimulation. Antigen penetrates the intestine's epithelium through these follicles and gathers there in organized lymphoid tissues (Rybicki, 2010; Santi, 2009). The vaccine antigen is then in contact with the M- cells and any deep invaginations or pockets in the intestinal luminal cells' basolateral plasma membrane. B-cells, T-cells, and macrophages, among other immune system cells, are concentrated in these pockets. M-cells in these lymphoid follicles can express class II MHC molecules and deliver antigens across the mucosal membrane, which can activate B-cells (Daniell *et al*., 2009; Hefferon, 2010). When the activated B-cells reach the diffuse mucosal associated lymphoid tissue (MALT), they go through plasma cell differentiation and release IgA subclass antibodies. These IgA antibodies enter the secretions of the lumen through the epithelial cells and potentially bind to the antigen there (Dus Santos & Wigdorovitz, 2005; Yuki & Kiyono, 2003). The mechanism of action for the plant-based consumable vaccines is schematically depicted in Figure 1.3.

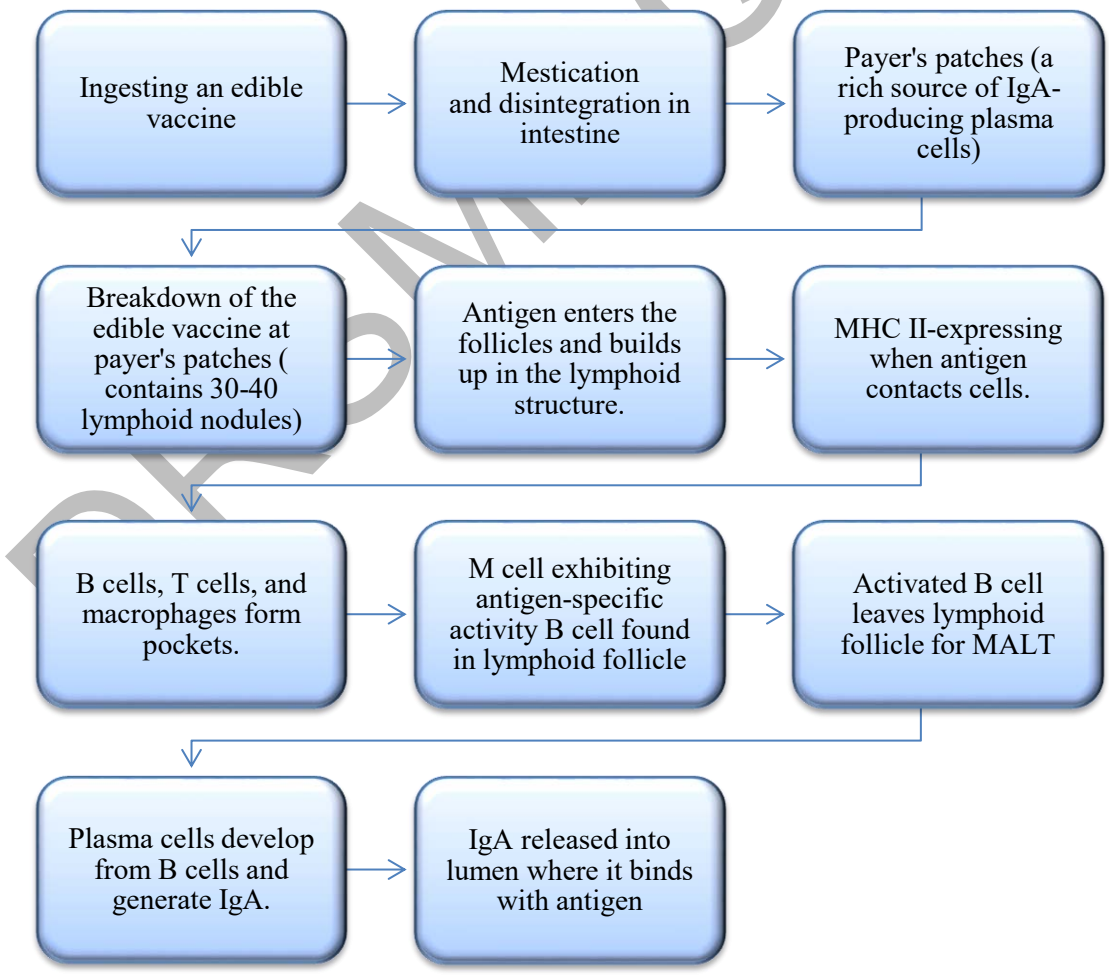


Figure 1.3: Plant-based vaccines' immunological mechanism.

1.7.2. Advantages of plant-based edible vaccines

The use of edible plants as a source of food and the creation of vaccinations for both people and animals has several benefits. The manufacturing of vaccines using a plant-based system can give a biotechnological solution since it allows for high-yield, cost-effective production while also reducing issues with distribution and storage (Concha *et al*., 2017). Plants can undergo post-transcriptional modification and appropriate folding of produced proteins, just like other eukaryotic species, and as a result, they can synthesise complex proteins, although they differ slightly from mammalian cells in terms of their glycosylation pattern (Kolotilin *et al*., 2014; Sohrab, 2020; Su *et al*., 2021).

The preservation of edible vaccines does not require a very cold environment. They can be supplied orally by directly consuming a specific transgenic plant part to protect against a particular infectious disease. In comparison to conventional vaccine production techniques like fermentation technology, plant-derived edible vaccines can be produced affordably in high bulk without requiring additional purification and processing stages. If natural edible plants are modified to produce a specific vaccine, the transport mechanism can also be abolished (Das *et al*., 2021; Michová *et al*., 2022). Transgenic seeds may be easily preserved for a long time period at room temperature and are also widely available as a source of vaccines. A big tribute to the nuclear transformation technology for plant transformation, which has reportedly been responsible for producing vaccine antigens against a number of disease-causing agents, including viruses and bacteria that are dangerous to human health and animals (Cardi *et al*., 2010; Dadar *et al*., 2016).

1.7.3. Disadvantages of plant-based edible vaccines

There isn't a single plant-derived edible vaccination for humans and other animals that is commercially available, despite numerous publications showing the successful generation of vaccine antigens in plants (Waheed *et al*., 2016). Because antigen expression levels can change in different areas of the plant, the fundamental constraint is the issue of optimal dose. Therefore, it is challenging to predict how much vaccine would be consumed by consuming transgenic plants in their raw form (Appaiahgari *et al*., 2017; Desai *et al*., 2010). If the patient consumes too much plant-derived vaccine, it may result in hypertension and allergic responses. Major obstacles to the development of this technology include cross-pollination between transgenic plants and other crop

plants, gene silencing issues, effects on soil microbes and insects, and disruption of the food chain (Tiwari *et al*., 2009).

1.8. Biotechnology and genetically engineered plant

Biotechnology is described as the use of a variety of methods to utilize and exploit biological organisms, as well as their systems and processes, in order to produce valuable products (Gibbs *et al*., 1983). Hungarian engineer Karl Ereky is credited with creating the term "biotechnology" (Ereky, 1919). The fundamental idea behind biotechnology is that researchers take particular genes of interest from animals' cells and also create them chemically or enzymatically using reverse transcriptase. A vector is then used to introduce the selected gene of interest into the expression system, such as an animal, plant, or microbe. As a result, this method, also known as genetic engineering or recombinant DNA technology, creates new genetic combinations that aid in the synthesis of new products. Transgenic organisms are those that have the gene of interest (Squires & Slotin, 1984).

The most dynamic and exciting area of genetic engineering is plant biotechnology. The first transgenic plant was created 30 years ago, and in 1996, genetically modified (GM) crops were first harvested. Since then, plant biotechnology is used to generate hormones, antibiotics, and vaccines for a variety of creatures, including humans, fish, and poultry animals. It is not only utilized to produce genetically modified crops (Laere *et al*., 2016).

1.8.1. Plant Tissue Culture

The practice of growing and replicating plant cells, tissues, and organs is known as plant tissue culture, on specific solid or liquid media in a sterile, controlled environment. Cell, in vitro, axenic, or sterile culture are other terms for plant tissue culturing (Thorpe, 2007). The theory of totipotency, underlying the regeneration of plants by tissue culture, was initially put forth in 1902 by the German researcher Haberlandt (Krikorian & Berquam, 1969). In many biochemical and growth experiments, the development of calluses, or masses of unorganized cells, on agar or in liquid suspension, is used. It is possible to examine differentiation, morphogenesis, and plant regeneration using segments of cultured stems, roots, leaves, or calluses (Gamborg *et al*., 1976).

Tissue and organ culture, protoplast culture, cell suspension culture, anther or pollen culture, and meristem culture for plant propagation without viruses are just a few examples of the many varieties that fall under this term (Kumar & Loh, 2012). The majority of the commercial method is based on micropropagation, which produces swift proliferation from microscopic stem cuttings, axillary buds, and to a lesser extent from somatic embryos, cell clumps in suspension cultures, and bioreactors (Meeting *et al*., 2004). Techniques for cultivating plant tissues have grown to be crucial for investigating a variety of theoretical and practical issues in research and development (Gamborg *et al*., 1976).

1.8.2. Preparation of explants for culturing

The explant is the portion of the plant that is removed and raised in culture. This could be axillary buds, cotyledons, hypocotyls, roots, shoot tips, zygotic embryos, or leaf discs. For this reason, these explants should be sterilized or handled with an aseptic technique; there are various straightforward ways to sterilize seeds. The aseptic plant that is created when these sterilized seeds germinate will be used as an explant for tissue culture (Gasic & Korban, 2006). Plants are not directly treated with chemicals to sterilize them through this process. The age and physiological state of the donor plant's explant may have a substantial impact on plant regeneration. For best outcomes in adventitious shoot regeneration in the case of cotyledon explants, the plant should be 3-6 days old. The first completely grown leaf is the preferred material for regeneration when leaves are the source of explant (Goh *et al*., 1990; Sharma *et al*., 1990).

1.8.3. Tissue culture media

Regeneration of tissue-cultured plants requires a certain type of culture medium. The particular culture media's composition is utilized to promote the growth and germination of shoots. For the cultivation of plant tissues, many mineral formulations are available. Gamborg's B5 medium (Gamborg *et al*., 1968), MS medium (Miller *et al*., 1955), and LS (Linsmaier & Skoog, 1965) are examples of the major media. Typically, the plant tissue culture media contain sucrose (2-3% w/v), vitamins, phytohormones, and other adjuvants such as coconut water. The manufacturers may recommend using ready-mixed powder sold commercially or preparing the nutritional medium by combining stock solutions of various chemical constituents (Altman & Hasagawa, 2012).

1.8.4. Plant growth regulators (PGRs)

Plant growth regulators also referred to as plant hormones, are organic compounds produced by plants that have an impact on physiological functions. In order to communicate between cells, plant growth regulators serve as chemical messengers (Sabagh *et al*., 2021). Plant growth regulators are chemicals that have an impact on many different aspects of a plant's life and parts, including flowering, aging, root growth, organ deformation and death, stem lengthening, fruit color enhancement, preventing leafing and leaf fall, mobilizing and translocating nutrients, stress tolerance and moisture relations in plants, maturity, disease resistance, and many other parameters (GRAHAM & Ballesteros, 1980; Harms & Oplinger, 1988).

Auxins, cytokinin, gibberellins, abscisic acid, and ethylene are the five currently identified types of plant hormones. They collaborate to control how cells grow and evolve. They can be broadly divided into two classes, plant growth promoters and inhibitors, based on how they function. Auxins, gibberellins, and cytokinin are plant growth promoters that encourage cell division, cell expansion, flowering, fruiting, and seed development. Abscisic acid, which is present in plant growth inhibitors, promotes plant dormancy and abscission while inhibiting plant development (Bons & Kaur, 2020; J. A. Roberts & Hooley, 1988). Auxins and cytokinin, the first two categories, are regarded as the "classical" types of PGRs (Rademacher, 2015).

1.8.4.1. Auxins

The Greek word auxins, which means "to grow," is the source of the English name auxin. Plants need auxins for a variety of developmental activities, including the creation of adventitious roots, somatic embryogenesis, and tissue swelling. They are also in charge of callus development (Tivendale & Cohen, 2015). Additionally, auxin concentration has been connected to a rise in callus formation and a decline in root growth (Bhatla, 2018; Bielach *et al*., 2017). Both synthetic and natural auxins are acceptable. Naturally occurring auxins include indole-acetic acid (IAA) and indole-butyric acid (IBA), whereas manufactured auxins include naphthalene acetic acid (NAA) (Rademacher, 2015).

1.8.4.2. Cytokinin

Isoprenoid-substituted adenine residues make up cytokinin. In addition to controlling root cell elongation, they also control apical dominance, fruit development, blooming,

leaf senescence, and other physiological and metabolic processes in plants (Bielach *et al*., 2017; Kamnek, 1992; Werner & Schmülling, 2009). The first cytokinin to be identified was zeatin (Letham, 1973). Other examples include kinetin and 6-benzylaminopurine (BAP) (Werner *et al*., 2001).

1.9. Plant Transformation

Foreign genetic material is introduced into plant cell during transformation. It can enter the cell either artificially or naturally. Three research teams reported the first evidence of plant modification utilizing the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid in 1983 (White *et al*., 1985). The first tobacco plant transformation case was reported later 1984 (De Block *et al*., 1984; Potrykus *et al*., 1985).

Many different ways have been used to transform plants. Figure 1.4 depicts the two main categories of gene transfer techniques: indirect gene transfer and direct gene transfer. In the indirect method, biological vectors like *Agrobacterium* and viral vector-mediated transformation are in charge of introducing foreign gene into plant genome. While in the direct technique, foreign gene transfer is accomplished through chemical and physical mechanisms. To deliver bare DNA into a protoplast, for instance, lipofection, microinjection, and electroporation are utilized. Other non-biological gene transfer techniques, such as silicon carbide whiskers, laser microbeam (UV) driven gene transfer, and biolistic, are used to introduce DNA into plant tissues (Birch, 1997). The nucleus, plastids, and mitochondria of a plant cell are all sites where genetic material can be found. Thus, for nuclear and chloroplast transformation, numerous new and better methods of plant transformation are applied (Keshavareddy *et al*., 2018b).

Plant cells can be genetically modified to express genes either temporarily or permanently. Transient gene expression usually lasts for a very short time. The inserted foreign genes are integrated into the host cell's genome during the stable transformation and passed down to succeeding generations. For the acquisition of several agronomic, horticultural, food, and industrial features, as well as new and modified proteins and pharmaceutical substances including human therapeutic proteins, vaccines, and antibodies, plant transformation is crucial. Plant transformation is very beneficial for controlling metabolic pathways, increasing plant yield, and phytoremediation (Gheysen *et al*., 1998; Hansen & Wright, 1999; Newell, 2000).

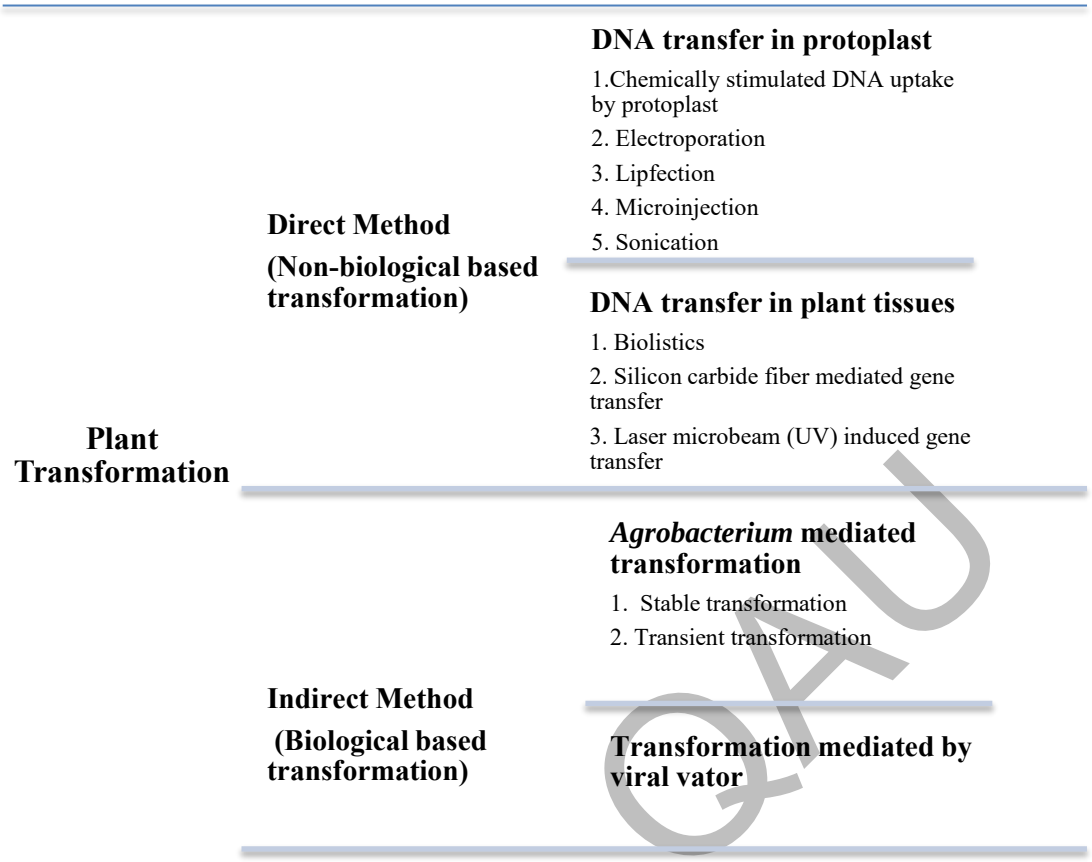


Figure 1.4: Techniques for transforming plants.

1.9.1. *Agrobacterium*-mediated Transformation

In the early 1980s, researchers successfully delivered particular DNA fragments into plant cells using the bacteria that causes crown gall disease, *Agrobacterium tumefaciens*, which initiated plant transformation as we know it today. By utilizing the soil bacterium *Agrobacterium tumefaciens'* inherent ability to modify host plants, transgenic plants have been developed. The most logical and natural candidate for transformation to consider was *Agrobacterium*, as it naturally transfers DNA (T-DNA) from the tumor-inducing (Ti) plasmid into the nucleus of plant cells, stably incorporating it into the plant genome (Chilton *et al*., 1977). Transient transformation and stable transformation have recently been proposed as two potential pathways for T-DNA expression during agroinfection (Janssen & Gardner, 1990; Krenek *et al*., 2015).

1.9.1.1 Stable Nuclear Transformation

Host cells are transformed by an *Agrobacterium*, and as a result, T-DNA is integrated into host DNA (Gelvin, 2010). Sensitivity of plants to transformation caused by *Agrobacterium* varies widely. These variations exist not only between species but also

between cultivars and ecotypes of same species. In comparison to other direct gene delivery techniques, the stable transformation of the plant by *Agrobacterium*, results in reduction in copy number of integrated genes in transformed plant. By doing this, the issues caused by the co-suppressive behaviour of transgenes are diminished. Therefore, for more stable expression of an important gene in transgenic plants, a simple and low-cost method is applied. For the purpose of identifying plants that have effectively undergone transformation, an active selection technique is also used. During the creation of the final vector, selectable marker genes are primarily used for herbicide or antibiotic resistance (Bhattacharjee *et al*., 2008; Hansen & Wright, 1999).

1.9.1.1.1. *Agrobacterium tumefaciens*

Crown gall disease is brought on by the gram-negative plant pathogen *Agrobacterium tumefaciens*, which affects a variety of plants. This phytopathogenic bacteria (figure 1.5) contains a tumor inducing (Ti) plasmid that causes virulence in plants. This pathogenic DNA integrates into the host genome after entering the host cell and causes a tumor. The Ti plasmid of *Agrobacterium* controls the bacteria's capacity to integrate DNA (Gelvin, 2010).

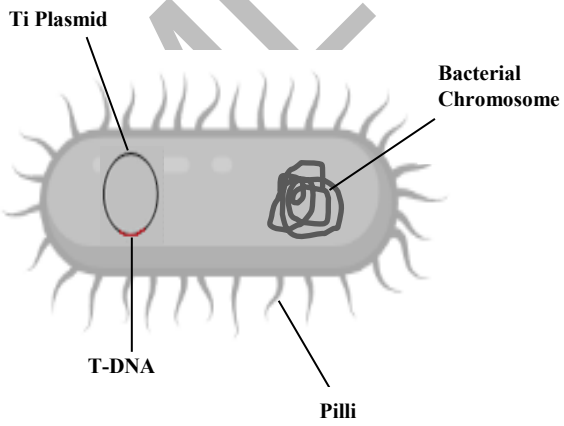


Figure 1.5: *Agrobacterium tumefaciens.*

1.9.1.1.2. T-DNA in *Agrobacterium tumefaciens*

The basic foundation for *Agrobacterium*-mediated transformation is the transfer of genetic material from the bacterial cell to the host cell. As the *Agrobacterium* infects the host cell, cleaved T-DNA enter plant cell, migrates into the nucleus via the nuclear pore complex and induces infection (Chilton *et al*., 1982). Inside the nucleus, the nuclear genome of plant cells combines with the pathogenic T-DNA. The complete T-DNA transfer mechanism is controlled by 35 virulent (vir) genes (De Framond *et al*., 1983; Yadav *et al*., 1980).

1.9.1.1.3. Ti Plasmid in *Agrobacterium tumefaciens*

T-DNA area and vir (virulence) region are two separate portions that make up the Ti plasmid, with the former region being surrounded by repetitive sequences of 25 base pairs known as left and right borders (Zhu *et al*., 2003); this is depicted in figure 1.6. Additionally, it has genes that control the production of auxin, cytokinin, and opine synthase.

There are various steps in the technique for T-DNA delivery by *Agrobacterium* infection. According to figure 1.7, these are: (1) signal from plant cell recognized by *Agrobacterium tumefaciens*, (2) processing of T-DNA, (3) migration of T-DNA into the host cell, (4) integration of T-DNA into the plant nuclear genome, and (5) expression of inserted T-DNA in the plant host cell.

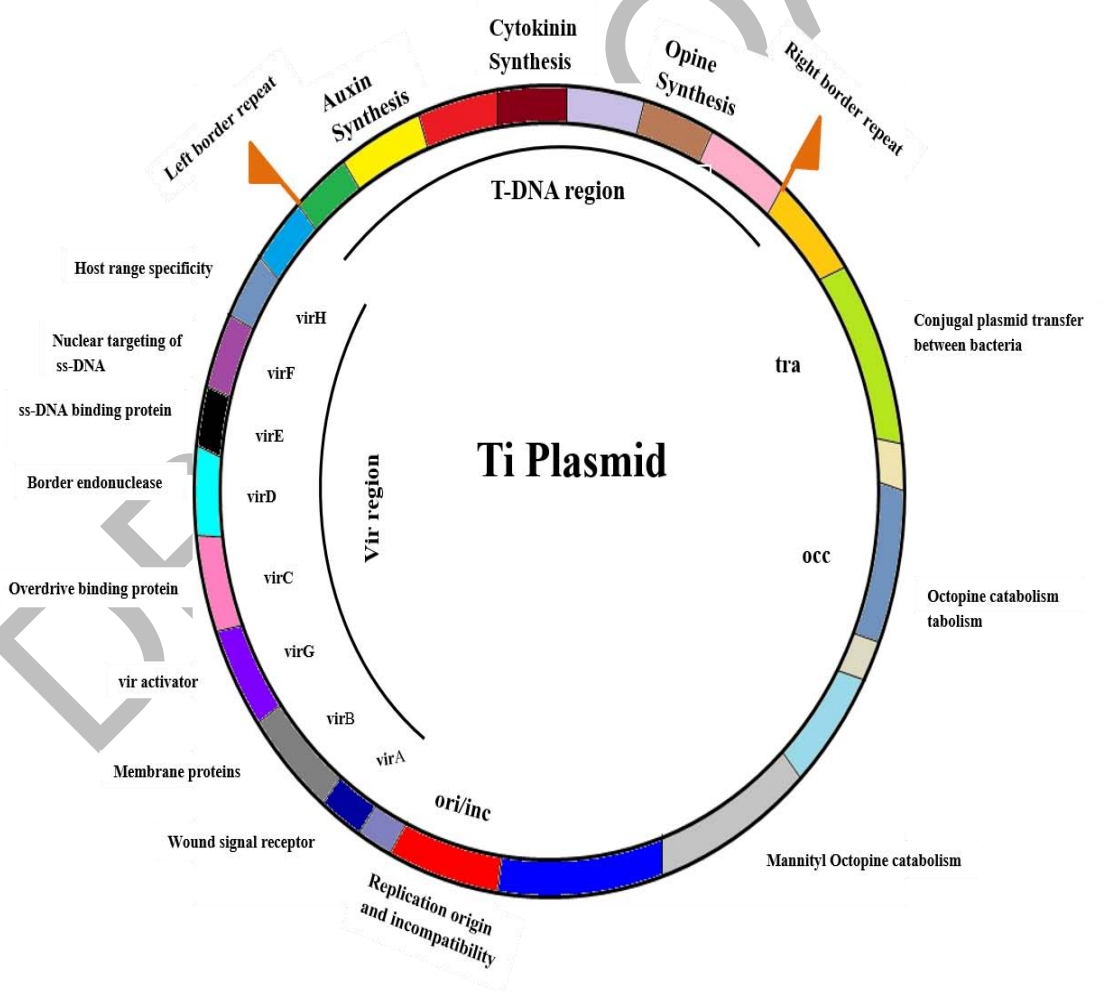


Figure 1.6: Ti plasmid in *Agrobacterium tumefaciens*.

1.9.1.1.4. Signal Recognition through *Agrobacterium tumefaciens*

The interaction between plants and *Agrobacterium* results in the release of chemicals. These substances include phenolic compounds and organic acid compounds (pH 5–5.8). The synthesis of phenolic chemicals from wound sites, such as acetosyringone and -hydroxy acetosyringone, affects bacterial chemotaxis (Zupan & Zambryski, 1995). Transmembrane receptors (VirA) on bacterial cells recognize these signals, which causes the phosphorylation of the VirG protein (Hood *et al*., 1993; Van Roekel *et al*., 1993). Figure 1.7 illustrates how VirG phosphorylation activates other vir genes (Stachel *et al*., 1986; Stachel & Zambryski, 1986).

1.9.1.1.5. T-DNA Processing

The T-DNA from Ti-plasmid must be cleaved by VirD1 and VirD2. The 25 bp border regions on the bottom strand of T-DNA serve as the excision site for VirD1 and VirD2. While VirD2 cleaves T-DNA from both border sequences, VirD1 is a site-specific helicase that unwinds the helical DNA strands. As a result, a single strand of DNA known as the T-strand is created (Martineau *et al*., 1994). The 3' end of the T-strand serves as the priming point for the regeneration of the bottom strand of T-DNA, while VirD2 caps the 5' end of the T-strand. Finally, VirC1 binds the appropriate T-DNA border sequence (De La Riva *et al*., 1998; Karthikeyan *et al*., 1996; Machida *et al*., 1993).

1.9.1.1.6. T-DNA Migration

Major roles in delivering the T-DNA to the host cell nucleus are played by VirD2 and VirE2. The nuclear localization signal (NLS) sequence found at the C-terminus of the VirD2 and VirE2 proteins directs the T-strand to the nucleus of the host cell (Pitzschke & Hirt, 2010). Through the type IV secretion system (T4SS), the VirD2/T-strand complex exits the bacterial cell through the pilus. The 11 VirB and VirD4 proteins make up the T4SS (Baron & Zambryski, 1996). Furthermore, the T-strand is shielded from nucleases by these two vir proteins. T-DNA that has both VirD2 and VirE2 is referred to as Ti-complex during transfer. The endoplasmic reticulum moves the Ti-complex from the cytosol to the nucleus (Guo *et al*., 2019). Figure 1.7 depicts the general mechanism of T-DNA migration toward the plant cell nucleus.

1.9.1.1.7. Nuclear Integration of T-DNA in plant cell

Stable transformation refers to the random integration of T-DNA into the nuclear genome of plant cells, where it permanently expresses the transgenes. It is still unclear exactly how T-DNA gets integrated into the nucleus at the molecular level.

VirE2 interacts with the bZIP transcription factor VIP1, which facilitates the binding of the VirE2/T-strand with mono-nucleosomes (Iwakawa *et al*., 2017). When the ubiquitin-proteosome system reaches the nucleus, it breaks down the components of the proteins to reveal the T-strand. VirF facilitates the T-disassembly complex's process (Lacroix & Citovsky, 2019; Tzfira *et al*., 2004).

The T-strand cannot bind to the host genome directly because VirD2 lacks ligase function. Additionally, site breakage can occur in the host genome as a result of metabolic processes or environmental pressures such being exposure to *Agrobacterium*. As illustrated in figure 1.7, it is possible that the host cell's DNA polymerase converts the single-stranded T-DNA into a double-stranded structure that then joins with the site breaks of the host DNA by a process known as non-homologous end joining (NHEJ) repair. This would allow the T-DNA to successfully integrate into the host nuclear genome (Nishizawa-Yokoi *et al*., 2021; Saika *et al*., 2014).

1.9.1.1.8. T-DNA Expression in plant cell

T-DNA has two potential outcomes upon integration into the nuclear genome of a plant cell. Figure 1.7 shows that it is either expressed with varying levels of expression within the plant cell or it has integrated but isn't expressed at all (Ozyigit, 2012; Primrose & Twyman, 2013).

1.9.1.1.9. Mechanism of stable Transformation

The process of transformation mediated by *Agrobacterium* is intricate. As it enables the integration of stable and single-copy sequences into the plant genome, it is utilized to genetically alter many different plant species. Two crucial points were emphasized to achieve successful transformation: (1) acetosyringone addition during co-cultivation processes and (2) Using cells that are actively dividing.

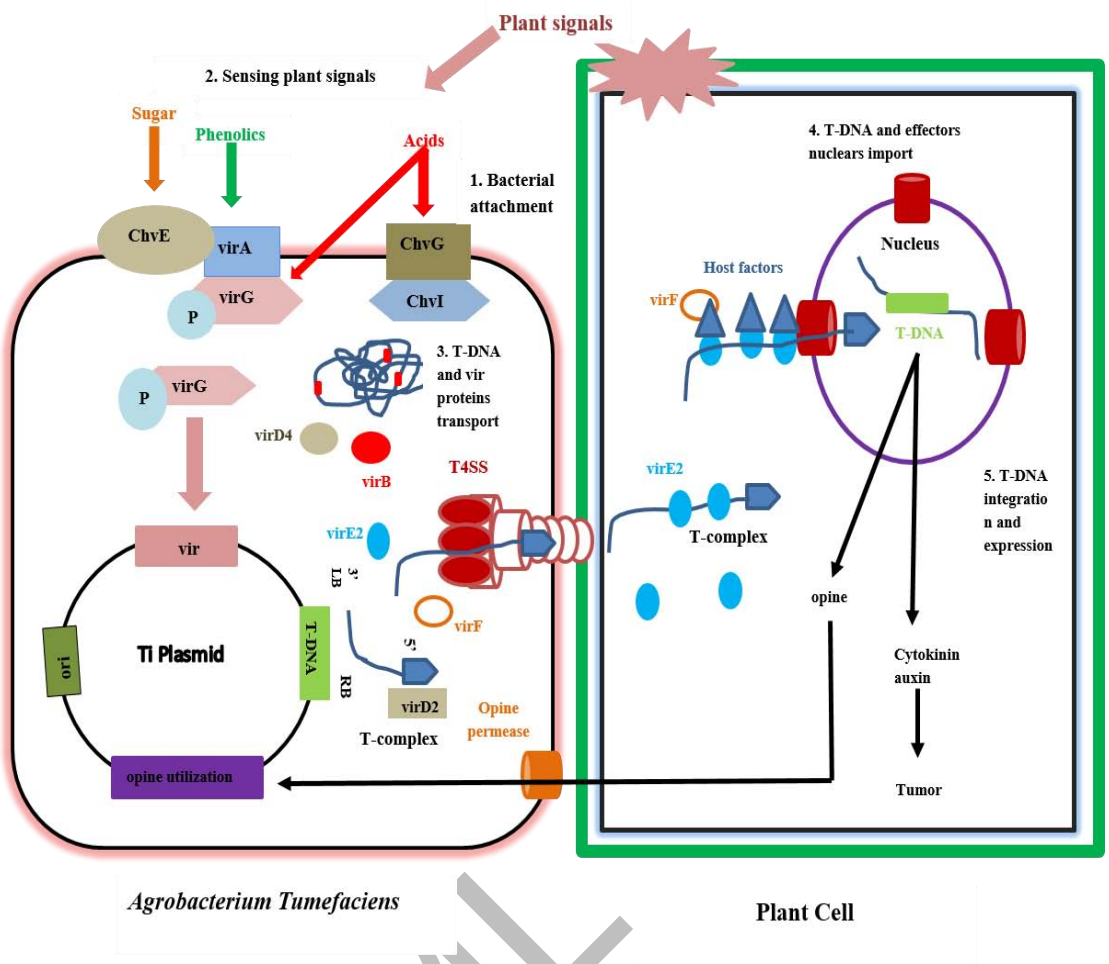


Figure 1.7: Process of *Agrobacterium*-mediated T-DNA delivery and its integration into the plant genome.

A schematic illustration of the many processes involved in *Agrobacterium*-mediated transformation is shown in figure 1.8. These include the following: (1) seed sterilization and inoculum preparation; (2) explant selection and preparation; (3) selection of transformed explants using optimal antibiotic concentrations; (4) regeneration of explants; and (5) acclimatization and molecular identification (Keshavareddy *et al*., 2018a; Pratiwi & Surya, 2020).

1.9.1.1.10. Advantages and disadvantages of stable transformation

The main benefits of this procedure are that plants changed with it have a higher transformation efficiency than plants transformed with other techniques. Transgene expression lasts a very long time. This approach is economical (Saba *et al*., 2019; Waheed *et al*., 2015). The only drawback of stable transformation is that, in comparison to other methods, overall plant regeneration is relatively challenging.

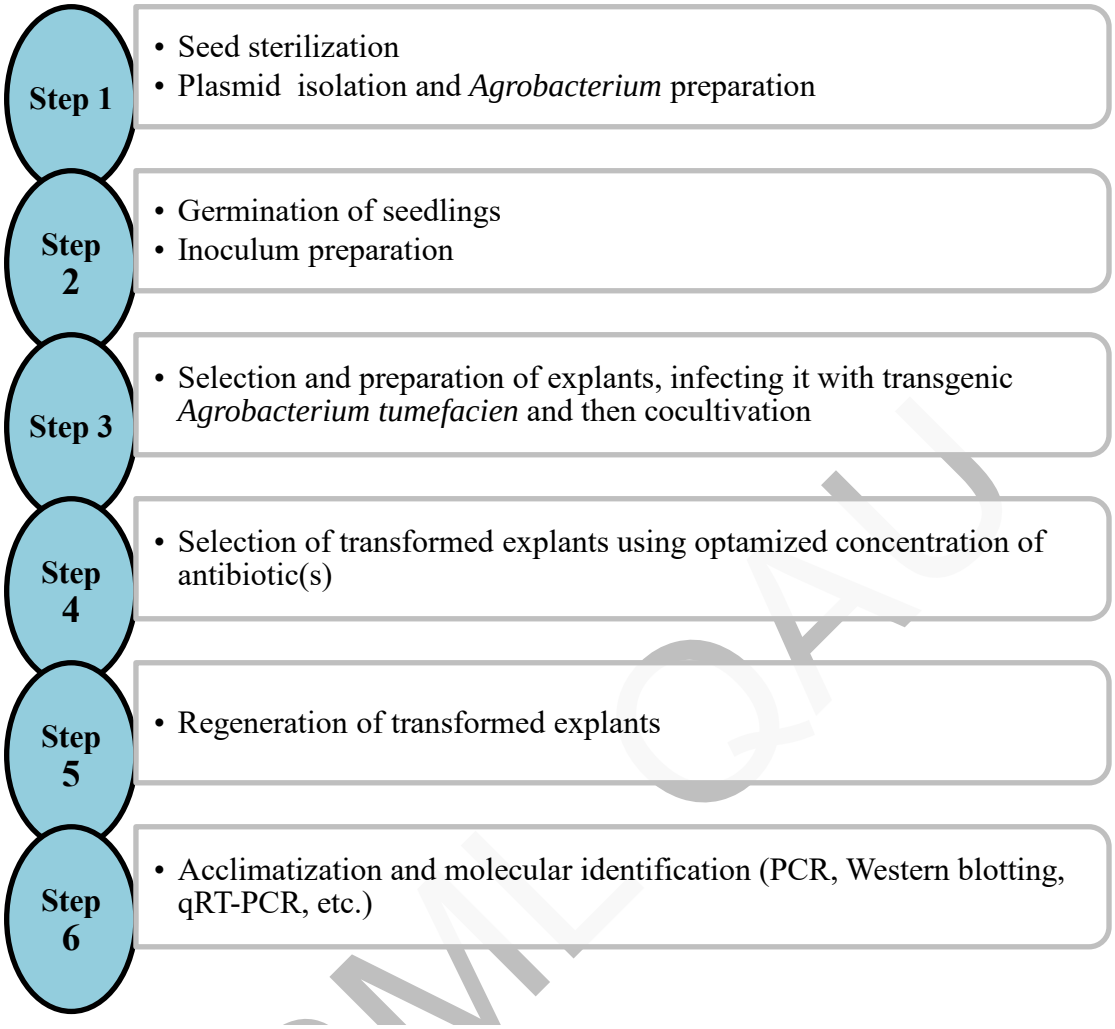


Figure 1.8: Schematic representation of *Agrobacterium*-mediated stable transformation.

1.9.1.2. Transient Transformation

Transgene transfer and expression without T-DNA integration occur as a result of cells being transformed by an *Agrobacterium*-mediated transient transformation (Gelvin, 2010). When *Agrobacterium tumefaciens* interacts with plant cells or tissues, a single-stranded copy of T-DNA (transfer DNA) is complexed with proteins and transferred into the nucleus of the plant tissues (Sainsbury, 2020). The inserted T-DNA copies are only momentarily present in the cell nuclei and do not integrate into the plant genome. The nuclear transcription of the non-integrated T-DNA copies results in a temporary expression for a few days (Janssen & Gardner, 1993; Joh *et al*., 2005). The transient expression after *Agrobacterium* infection in plant cells typically peaks between 2-4 days after infection and lasts for 10 days, declining gradually for each transformed plant cell. A high level of transient expression in infected plant cells or

tissues may be observed; it appears that initially, plant cells acquired T-DNA with large copy numbers from *Agrobacterium*, cells show maximal transient expression, and then expression peak is gradually diminished due to instability of injected non-integrated T-DNA copies (Kapila *et al*., 1997; Sánchez-Alvarez *et al*., 2019). The PEG approach, electroporation, the biolistic method, and the agroinfiltration technique are among the transient ways for transgenic expression.

1.9.1.2.1. Agroinfiltration

Agroinfiltration is a straightforward but efficient technique for transgene delivery into the host cell (Shen *et al*., 2014; H. Wang & Jiang, 2011; Zhao *et al*., 2017). Through the use of physical or vacuum infiltration procedures, direct transfer of T-DNA is involved (Kaur *et al*., 2021; Sainsbury & Lomonossoff, 2014). After infiltration, chaperones transport the T-DNA to the nucleus, where a small amount of it fuses with the host nuclear genome, causing stable transformation. In contrast, transgenic expression is high but transient when T-DNAs do not bind to the host genome. Furthermore, leaf tissues are the primary target of this technique (Nishitani *et al*., 2021).

1.9.1.2.2. Advantages and disadvantages of Transient agroinfiltration Transformation

Transient expression systems have several benefits, including cost-effectiveness and speedy and high transgene expression in a limited amount of time. The only place where cells are damaged with this non-invasive process is where the infiltration occurred. This technique is highly flexible because different parts of the sample can be infiltrated with one or more constructs, allowing the same infiltrated sample to be used for multiple experiments (Heenatigala *et al*., 2018; Nosaki & Miura, 2021).

The technique has a few drawbacks, including transgene loss due to host cell replication and the need for re-infection because the expression is transient (Li *et al*., 2021; Zhang *et al*., 2020).

1.10. Edible Plant

Every human needs food to survive, and they either rely on plants directly or indirectly. For practically all animals that cannot manufacture their own food, plants are the best source of food and metabolic energy. Edible plants are those that have portions that humans can comfortably eat. 20,000 species are known to be edible (Parodi *et al*., 2018). The most popular edible plants are raised all over the world in a variety of

habitats and temperatures, both commercially and in backyard gardens. Garlic, cabbage, and fruit crops like oranges, apples, bananas, and pineapple are examples of small-scale crops, whereas crops farmed on a big scale often include maize, rice, wheat, oats, and potatoes (Blancke, 2016). Numerous vegetable varieties are grown specifically for their leafy sections. Many plant leaves, including those of cabbage, spinach, mustard greens, Swiss chard, and turnip greens, are eaten as vegetables as well as spices like oregano. Leafy vegetables are any raw plant leaves that are consumed, sometimes along with petioles and shoots. Although they originated from various plant species, most of them are similar to other leaf vegetables in terms of nutrition and preparation. There are more than a thousand edible species of short-lived herbaceous plants, including spinach and lettuce (French, 2015).

1.11. Primary and Secondary metabolites

Numerous studies are being conducted to determine whether phytochemicals, which are plant components with specific bio-activities toward animal metabolism and biochemistry, have health-promoting properties. As potential nutritionally active substances, it is crucial to establish the scientific basis for their usage in foods (King & Young, 1999). Phytochemicals may have health benefits as substrates for biochemical reactions, cofactors of enzymatic reactions, enzyme inhibitors, absorbents that bind to and remove undesired elements in the intestine, ligands that agonize or antagonize cell surface or intracellular receptors, scavengers of reactive or toxic chemicals, substances that promote the absorption and or stability of important nutrients, and selective growth factors for beneficial cells. These phytochemicals include fiber, terpenoids, phenolics, and alkaloids (Rochfort & Panozzo, 2007; Winston & Beck, 1999). The scientific evidence for phytochemicals' protective effects against diseases like cancer, coronary heart disease, diabetes, high blood pressure, inflammation, microbial, viral, and parasite infections, mental illnesses, spasmodic disorders, ulcers, etc. is based on chemical mechanisms employed in vitro and cell culture systems, various disease states in animals, and human epidemiology (Craig, 1997).

Flavonoids, phenolic acids, and polyphenols are the three most significant categories of dietary phenolics. The most studied and largest group of plant phenols are flavonoids. Hydroxybenzoic and hydroxycinnamic acids, which are both commonly used, are within the broad group of phenolic acids. Tannins also referred to as phenolic polymers, are high molecular weight molecules that fall into two categories, hydrolyzable tannins,

and condensed tannins. Even within a single food, there is a substantial degree of diversity in the quantification of food phenolics, according to early findings. Phenolics are biologically active substances with potential anti-disease effects (King & Young, 1999).

1.12. *Spinacia oleracea*

A green annual plant in the Amaranthaceae family that is grown for its edible leaves is known as spinach (round leaf spinage). The name "spinach" is derived from the Persian word "ispanai" which means "green hand." It eventually became the Latin word "spanachia" which later became the English words "spinage" and "spinach" (Madhvi & Manju, 2014). Spinach is a temperate-area plant, and at 15 to 18 degrees Celsius in the spring or the fall, the maximum leaf is produced. The germination temperature for seeds is 4 °C. With a critical day length of 13–14 hours, spinach is a plant that must grow under lengthy days. It appears that four to eight photoperiods are necessary for induction (Chitwood, 2016). The minerals found in spinach leaves, which make up to 1.8 percent of the dry weight, are present in all leafy vegetables and are crucial sources of nutrition. Due to relatively high iron content of spinach leaves (4-6 mg per 100g dry weight), which gives the vegetable its unique nutritional value, spinach is advised for people with anaemia and young children (Rashid *et al*., 2020).

1.12.1. Taxonomic Classification

The leafy vegetable spinach (*Spinacia oleracea L.*), which is consumed throughout the world, is significant economically. The Amaranthaceae family includes the green vegetable spinach. Other significant agricultural products in this family of the Caryophyllales order include beet, quinoa, and amaranth.

Table 1.1: Scientific classification of *Spinacia oleracea L.* (https://www.gbif.org/species/3083647)

Domain	Eukaryota
Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Caryophyllales
Family	Amaranthaceae
Genus	*Spinacia*
Species	*oleracea*

1.12.2. Morphology

Annual plants like spinach have distinct vegetative development and reproductive stages. Usually, seeds are sown near the end of winter or the early spring. The rosette-shaped leaves can have crinkly or flat appearances (Ma *et al*., 2016). Simple leaves of the spinach plant have an approximate length and width of 2–30cm (0.8–12.0in) and 1–15cm (0.4–6.0in) respectively. These leaves grow from the center of the plant. The shrub bears 3–4 mm (0.1in) wide, yellow-green, little blooms (Ebadi-Segheloo *et al*., 2014). The flowers grow little fruit clusters with seeds in them. Spinach plant that lives for just one growing season and grows to a maximum height of 30cm (12 in). Originally from Iran, spinach is a product of ancient Persia. The two main forms of spinach are the smooth leaf variety and the crinkle leaf variety, and they range in color from light to dark green (Eftekhari *et al*., 2010; Meng *et al*., 2017).

1.12.2.1. Types of spinach

There are three main types of spinach based on morphology. The most popular type of spinach, flat-leaf spinach, comes in first, spatulas, spade-shaped leaves, a delicate texture, and a somewhat sweet flavor set flat-leaf spinach apart. The second type of spinach has very dark green, curled, crinkly leaves and is also known as curly leaf spinach. The third kind, Semi-savoy spinach, has somewhat crinkly leaves that are nonetheless crisp. Figure 1.9, is depicted (Grevsen & Kaack, 1997).

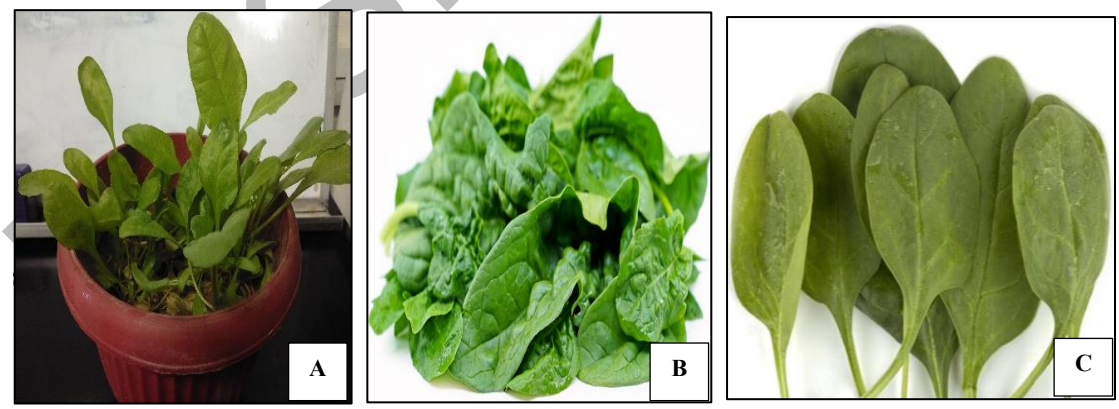


Figure 1.9: Different types of *Spinacia oleracea*. A. flat-leaf spinach, B. savoy spinach, C. Semi-savoy spinach (Grevsen & Kaack, 1997).

1.12.3. Center of Origin and World distribution

Although its exact origins are unknown, it is thought that domesticated spinach was first developed in the region of Iran, former Persia, some 2,000 years ago (Frye, 1962).

Since no references to spinach have been discovered in Greek or Roman writings, and since the earliest known written accounts of the crop date from the fourth century A.D in Mesopotamia, it is assumed that the crop has only recently become widespread (Dandamaev, 1989; El Faz, 1995). Spinach likely entered China via Nepal because the earliest written record of it is from China and dates to the 7th century. Although accounts claim that the Saracens brought spinach to Sicily in the ninth century, the first recorded accounts of its cultivation in Muslim lands originate from the tenth century, and the first documented accounts of its cultivation in continental Europe date from Moorish Spain in the twelve (Heine, 2018).

According to the historical data cited above, spinach traveled on two different paths, first to Southern and Eastern Asia and then to Africa, the Mediterranean, and Northern Europe, from where it was later brought to the Americas (Hallavant & Ruas, 2014; Laufer, 1915). Two major groupings of spinach cultivars, known as Asian-type and Western-type cultivars, are frequently distinguished from one another. These differences are likely due to the various selection regimes used in the two regions (She *et al*., 2018). Recent phylogenetic analyses have demonstrated a relationship between spinach accessions' genetic relationship and geographical origin, with recurrent distinctions between Asian and Western cultivars (Ribera *et al*., 2021a). Asian cultivars maintained the wild spinach's long petioles, narrow, hastate, and smooth leaf shape, while Western cultivars modified the hastate leaf shape to a round form, enlarged the leaves, and introduced the savoy leaf texture (Ribera *et al*., 2021b; Xu *et al*., 2017).

1.12.4. Nutrition significance and phytochemical properties of spinach

The mineral and vitamin content of spinach is high. Spinach has a far wider range of minerals and vitamins than other popular leafy green veggies (Morelock & Correll, 2008). Amazingly, it contains significant levels of Vitamin B9 (folate), one of the essential medicines recognized by WHO and used as a supplement for pregnant women and to prevent anaemia. Additionally, spinach contains significant amounts of carotenoids, such as vitamin A, lutein, and zeaxanthin, which are known to be effective antioxidants and scavengers of reactive oxygen species. Additionally, spinach includes additional compounds with strong antioxidant capabilities, such as vitamin C and vitamin E (de Benoist, 2008; J. L. Roberts & Moreau, 2016).

The crop also contains notable amounts of other phytochemicals in the form of phenolic compounds. Many phenolic substances, including flavonoids and polyphenols, have

antioxidant effects (Lin *et al*., 2016). Studies on the antioxidant activity of spinach in model animals have revealed anti-aging, anti-proliferative, and decreased oxidative-stress benefits. Consuming spinach has antioxidant benefits in humans as well, as numerous studies have demonstrated (Chun *et al*., 2005).

Additionally, the research demonstrated that eating spinach has other benefits, such as anti-inflammatory, anti-obesity, hypoglycemic and lipid-lowering effects in both human and animal models (Maeda *et al*., 2008). Additionally, data on anti-cancer activities in mouse and in vitro research was accumulated, with the main theme being the suppression of cancer cell development (Fiedor & Burda, 2014; Issa *et al*., 2006). Table 2 lists the nutritional benefits and phytochemical characteristics of spinach (Ribera Tort, 2019).

Table 1.2: Nutritional value on average per 100g of raw spinach and the corresponding Dietary Reference Intake (DRI)

Nutritional Profile	Content	% DRI
Energy	23kcal	1.1
Macronutrients		
Carbohydrates	3.63g	2.8
Dietary Fiber	2.2g	8.8
Sugars	0.42g	0.8
Lipids (fat)	0.39g	1.4
Protein	2.86g	6.7
Water	91.4g	
Minerals		
Calcium	99mg	9.9
Iron	2.7mg	15.1
Magnesium	79mg	25.1
Manganese	0.9mg	49.8
Phosphorus	49mg	7.0
Potassium	558mg	11.9
Sodium	79mg	5.3
Zinc	0.53mg	6.6
Vitamins		
Vitamin A	496µg	67.0
Vitamin B9	194µg	48.5
Vitamin C	28.1µg	37.5
Vitamin E	2.21µg	14.7
Vitamin K	482.9µg	536.6
Others		
Lutein + zeaxanthin	12.2mg	-
Total phenolics	32.5mg	-
Oxalate	2.08mg	-
Nitrate	146mg	-

1.12.5. Medicinal significance of *Spinacia oleracea*

Green leafy vegetables include biological elements that have significant pharmacological or therapeutic value. Green leafy vegetables include phytonutrients that provide numerous general health advantages, including defense against eye problems, oxidative stress, iron deficiencies, etc. Green leafy foods are beneficial for human health since they increase nutritional status and lower the chance of developing certain diseases like diabetes, cancer, and hepatotoxicity. The current study identifies the phytochemicals that are present in vegetables and their pharmacological advantages. Since ancient times, *Spinacia oleracea* has been used as a food source. It has a lot of nutrients and minerals that are beneficial for preserving human health. The nutrition and health of the growing global population is a significant forthcoming concern, particularly in poorer nations. Plant food serves as an energy source and provides macronutrients and micronutrients that are vital for good health. Additional health advantages like antioxidant activity are also included (Mane *et al*., 2015).

1.12.5.1. Strengthens the Immune System

Vitamin E and magnesium, two vitamins and minerals found in spinach help to enhance your immune system. You are protected from disease-causing viruses and bacteria by this system. Additionally, it protects your body from other dangers including contaminants or toxins (Mukherjee *et al*., 2016).

1.12.5.2. Keeps Eyes Healthy

Spinach contains carotenoids called lutein and zeaxanthin which reduce the risk of developing chronic eye diseases. For instance, they reduce the risk of developing cataracts, just like vitamin C. Spinach is a great source of vitamin A, which helps with vision (Tewani *et al*., 2016).

1.12.5.3. Fights Free Radicals

The majority of fruits and vegetables, including spinach, contain antioxidants. Free radicals are metabolic by-products. They may result in oxidative stress, which speeds up aging and raises your risk of diabetes and cancer. Antioxidants included in spinach, however, serve to combat oxidative stress and lessen the harm it causes (Jiraungkoorskul, 2016; Nayak *et al*., 2010).

1.12.5.4. Wound Recovery

Spinach contains a lot of vitamin C, which is good for your body because it helps it build collagen, which is necessary for healing wounds. In addition to supporting the healing process, vitamin C also increases the amount of iron that the body absorbs from plant-based diets (Güler *et al*., 2015).

1.12.5.5. Lowering blood pressure

High quantities of nitrates found in spinach have been demonstrated to help regulate blood pressure and lower the risk of heart disease (Liu *et al*., 2013).

1.12.5.6. Aids in Good Bone Health

Vitamin K, which is included in spinach and helps maintain strong bones, is proof that getting enough vitamins is excellent for your health. It also helps the body better absorb calcium. Each cup of spinach has 250mg of calcium, which the bones and teeth need. Calcium acts as a bone-strengthening agent and maintains bone health (Adhikary *et al*., 2017).

1.12.5.7. Anti-inflammatory Properties

Neoxanthin and violaxanthin, two anti-inflammatory components that control inflammation, are present in this superfood. Due to its strong anti-inflammatory content, it can aid in the prevention of osteoporosis, migraine, asthma, arthritis, and headaches (Garg *et al*., 2010).

1.13. Selection of *Spinacia Oleracea*

Spinacia oleracea is a variety of leafy vegetables. Spinach is the world's healthiest vegetable, according to WHO. It is rich in vitamins and minerals and also contains beta-carotene, lutein, xanthin, and flavonoids. Spinach is distinctive among green vegetables due to its high nutritional value, antioxidant qualities, and vitamin content (Swiader *et al*., 1992). Spinach is utilized as a natural and non-toxic antibacterial agent for fish (Krishnan & Joice, 2018).

Genetically modified spinach is regarded as a good source for the creation of edible vaccines due to its nutritional and therapeutic importance. Both the HIV-1 Tat protein (a potential vaccine candidate) and the anthrax vaccine are being tested in spinach, a plant-derived, edible carrier (Saxena & Rawat, 2014). In many human diseases, the spinach plant has also been employed as an edible vaccination. As a result of its various benefits, which include the ability to produce fish vaccines against vibriosis, it is a

beneficial and reliable eating plant.

1.14. Aims and Objectives

The current study was designed to cure a bacterial disease causing vibriosis in fish. We were interested in producing the antigen at a large scale that is involved in this particular disease. The plant-based vaccines are currently known in the market because of their use and cost-effective procedure. Hence, we have expressed OmpK gene in *Spinacia oleracea* by *Agrobacterium*-mediated nuclear stable transformation and transient agroinfiltration transformation. The present study consisted of the following objectives:

- To optimize the best seed sterilization method for *Spinacia oleracea*.
- To optimize the best-regeneration media for *Spinacia oleracea.*
- To optimize the concentration of hygromycin for nodal explants of *Spinacia oleracea* for the identification of transformed plants.
- To carry out *Agrobacterium*-infiltration on the leaves of *Spinacia oleracea* for testing the transient expression of the OmpK gene.
- To carry out *Agrobacterium*-mediated stable nuclear transformation on the nodal explants of *Spinacia oleracea* for testing the stable expression of the OmpK gene.
- To confirm the transformations by performing different molecular biology techniques such as PCR and qRT-PCR.
- To estimate and quantify the OmpK protein by Dot blotting, Western blotting, and ELISA.

2. Materials and Methods

This research study aimed to optimize the best shooting regeneration medium after seed germination, optimization of antibiotic hygromycin for wild-type *Spinacia oleracea* plants, and efficient genetic transformation of *Spinacia oleracea* with *Agrobacterium tumefaciens* with plasmid-containing OmpK gene from *Vibrio anguillarum*. Additionally, to confirm the integration and expression of the OmpK gene via the polymerase chain reaction (PCR) and by using other molecular biology techniques in transformed plants. All utilized materials and followed methods in this present study are illustrated in this chapter. The current research study was performed in the Plant Biotechnology Laboratory, Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, and Pakistan.

2.1. Materials

2.1.1. Laboratory Instruments

All laboratory instruments were used after getting information and proper guidelines from the instruction book of each relevant instrument. The information on various instruments which were used for this research work is given in table 2.1.

Table 2.1: Laboratory Instruments

Appliances	**Company**
Autoclave	Yamato, USA
Balance	Ohaus Corp, USA
Blotting apparatus	Major Science, USA
Centrifuge	Eppendorf, Germany
Conventional PCR	Applied biosystems,USA
Electroporator	Biorad, USA
Freezer	Dawlance, Pakistan
Freezer (-70°C)	VWR, USA
Gel casting device	Cleaver Scientific, USA
Gel documentation system	Alpha View SA Version 3.4.0.0, USA
Colibri microvolume spectrometer	Titertek Berthold, Germany
Hot plate stirrer	IKA Labortechnik, Germany
Magnetic stirrer	VWR, USA
Minitron Incubator	VWR, USA
Micropipettes	Eppendorf, Germany
Orbital shaker	VWE, USA
Power supply	Biometra, USA
Real-Time PCR	MYgo, Ireland
Spectrophotometer/Microplate reader	ThermoScientific multiskan GO
Vertical Gel electrophoresis apparatus	Cleaver Scientific, USA
Vortex	Scientific Industries, USA
Water bath	Precision, 180 Series, USA

2.1.2. Laboratory Glass and Plastic wares

The glass wares were reused, for example, glass plates (9cm), magenta boxes, flasks, beakers, measuring cylinders, etc. The glass wares were washed by using commercial liquid detergent (Max), later they were dipped in bleach solution for 3 to 4 hours and dried in a dry oven at 150ºC. For sterilization, then they were autoclaved by keeping the pressure 15psi (pound per square inch) at 121ºC for 20 minutes. In the current research study, different plastic wares and glassware were used which had been purchased from various manufacturing and supplying companies. The detail of these materials is given in table 2.2. Almost all plastic wares were discarded after their first usage, for example, petri plates (plastic 6cm), Eppendorf tubes, micropipette tips, microcentrifuge tubes, syringes, syringe filters, etc.

Table 2.2: Consumables

Consumables	**Company**
Glassware	Pyrex, USA
1.5/02 ml Eppendorf tubes	Axygen Scientific, USA
15/50 ml Falcon tubes	The corning® USA
PCR tubes	Thermo Scientific USA
Petri plates	Pyrex, USA
Gloves	Qualtex Malaysia
Nitrile gloves	Gen-X, Malaysia
Magenta-boxes (Jars)	Pakistan
Pipette Tips	Extra Gene, USA
Parafilm PM-96	Bemis, USA
Surgical blades	XINDA, China
Syringe filter (0.2 µm)	Sartorius, Germany
Microtiter plates	Costar, USA
Whatman filter paper	GE Healthcare UK

2.1.3. Chemicals and Reagents

Chemicals and reagents of high-grade purity were used in this experimental work. They were purchased from different chemical manufacturers and suppliers. The most important media Murashige and Skoog (MS) and Agar and Luria Broth (LB) were obtained from Sigma Chemical Company, USA, and "DIFCO" Laboratories, USA respectively. All solutions of chemicals and reagents were made by using distilled water as solvent. The detail of chemicals and reagents is summarized in table 2.3.

Table 2.3: Chemicals and Reagents

Chemicals	**Company**
Agar (Bacteriological grade)	Bioworld, Dublin, Ireland
Ammonium persulphate (APS)	AnalaR^{TM}, England
Ammonium sulfate ($(NH_4)_2SO_4$)	GPR, BDH laboratory supplies, UK
Agar Plant TC	Phytotech lab, US
Bovine serum Albumin (BSA)	MP Biomedical, USA
Boric Acid	AnalaR^{TM} England
Bromophenol Blue	Sigma-Aldrich, USA
β-mercaptoethanol	Biochem, USA
Calcium Chloride	AnalaR^{TM}, England
Cefotaxime	Sanofi-aventis Pakistan
Chloroform ($CHCl_3$) Analytical grade	Sigma-Aldrich, USA
Coomassie	AnalaR^{TM} England
CTAB	Oxford lab chem,India
Dimethyl sulfoxide (DMSO)	Biochem, USA
Ethanol 99.7-100% pure v/v	Merck, Germany
Ethidium Bromide	Sigma-Aldrich, USA
Ethylenediamine tetra-acetic acid (EDTA)	AnalaR^{TM} England
Glacial Acetic acid	Merck, Germany
Glucose	GlaxoSmithKline, UK
Glycerol (87%)	AnalaR^{TM} England
Glycine	Merck, Germany
HEPES, Free Acid ($C_8H_{18}N_2O_4S$)	PhytoTechnology Laboratories, USA
Hydrochloric Acid	Sigma-Aldrich, USA
Imidazole	Sigma-Aldrich, USA
Isoamyl-alcohol	AnalaR^{TM} England
LB Broth (Miller)	Microgen India
Magnesium chloride 6-hydrate ($MgCl_2.6H_2O$)	AnalaR^{TM} England
Methanol	Sigma-Aldrich, USA
Phenol	Merck, Germany
Potassium Chloride (KCl)	AnalaR^{TM} England
Propanol-1 (Propa-1-ol)	AnalaR^{TM} England
Propanol-2 (Propa-2-ol)	AnalaR^{TM} England
Sodium dodecyl Sulfate (SDS)	PhytoTechnology Laboratories, USA
Sodium acetate (CH_3COONa)	AnalaR^{TM} England
Sodium Chloride (NaCl)	Applichem, USA
Sodium Hydroxide Pellets	Merck, Germany
Sodium metabisulphite	Sigma-Aldrich, USA
Sucrose	AnalaR^{TM} England
Sulphuric acid	Sigma-Aldrich, USA
Tris base	Invitrogen, USA
Tris-(hyroxymethyl) aminomethane	PhytoTechnology Laboratories, USA
Triton X-100	AnalaR^{TM} England
Tween-20	Sigma-Aldrich, USA

2.1.4. Ready to use Laboratory Kits and Reagents

A section of high-quality, cost-effective, convenient supply of ready-to-use laboratory solutions and kits was also used for this experimental work. These reliable reagents and kits were purchased from different companies as shown in table 2.4.

Table 2.4: Ready to use Laboratory Kits and Regents

Reagents	**Company**
Anti-His antibody	Abcam, USA
Chemiluminescence system	ECL® PLUS; GE Healthcare
dNTP Set	Thermofisher, USA
Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG	Abcam, USA
1 Kb DNA ladder	Thermofisher, USA
Ni-NTA Agarose	Thermofisher, USA
4-10% NuPAGE® Bis-Tris Gel	Thermofisher, USA
Protein ladder	Thermofisher, USA
RNase A	Bioworld
SYBR green	Thermofisher, USA
3,3´,5,5´-tertamethylbenzidine (TMB)	Thermofisher, USA
Taq DNA Polymerase (5U/µl)	Thermofisher, USA

2.1.5. Primers

A primer is a short nucleic acid sequence that acts as the preliminary to DNA synthesis. Primers are composed of short RNA strands in living things. An enzyme called primase, a subtype of RNA polymerase, produces a primer before DNA replication. In this study, the Ompk nuclear forward and reverse primers were employed, as described in table 2.5.

Table 2.5: Primers

Primers	**Sequence**
OmpK-nuclear forward primer	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCGTAAATCACTTTTAGCTCTAGGCC3'
OmpK-nuclear reverse primer	5'GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTGATGGTGATGGTGATG3'

2.2. Methods

2.2.1. Workplace safety measures

It was necessary to maintain sterilized conditions throughout the whole experimental work especially during seed sterilization, seed germination, and plant tissue culturing, streaking of *Agrobacterium* culture on Luria Agar plates and during plant

transformation procedure for achieving the end goal of disinfection, to reduce contamination and cross-contamination. Following actions were taken for this purpose:

- Laboratory coat was worn during lab work.
- Nitrile gloves were used during solutions preparation, gel formation, working with plant materials and bacterial cultures.
- All materials and media, such as: MS media, LB media, distilled water, glass wares, and surgical instruments (forceps, scalpels, blades) were sterilized before use by autoclaving for 20 minutes at 121ºC.
- For sterilization purposes, every time Ultraviolet (UV) radiations were exposed for 15 to 20 minutes to all materials that were used in LFH (Laminar Flow Hood) except seeds, antibodies, hormones, and some other photosensitive reagents during experimental work.
- The working surface was swapped with 70% ethanol or 95% methanol.
- Hands were washed and cleaned with a disinfectant commercial soap or 70% ethanol before and after working in Laminar Flow Hood (LFH).
- All waste material was cleaned either by bleaching or autoclaving before disposing of.

2.2.2. Sterilization of Laminar Flow Hood

Seed sterilization, plant tissue culturing, inoculation, and streaking of *Agrobacterium* culture and all plant transformation steps were taken in Laminar Flow Hood (LFH). Sterile working conditions were maintained by the LFH which contains a High Efficiency Particulate Absorption (HEPA) filter. Every time before working in LFH, it was sterilized by swapping the working surface gently with spirit or 70% ethanol. All apparatus and material except hormones, antibodies, plant seeds, plant tissues bacterial cultures, and other photosensitive reagents, was sprayed carefully with spirit and then put into LFH under ultraviolet (UV) light exposure for 15 to 20 minutes. After 30 minutes, UV was turned off and hands were sprayed with 70% ethanol before taking them into the sterile environment of LFH. Instruments like forceps, scalpels and blades they were sterilized by dipping in 70% ethanol and flamed till they are red-hot then cooled before use. After finishing the work in LHF, all discarding material was discarded carefully, either after autoclaving or bleaching them off in a 10% bleaching solution if needed.

2.2.3. Different types of culture media preparation

Various distinctive media were used at different stages of current experimental work. For example, half Murashige and Skoog (½MS) media, full Murashige and Skoog (Full MS) media, Luria Broth (LB) media, and Luria Agar (LA) media were mostly used under optimized conditions. Tables 2.6 and 2.7 provide the chemical composition of various plant culture media and bacterial culture media, respectively.

2.2.3.1. Plant culture media

Different media were used for different purposes. MS media were used for seed germination. Different strengths of MS medium specifically optimized with plant growth regulators (PGRs) were used for the regeneration of plants.

2.2.3.1.1. Murashige and Skoog media

Different strengths of MS media (Full MS and half MS) were used for seed germination and plant regeneration (table 2.6). Full MS media was utilized for the germination of spinach seeds as well as for the regeneration of spinach plant tissues. For its preparation, MS and sucrose were weighed (table 2.6) and dissolved in the required amount of distilled water in a media bottle. The pH was adjusted in the range of 5.75 to 5.85 via a pH meter with the help of 0.1 N HCl or NaOH solutions. Lastly, agar was weighed (table 2.6) and added to the bottle. The medium was sterilized under controlled conditions, 121°C temperature, and 15 psi for 20 minutes, in an autoclave. Finally, 30 ml Petri plates in LFH were filled with moderately warmed MS Medium, which was then let to set. Jars and plates were tightly sealed with parafilm and kept at 25°C in the growth chamber.

Table 2.6: Chemical composition of plant culture media

Components	Concentration (g/l)
½ MS Media	
MS medium w/ vitamins, glucose, & sucrose	2.2g
Sucrose	30g
Agar	0.8%
Liquid 1/2 MS Media	
MS medium w/ vitamins, glucose, & sucrose	2.2g
Sucrose	30g
Full MS Media	
MS medium w/ vitamins, glucose, & sucrose	4.4g
Sucrose	30g
Agar	0.8%

2.2.3.2. Bacterial culture media

Luria Broth (LB) media was used for bacterial inoculations and Luria Agar (LA) media was used for streaking the bacterial inoculum.

2.2.3.2.1. Luria Broth media

Luria Broth media was utilized for bacterial growth (inoculation) in flasks. For its preparation, in a flask, LB was weighed (table 2.7) and dissolved in the required amount of distilled water. The flask was tightly covered with aluminium foil with the help of paper tape. At standard conditions, the prepared media was sterilized through autoclave and stored at room temperature.

2.2.3.2.2. Luria Agar media

Luria Agar (LA) was utilized for bacterial growth (streaking) in a petri plates. For its preparation, LA was weighed (table 2.7) and dissolved in the required amount of distilled water in a flask. The flask was tightly covered with aluminium foil with the help of paper tape. At standard conditions, the prepared media was sterilized through the autoclave. In the LFH, the exact calculated concentration of antibiotic was added in slightly warmed LA media and then poured into Petri plates (30ml). After complete solidification, plated was sealed by parafilm and stored in a growth room at 25ºC.

Table 2.7: Chemical composition of bacterial culture media

Components	**Concentration (g/l)**
LB Media	
Tryptone	10g
Yeast Extract	05g
Sodium Chloride	10g
LA Media	
Tryptone	10g
Yeast Extract	05g
Sodium Chloride	10g
Agar	0.1%

2.2.4. Preparation of stock solutions

Various hormones and antibiotics were utilized in this research. Their compositions are described in table 2.8.

Table 2.8: Composition of stock solutions of hormones and antibiotics

Stock solutions	Concentration
Hormones	
IAA	1mg/ml
BAP	1mg/ml
Antibiotics	
Hygromycin	50mg/ml
Kanamycin	50mg/ml
Rifamycin	20mg/ml
Cefotaxime	250mg/ml

2.2.4.1. Stock solution preparation of plant hormones

2.2.4.1.1. Indole acetic acid (IAA)

For the preparation of 15mg/ml IAA stock solution, 15mg of IAA powder was weighed and added into a 15ml falcon tube. It was dissolved into a 2ml to 5ml solution of 1M NaOH because it was not completely soluble in distilled water. Once fully dissolved, distilled water from an autoclave was used to dilute it to a level of 15ml. A sterile syringe filter (0.2µm) was used to filter the IAA stock solution in LFH. 1.5ml aliquots were created in eppendorf tubes and kept at -20^{o}C.

2.2.4.1.2. Benzylaminopurine (BAP)

For the preparation of 15mg/ml BAP stock solution, 15mg of BAP powder was weighed and added into a 15ml falcon tube. It was dissolved into a 2ml to 5ml solution of 1 M NaOH because it was not soluble in distilled water. With autoclaved distilled water, the volume was increased to 15ml once it had completely dissolved. BAP stock solution was filtered in LFH using a 0.2µm sterile syringe filter. Eppendorf tubes were used to create 1.5ml aliquots, which were then kept chilled at -20^{o}C.

2.2.4.2. Stock solutions preparation of antibiotic

2.2.4.2.1. Hygromycin

Hygromycin powder 2.5g was weighed, dissolved in 25ml of autoclaved distilled water, and the volume was then increased to 50ml in a falcon tube to create a 50mg/ml hygromycin stock solution. After that, a syringe filter (0.2µm) was used to filter sterilize the stock solution in LFH. Aliquots of stock were made and kept at -20°C.

2.2.4.2.2. Kanamycin

2.5g of kanamycin powder was weighed, dissolved in 25ml of autoclaved distilled water, and the volume was then increased to 50ml in a falcon tube to create a 50mg/ml kanamycin stock solution. After that, a syringe filter (0.2µm) was used to filter sterilize the stock solution in LFH. Aliquots of stock were made and kept at -20°C.

2.2.4.2.3. Rifamycin

0.4g of rifamycin powder was weighed and mixed in 10ml dimethyl sulfoxide (DMSO) to create a 20mg/ml rifamycin stock solution. DMSO was then used to make the volume up to 20ml in a 50ml falcon tube. After that, a syringe filter (0.2µm) was used to filter sterilize the stock solution in LFH. Aliquots of stock were made and kept at -20°C.

2.2.4.2.4. Cefotaxime

To make a stock solution containing 250mg/ml of cefotaxime, 5g of the drug's powder was weighed and dissolved in 10ml of distilled autoclaved water, and the final volume was raised to 20ml in a 50ml falcon tube. After that, a syringe filter (0.2µm) was used to filter sterilize the stock solution in LFH. Stock was divided into aliquots and kept at -20°C.

2.2.5. Plant Material

In the research, spinach seeds were used. Their seeds were purchased from Awan seed store, Islamabad, Pakistan. The seeds were kept sealed in 15ml falcon with parafilm and stored at a dry place with humidity of 30% at room temperature.

2.2.5.1. Spinach seeds sterilization

Seeds surface were sterilized within the sterile conditions of LFH before inoculating them on the germination media. This step was done to minimize the possibility of bacterial and fungal contamination which would otherwise reduce the seed germination ability. Spinach seeds were surface sterilized using different chemicals. Following treatment, the seeds were three times rinsed with autoclaved distilled water and then allowed to air dry on sterile filter paper. Different chemicals treatment along with the treatment time is provided in table 2.9.

Table 2.9: Different methods of seed sterilization used

Sr. No.	Seed sterilization methods (SSM)	Chemical used	Time of treatment
1	SSM 1	70% ethanol, 20% bleach	5 mins, 20 mins
2	SSM 2		10 mins, 25 mins
3	SSM 3		15 mins, 30 mins
4	SSM 4		20 mins, 35 mins
5	SSM 5	70% ethanol, 30% bleach	5 mins, 20 mins
6	SSM 6		10 mins, 25 mins
7	SSM 7		15mins, 30 mins
8	SSM 8		20 mins, 35mins
9	SSM 9	70% ethanol, 40% bleach	5 mins, 20 mins
10	SSM 10		10 mins, 25 mins
11	SSM 11		15 mins, 30 mins
12	SSM 12		20 mins, 35 mins
13	SSM 13	0.2% mercuric chloride	1 min
14	SSM 14		3 mins
15	SSM 15		6 mins
16	SSM 16		9 mins
17	SSM 17		12 mins
18	SSM 18	0.5% mercuric chloride	40 secs
19	SSM 19		1 min
20	SSM 20		3 mins
21	SSM 21		6 mins
22	SSM 22		9 mins
23	SSM 23	70% ethanol, 3% sodium hypochlorite +0.1% tween 20	30 secs, 2 hours
24	SSM 24	Distill water, 5% sodium hypochlorite +1% tween 20, 70% ethanol	5 mins, 20 mins, 20 mins

2.2.5.2. Germination media for spinach seeds

Sterilized spinach seeds were germinated using full MS media. The media was prepared, autoclaved and poured in sterile jars. Before inoculation of seeds, jars containing media and other apparatus except seeds were exposed with UV light in LFH for 15 minutes. Seeds were inoculated in these jars. The jars were parafilm-sealed and put in the growth room at 25°C with a 14/10-hour light/dark cycle.

2.2.5.3. Seed Germination Efficiency

The seed germination efficiency was calculated for spinach seeds after using 24 different seeds sterilization methods and then their germination on full MS media. The

experiment was done in three batches for spinach seeds separately with two weeks gap between three successive batches. Further, three plates were used in each batch, each containing 10 seeds. The formula of seed germination is given below:

$$Seed\ germination\ efficiency\ (\%) = \frac{No.of\ seeds\ germinated}{Total\ no.of\ seeds\ inoculated} \times 100$$

2.2.5.4. Preparation of explants

A small fragment of node and leaf called explant was used for regeneration of the plant using tissue culturing. In this study, nodal explants were tissue cultured for regeneration and transformation.

2.2.5.5. Nodal explants preparation

Nodal explants were prepared by using 2-3 weeks old, in vitro grown plants. Using sterile forceps and scalpel, leaves were excised from nodes and the nodes were sliced into 1.5-2cm long pieces.

2.2.6. Spinach explant regeneration

For spinach nodal explants, different regeneration media were used for optimization purpose. Each regeneration media comprised of different strengths of MS media (table 2.6) with a combination of hormones. Different media along with their compositions are given in table 2.10.

Table 2.10: Composition of spinach regeneration media

Sr. No.	Spinach Regeneration Media (SRM)	Composition of SRM		References
		Strength of MS	Hormones and Working Concentrations	
1	SRM 1	½ MS	GA3 25mg/l IAA 1.5mg/l	(Shojaei *et al*., 2010)
2	SRM 2	Full MS	GA3 2.5mg/l IAA 1.5mg/l	(Shojaei *et al*., 2010)
3	SRM 3	Full MS	2,4D 0.5mg/l Kinetin 2mg/l	(Al-Khayri *et al*., 1992)
4	SRM4	Full MS	BAP 2.5mg/l	
5	SRM 5	½ MS	Kinetin 0.5mg/l BAP 0.5mg/l	(Rashid & Bal, 2010)

6	SRM 6	Full MS	BAP 1mg/l NAA 0.1mg/l	(Durrani *et al*., 2017)
7	SRM 7	½ MS	NAA 2mg/l IAA 2mg/l BAP 5mg/l Kinetin 4mg/l	
8	SRM 8	½ MS	IAA 0.2mg/l BAP 3mg/l Thiamine HCl 1mg/l	(Wurbs *et al*., 2007)
9	SRM 9	½ MS	IAA 0.2mg/l BAP 3mg/l Zeatin 2mg/l	(Ruf *et al*., 2001)
10	SRM 10	½ MS	IAA 2mg/l NAA 2mg/l BAP 5mg/l	
11	SRM 11	Full MS	IAA 2mg/l NAA 2mg/l BAP 9.4mg/l	
12	SRM 12	Full MS	Zeatin 2mg/l	
13	SRM 13	Full MS	Zeatin 1.7mg/l	
14	SRM 14	Full MS	BAP 1mg/l IAA 0.5mg/l	
15	SRM 15	Full MS	BAP 5mg/l IAA 0.5mg/l	
16	SRM 16	Full MS	Kinetin 2mg/l 2,4D 0.5mg/l GA3 1mg/l	(Al-Khayri *et al*., 1992)
17	SRM 17	Full MS	IAA 1.5mg/l GA3 2.5mg/l NAA 0.5mg/l	(Shojaei *et al*., 2010)
18	SRM 18	Full MS	BAP 2.5mg/l NAA 0.5mg/l	(Zahra, 2018)
19	SRM 19	Full MS	NAA 3.7mg/l GA3 1.7mg/l	(Knoll *et al*., 1997)
20	SRM 20	Full MS	GA3 10mg/l Kinetin 2mg/l NAA 10mg/l	(Naderi *et al*., 2012)
21	SRM 21	Full MS	IAA 4.165mg/l GA3 34.64mg/l	(Xiao & Branchard, 1993)
22	SRM 22	Full MS	NAA 10mg/l BAP 0.2mg/l GA3 3.5mg/l	(Leguillon et al., 2003)

2.2.6.1. Regeneration Efficiency of nodal explant

Nodal explants of spinach were cultivated on various regeneration medium in jars with various combinations of growth regulators as stated in Table 2.10 to discover the optimal media for regeneration of shoots. This experimental work was done in three different batches. Visual observation was done to check the effect of different parameters on the growth efficiency of nodal explants every 2ndday for 30 days. The shoot regeneration efficiency of spinach nodal explants was calculated by using the following formula:

$$Shoot\ regeneration\ efficiency\ (\%) = \frac{No.of\ shoots\ regenerated}{Total\ no.of\ nodal\ explants\ inoculated} \times 100$$

2.2.7. Optimization of Hygromycin for spinach nodal explants

As high concentration of hygromycin can affect the regeneration efficiency of nodal explants, therefore hygromycin concentration was optimized. This experiment was done to get the appropriate concentration of hygromycin for the selection of transformed explants and their regeneration in the following transformation experiments. Six different hygromycin concentrations 0, 5, 10, 15, 20 and 25mg/l were supplemented into spinach regeneration media 15 (SRM 15) under aseptic conditions in LFH. Untransformed spinach nodal explants were positioned horizontally on SRM 15 in jars and sealed with parafilm. Optimization was done in triplicates and each replicate had 5 nodal explants. These replicates were placed in growth room and were observed for 3-4 weeks. During the experiment, the medium was refreshed after 15 days. The images were taken for the number of regenerated spinach nodal explants in four weeks.

2.2.8. Media preparation for transformation

Three types of media were used for the transformation of *Spinacia Oleracea*. The details of the media are described below.

2.2.8.1. Co-cultivation media preparation

To prepare the co-cultivation media 200µl acetosyringone was added in autoclaved and slightly warmed spinach regeneration media 15 (SRM 15). Then, this prepared medium was poured into Petri plates (30ml per plate), allowed to solidify in LFH under aseptic conditions. Parafilm was used to seal the plates, which were then stored for future use at 25^{o}C in the growth chamber.

2.2.8.2. Washing media preparation

Liquid ½MS media (table 2.6) was used as a washing medium. It was prepared by using distilled water; pH was adjusted and sterilized by autoclaving. 1000mg/l, 500mg/l and 250mg/l of cefotaxime were used by separately adding in 50ml of liquid ½MS medium for the first three times washing, respectively. Lastly, distilled water was used for the fourth washing without using cefotaxime.

2.2.8.3. Selection media preparation

To prepare the selection media for nodes explant, 100mg/l of cefotaxime and 25mg/l of hygromycin were added in autoclaved SRM 15. Then, this prepared medium was poured into Petri plates, allowed to solidify in LFH under aseptic conditions. Parafilm was used to seal the plates, which were then stored for future use at 25ºC in the growth chamber.

2.2.8.4 Infiltration media

Infiltration media (table 2.11) was prepared as described by (Sparkes *et al*., 2006). A day before infiltration, stocks of all components were prepared and stored at 4°C. Infiltration media was freshly prepared each time.

Table 2.11: Infiltration media and its components

Components	**Concentration**
Infiltration media	
D- Glucose	250mg
MES stock (500mM)	5ml
Acetosyringone (1M)	5µl
$Na_3PO_4.12H_2O$ (20mM)	5µl
Distilled water	50ml
500 mM MES stock	
MES	4.88g
Distilled water	50ml
20 mM Na3PO4.12H2O	
$Na_3PO_4.12H_2O$	0.38g
Distilled water	50ml
1M Acetosyringone	
Acetosyringone	0.196g
DMSO	1ml

2.2.9. First Transformation: genetically modified *Agrobacterium tumefaciens* strain

In this bacterial transformation, *Agrobacterium tumefaciens* were transformed with binary vector pGWB5 having our gene of interest that is OmpK.

2.2.9.1. Preparation of *Agrobacterium tumefaciens* growth culture

The strain of *Agrobacterium* GV3101 was grown on LA plates containing 50mg/l rifamycins at 28ºC and kept in an incubator for 2-4 days until colonies of *Agrobacterium* appeared on the plate. A single colony of bacteria was picked by sterile loop and inoculated in a liquid LB medium containing 50mg/l rifamycin for selection. For bacterial growth, liquid LB medium was placed in a shaking incubator at 28ºC for 1-2 days until bacterial growth appeared in LB broth. After bacterial growth, 0.6-0.8 value of OD_{600} was measured by using a spectrophotometer (ThermoScientific multiskan GO).

2.2.9.2. Electro-competent cells preparation

For this study, we prepared electro-competent cells of GV3101 *Agrobacterium* strain. Electro-competent cells were prepared by transferring the bacterial inoculum (in LB media) into a 50ml falcon. The inoculant was centrifuged at 2400 rpm for 20 minutes at 4°C and the pellet was dissolved in 40ml chilled distilled water. Centrifugation at 2400 rpm for 20 minutes at 4°C was done and the pellet was dissolved in 20ml chilled distilled water. Centrifugation was done and the pellet was dissolved in 10ml chilled distilled water. Finally, centrifugation was done and the pellet was dissolved in 500µl of ice-cold 10% glycerol (10ml glycerol: 90ml distilled water). After mixing gently, aliquots of 60µl were made and stored at -80ºC.

2.2.9.3. Electroporation

Electro-competent cells were transformed with a plasmid (7µl) by electroporation using the Electroporator (Bio-Rad, USA), according to the protocol mentioned in the manual. The electro-competent cells were thawed on ice. 7µl plasmid was added to 50µl of these cells and the sample was transferred to a cuvette. The conditions for electroporation were set as listed in the manufacturer's manual and the PULSE button was pressed on the electroporator for the electric shock of 1.8kV. 400µl LB media (table 2.7) was added to the cuvette. The sample was then put into a new Eppendorf tube and was incubated at 37°C for 3 hours. 30µl from this sample was used for streaking on LA plates

containing suitable antibiotics and 100µl from the same sample was used for inoculation in LB containing corresponding suitable antibiotics (table 2.12). The plates as well as the inoculated LB were placed overnight at 28°C.

Table 2.12: Antibiotics for the selection of transformed *Agrobacterium tumefaciens*

Antibiotics	**Working concentration**	**Stocks prepared**
Kanamycin	50mg/l	50mg/l
Rifamycin	50mg/l	20mg/l

2.2.9.4. Binary vector pGWB5 containing OmpK

Agrobacterium tumefaciens strain GV3101 was transformed with pGWB5 binary vector containing our gene of interest that is OmpK. This transformed Agrobacterium was used for transformation of Spinacia Oleracea. T-DNA region of expression vector contained expression cassette (attB1-OmpK-6-His-attB2) with 35S promoter and NOS terminator as shown in Figure 2.1.

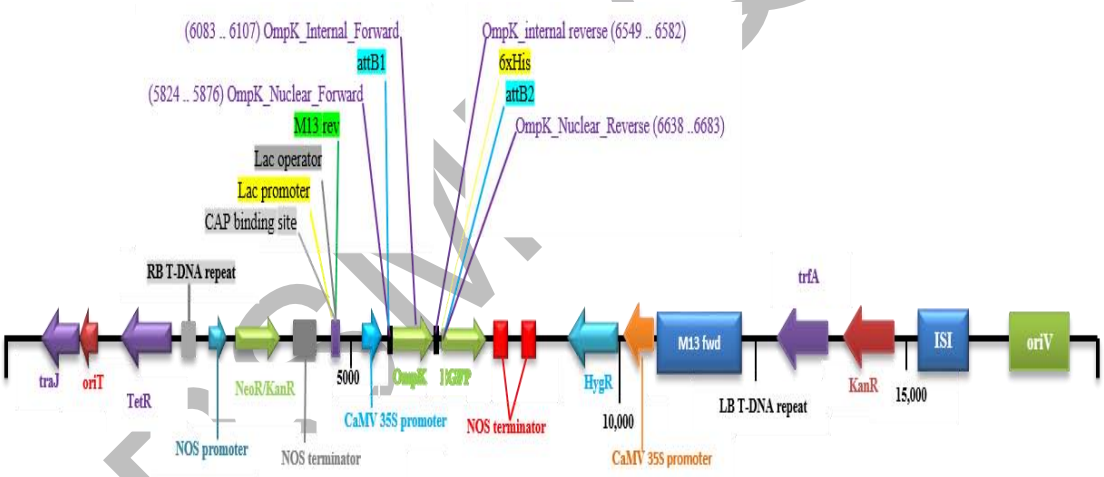


Figure 2.1: Pexp OmpK-PGWB5 (17,104 bp). **oriV** (incP origin of replication), **IS1** (insertion sequence: IS1), **KanR** (aminoglycoside phosphotransferase), **trfA** (trans-acting replication protein that binds to and activates oriV), **LB T-DNA repeat** (left border repeat from nopaline C58 T-DNA), **M13 fwd** (common sequencing primer, one of multiple similar variants), **CaMV 35S promoter** (strong constitutive promoter from cauliflower mosaic virus), **HygR** (aminoglycoside phosphotransferase from E. coli), **NOS terminator** (nopaline synthase terminator and poly (A) signal), **attB2** (mutant version of attB), **6xHis** (6xHis affinity tag), **attB1** (mutant version of attB), **M13 rev** (common sequencing primer, one of multiple similar variants, **lac promoter** (promoter for the E. coli lac operon), **lac operator** (lac repressor encoded by lacI), **CAP binding site** (E. coli catabolite activator protein), **NeoR/ KanR** (aminoglycoside phosphotransferase from Tn5), NOS promoter (nopaline synthase promoter), **RB T-DNA repeat** (right border repeat from nopaline C58 T-DNA), **TetR** (tetracycline resistance regulatory protein), **oriT** (incP origin of transfer), **TraJ** (oriT-recognizing protein), **EGFP** (the original enhanced GFP).

2.2.10. Second transformation-transgenic *Spinacia oleracea*

Two types of transformation strategies were utilized for the transformation of plants. *Spinacia oleracea* was transformed via stable transformation and transient transformation strategy.

2.2.10.1. *Agrobacterium*-mediated stable transformation of *Spinacia oleracea*

2.2.10.1.1. Co-cultivation

GV3101 strain of *Agrobacterium Tumefaciens* containing the binary vector pGWB5 was cultured in 50ml of LB media (table 2.7). This media was having 50mg/l kanamycin and 50mg/l rifampicin for selection. The flask was kept on an incubator shaker for 48 hours at 28°C. The O.D was adjusted at 0.8 with liquid LB broth. The inoculated LB media was transferred to a 50ml falcon and was centrifuged at 5500×g for 20 minutes at room temperature. The supernatant was discarded and the pellet was mixed in 50ml of liquid ½ MS (table 2.6), within the sterile condition of LFH. 40mg/l acetosyringone was added to this infection media. Explants (nodes) were prepared and dipped in this bacterial suspension for 8 minutes. After infection, they were blotted on autoclaved filter papers and were shifted to the co-cultivation media plates. In each plate 10-20 nodal explants were co-cultivated. The plates were sealed with parafilm and were placed in growth room at 25±2°C in dark for different time periods i.e., 2 and 3 days. A control (no infection) was also shifted to co-cultivation media. Whole work was done in duplicates.

2.2.10.1.2. Selection

After two day's incubation on co-cultivation media, nodal explants were washed thrice for 5 minutes, firstly by dipping in 50ml washing media containing 1000mg/l cefotaxime, secondly in 50ml washing media containing 500mg/l cefotaxime and thirdly in 50ml washing media containing 250mg/l cefotaxime and lastly washed with sterile distilled water. As illustrated in figure 2.2, nodal explants were next dried on sterile filter paper before shifting to petri plates containing selection media supplemented with 10mg/l hygromycin and 100mg/l cefotaxime. The selection plates having 4-5 infected nodal explants were placed in the growth room under light at 25-28ºC. After every one week, these infected explants were washed thrice by dipping in washing media for 5 minutes and shifted on petri plates having fresh selection media. The whole work was done in LFH under aseptic conditions. Transformed nodal

explants from spinach plant were regenerated; their shoot regeneration efficiency and callus formation efficiency was calculated by the following formulas:

$$Callus\ formation\ efficiency\ (\%) = \frac{No.of\ calli\ formation\ from\ explant}{Total\ no.of\ nodal\ explants\ inoculated} \times 100$$

$$Shoot\ regeneration\ efficiency\ (\%) = \frac{No.of\ shoots\ regenerated}{Total\ no.of\ nodal\ explants\ inoculated} \times 100$$

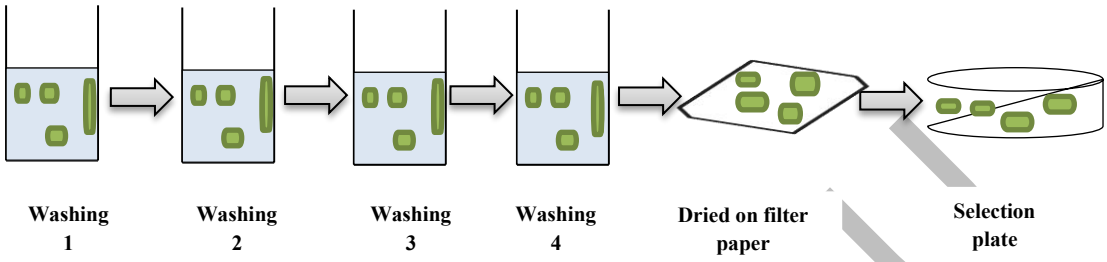


Figure 2.2: Washing steps after co-cultivation: Washing 1: Liquid ½ MS with 1000mg/l cefotaxime; Washing 2: Liquid ½ MS with 500mg/l cefotaxime; Washing 3: Liquid ½ MS with 250mg/l cefotaxime; Washing 4: Autoclaved distilled water and dried on filter paper then transfer on selection plate.

2.2.10.2. *Agrobacterium* infiltration for transient plant transformation

Transient transformation of spinach was done by following the protocol described by (Sparkes *et al*., 2006) with few modifications. For transient infection, we used a tissue cultured plant, which was 2-3 weeks old. The plant was acclimatized and was ready for infection as the leaves developed on it, shown in figure 2.3.

Figure 2.3: Agroinfiltration process.

2.2.10.2.1. *Agrobacterium* suspension preparation for infiltration

Agrobacterium suspension was prepared in infiltration media. About 1-1.5ml of inoculum (LB+GV3101 containing pGWB5 vector) was transferred to a sterile eppendorf. The bacterial cells were centrifuged at 1,000g for 10 minutes at 24 °C (room temperature). After discarding the supernatant, the pellet was suspended in 1 ml of infiltration media (table 2.11). This washing step was repeated twice with the same conditions to remove traces of any antibiotic that could have an adverse effect on the infiltrated plant. Mixed the pellet in 1ml of infiltration media. Invert falcon few times to dissolve the pellet completely. Once dissolved, 1:10 (1 part bacterial suspension: 9 parts infiltration media) dilutions were prepared and absorbance was measured at 600nm using a spectrophotometer. The final $O.D._{600}$ of the suspension should not be higher than 1.5-2.

2.2.10.2.2. Plant inoculation

The leaves were infiltrated with bacterial suspension by using 1ml syringes (figure 2.3). Agro-injection was performed by injecting 600µl of this suspension. The infiltration solution was injected in the leaves through abaxial (lower) domain. The infiltration solution was injected gently to avoid it from splashing out. Once all of the leaves (12–15 leaves) had been infiltrated, a few drops of the infiltration solution seemed to be dripping from the hydathodes. Only those leaves that were completely infiltrated were used in this study.

2.2.10.2.3. Collection of leaves

Different co-cultivation time was provided to leaves. Few leaves were picked at 1st, 2nd, 3rd and 4th days post agro infiltration. They were then kept at -20°C while being wrapped in aluminum foil.

2.2.11. Techniques of molecular biology to verify transformation

In this present study, transformation of plants with OmpK were confirmed by conventional PCR analysis. Copy number of integrated OmpK gene was determined by performing quantitative real-time PCR (qRT-PCR) technique. Bradford assay was performed for protein estimation after protein purification from plants. In transgenic plants, expression of OmpK protein was confirmed by performing Dot blot assay and western blotting. Indirect Enzyme-Linked Immunosorbent Assay (ELISA) was

performed to quantify the OmpK protein in total soluble protein, extracted from transgenic plants.

2.2.11.1. Plasmid isolation from transformed *Agrobacterium Tumefaciens*

Plasmid isolation was done by the protocol reported by (Russell & Sambrook, 2001) with few modifications. The inoculated LB media (with bacterial growth of 0.6-0.8 $O.D._{600}$) was transferred to a fresh 50ml falcon. The culture was centrifuged for 10 minutes at 4°C at 4000 rpm, and the supernatant was discarded. A 200µl solution I was used to dissolve the pellet (Annexures 2.1) which was then transferred to a fresh Eppendorf tube. 400µl of solution II (Annexures 2.1) was added. The tube was placed on ice for 3-5 minutes after being gently inverted numerous times. 300µl of lysis solution III (Annexures 2.1) was added to it. By repeatedly inverting the tube, all of the components were combined. The tube was once more incubated for 5 minutes on ice before being centrifuged for 5 minutes at 4°C at 14000 rpm. A fresh tube was filled with 600µl of the supernatant after it had been collected. It was then mixed with an equivalent volume of phenol-chloroform (Annexes 2.1). The tube was once more centrifuged for 5 minutes at 4°C at maximum speed. There were two layers visible. A new Eppendorf tube was used to transfer the top aqueous layer. This was mixed with 600µl of isopropanol, and the tube was placed on ice for an hour. It was centrifuged for 5 minutes at maximum speed at 4°C after an hour. The supernatant was discarded. The pellet was washed using 70% ethanol (1ml) followed by centrifugation at the maximum speed for 5 minutes at 4°C. Ethanol was removed and the pellet was allowed to dry. Depending on the size of the pellet, T.E buffer (Annexeures 2.1) with RNase A was added, and it was then kept at -20°C.

2.2.11.2. Isolation of plant genomic DNA

Genomic DNA was extracted by cetyltrimethylammonium bromide (CTAB) procedure reported by (Russell & Sambrook, 2001). DNA was isolated from both wild-type and transgenic plants. CTAB buffer (Annexures 2.2) was prepared one day before the DNA extraction from plants. The sample (nodes or leaves) was placed overnight at -20°C. Next day, it was ground into fine powder with liquid nitrogen. The powdered sample was transferred to freshly autoclaved eppendorf. The temperature of the water bath was pre-adjusted at 65°C. Before use, CTAB was placed in water for 30 to 60 minutes. To the sample in Eppendorf, 700µl of CTAB buffer was added. The homogenate was vortexed and left in a preheated water bath for an hour. 600µl of cold phenol-

chloroform-isoamyl alcohol (Annexeures 2.2) were added to each sample after an hour. By repeatedly inverting the Eppendorf over the course of 30 minutes, the components were well homogenized. It was then centrifuged at 14,000 rpm for 15 minutes. The pellet was discarded and the supernatant was collected in a new Eppendorf. The sample was then incubated at -20°C overnight with an equal volume of cold isopropanol. The sample was centrifuged for 5 minutes at 14,000 rpm the very next day, and the pellet was obtained. After adding 1ml of chilled70% ethanol (Annexures 2.2), the pellet was centrifuged at 14,000 rpm for two minutes. After discarding the supernatant, the washing process was repeated. The supernatant was once more discarded. The pellet was allowed to air-dry by inverting it on a paper towel for 10-20 minutes. Finally, depending on the size of the pellet, the pellet was dissolved in an appropriate quantity of T.E. buffer (Annexeures 2.2) and RNase A, and then it was stored at -20°C.

2.2.11.3. Micro-volume quantification of DNA concentration

The concentration of DNA isolated from spinach was measured using a Colibri microvolume spectrophotometer using a highly sensitive approach called NanoDrop micro-volume quantification (Desjardins & Conklin, 2010). Before analysing the DNA sample, the bottom and top optical panels of the micro-volume spectrophotometer were cleaned by using 2-3µl of de-ionized H_2O onto the bottom optical panel. The upper panel got in contact with deionized water when the lever arm was fully closed. After that, the lever arm was raised and both optical panels were cleaned with dry, clean lab wipes free of fur. "Nucleic Acid application" was selected after opening the NanoDrop software. By distributing 1µl of TE buffer over the lower optical panel, a blank measurement was carried out. After lowering the lever arm, the option labelled "Blank" was chosen. Following the completion of the blank measurement, a lab wipe was used to clean both optical panels. For the purpose of quantifying DNA samples, the right constant was selected. Onto the lower optical panel, 1µl of the DNA sample was poured. The “measure” was selected in the software application after closing the lever arm. The concentration and purity ratio of DNA was automatically calculated by the software.

2.2.11.4. Polymerase Chain Reaction (PCR)

For the confirmation of transformation and integration of the OmpK gene in the nuclear genome of spinach, PCR was performed. CTAB method was used for the extraction of DNA from spinach wild type or transformed explants (nodes and leaves). A total of 25 µl volume of the master mixture was prepared for 1X PCR. Positive control (OmpK

containing plasmid) and wild-type plant was used as a negative control. The expected size of the amplicon was 876 bp by using OmpK nuclear primers in this PCR. The compositions of the master mixture and standard conditions for PCR are given below in Table 2.13 and 2.14 respectively.

OmpK-nuclear forward primer	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCGTAAATCACTTTTAGCTCTAGGCC-3'
OmpK-nuclear reverse primer	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTGATGGTGATGGTGATG-3'

Table 2.13: For 1X PCR, composition of master mixture

Reagents	**Concentration (µl)**
Taq. Buffer	2.5
$MgCl_2$	1.5
dNTPs	0.5
Forward primer	0.5
Reversed primer	0.5
Taq. Polymerase	01
Template	01

Table 2.14: For PCR, standard conditions

Steps	**Temp. (˚C)**	**Time (min:sec)**	
Initial denaturation	95	05:00	
Final denaturation	95	00:45	**35 cycles**
Annealing	66	01:00	
Initial extension	72	02:00	
Final extension	72	10:00	
Hold	04	∞	

PCR profile with temperature and time at different stages of reaction is given in figure.2.4. The annealing temperature was 66°C. The total PCR reaction had 35 repeated amplification cycles.

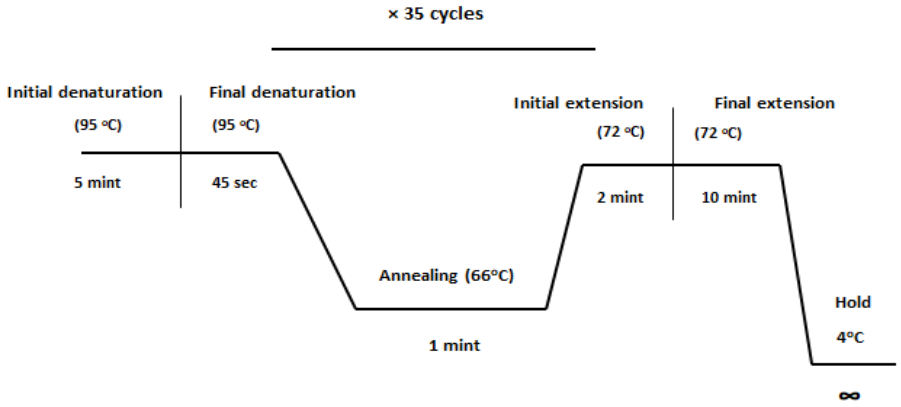


Figure 2.4: Profile of PCR program conditions required in different steps of a PCR reaction.

2.2.11.5. Agarose Gel Electrophoresis and imaging

Agarose gel electrophoresis was used to confirm the PCR results. All PCR samples including the transgenic samples as well as the wild-type samples were loaded into wells. Before loading gel, 7µl of PCR products were mixed with 3µl of loading dye (Annexures 2.3). 1kb ladder (Cat No: SM0314, Thermo Scientific, USA) (figure 2.5) was used for checking the size of DNA sample. Each sample was carefully loaded into distinct wells. Following the loading of the samples, the gel tank's lid was sealed, the power source was attached, and electrophoresis was carried out for 70 minutes at 110 volts and 500mA. DNA bands were seen under UV light after a successful run using the gel documentation technique.

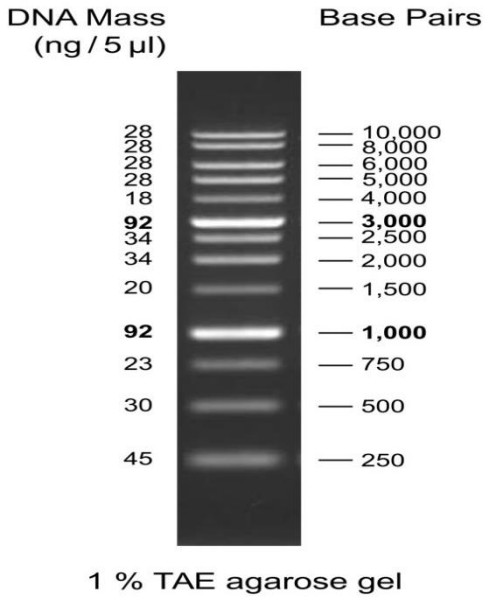


Figure 2.5: 1kb DNA ladder for Agarose Gel Electrophoresis.
(Taken from: www.gentechbio.com)

2.2.11.6. Quantitative Real Time (qRT) PCR

After successful transformation, the level of expression of a transgene within a plant cell was checked through qRT-PCR by following the protocol described by (Kang *et al*., 2012). The qRT-PCR was carried out using MyGo Pro Real time PCR (Stokesley Middlesbrough, UK). DNA samples of both wild-type and transgenic were used. Three dilutions i.e., 1:10, 1:100 and 1:1000 were prepared in PCR water for each DNA sample. A fluorescent dye called SYBR Green (Cat No: K0221 Thermo Scientific, USA) was used in this technique. Master-mix was prepared as given in Table 2.15. The primers used are provided as follow

OmpK-nuclear forward primer	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCGTAAATCACTTTTAGCTCTAGGCC-3'
OmpK-nuclear reverse primer	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTGATGGTGATGGTGATG-3'

Table 2.15: For qRT-PCR, composition of reaction mixture

Components	**Concentration for 1X (µl)**
Forward primer	0.25
Reverse primer	0.25
DNA	0.5
SYBR Green	5
PCR water	4

The conditions for qRT-PCR reaction with program names are listed in table 2.16

Table 2.16: Condition for qRT-PCR

Programs name		**Temperature (°C)**	**Ramp (°C/s)**	**Hold (s)**
Hold		95	4	600
3 step amplifications	**Denaturation**	95	5	10
	Annealing	59	4	45
	Extension	72	5	15
Pre-melt hold		95	5	15
High resolution melting	**Initial stage**	59	4	60
	Final stage	95	0.05	15

2.2.11.7. Protein extraction

Total soluble protein was extracted from regenerated nodes of transformed and untransformed spinach plants. Approximately, 100mg of node material and leaves was ground in liquid nitrogen by using pestle and mortar. To this powder, 500µl of extraction buffer (Annexures 2.4) was added. The mixture was mixed properly. The slurry was transferred to a freshly autoclaved Eppendorf tube was centrifuged at 20,000

g for 10 minutes at 4°C. The pellet was discarded and the supernatant was collected in a new Eppendorf tube. Total Soluble Protein (TSP) fraction of this collected supernatant was centrifuged once more for 10 minutes at 20,000g and 4°C to eliminate cellular debris. The supernatant was gathered, transferred to a new Eppendorf tube, and kept at -20°C further use.

2.2.11.8. Bradford assay (Protein quantification)

After extraction of the protein, concentration of TSP was done by using Bradford's assay. Bovine Serum Albumin (BSA) was used as a standard to plot the standard curve. 2mg/ml stock solution of BSA was prepared which was used to prepare five different dilutions of BSA (Annexures 2.5). Protein extraction buffer (Annexures 2.4) was used as a blank. 20µl of blank, BSA dilutions and samples were loaded into separate wells of a 96 well plate. Afterwards, 200µl of Bradford's reagent was added to each well having samples. The plate was covered with aluminum foil and was incubated in dark for 30-60 minutes. Following incubation, absorbance was taken at 595nm using a spectrophotometer. The absorbance values of BSA dilutions were then used to plot a standard curve as shown in figure 2.6. The concentrations of each protein sample was calculated from this standard curve using the TREND formula on excel sheet.

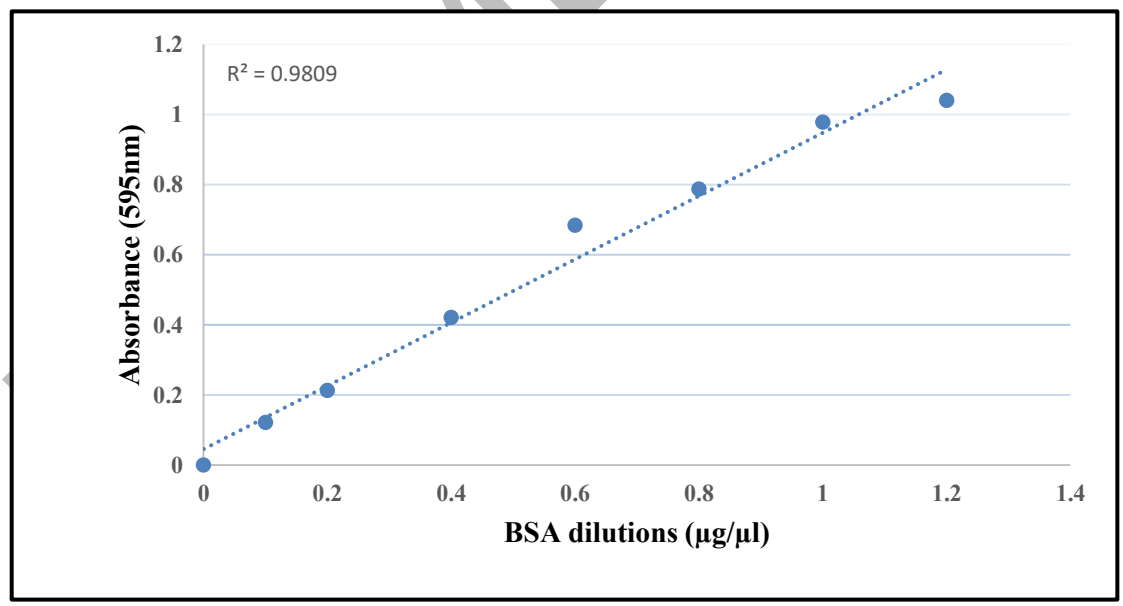


Figure 2.6: Standard curve of BSA dilutions for protein estimation.

2.2.11.9. Dot blot assay

A Dot blot is a simple and quick assay. It is performed on the same principle as many other immunological techniques; a particular antigen is recognized and bound by a particular antibody. In transgenic plants, the expression of the Ompk gene was

confirmed by performing a Dot blot assay. Different steps such as blotting, blocking, primary antibody treatment, secondary antibody treatment, and visualization were performed by using the standard protocol mentioned by the biotechne® R&S system.

2.2.11.9.1. Blotting

For dot blotting, a nitrocellulose membrane was used. After removing the protective covering with the help of forceps, transgenic protein samples of 5-10µl were blotted on the membrane. The blotted sites were marked by lead pencil. The membrane was incubated for one hour at 25ºC.

2.2.11.9.2. Blocking

For blocking the non-specific sites, 20ml of blocking buffer (Annexure 2.6) was loaded on the membrane and incubated for 30-60 minutes with gentle shaking at 25ºC. After incubation, the blocking buffer was discarded. To avoid excess BSA binding, the membrane was washed with TBST buffer (Annexure 2.7).

2.2.11.9.3. Primary antibody treatment

His tag antibody (Abcam USA) was used as the primary antibody. Working dilution 1:1000 of primary antibody (Annexure 2.8) was made from stock in TBST buffer (Annexure 2.7). At 25°C, the membrane was incubated with the primary antibody for an hour while being gently shaken. The membrane was rinsed with TBST buffer three times after incubation.

2.2.11.9.4. Secondary antibody treatment

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Abcam, USA) was used as the secondary antibody. Working dilution 1:1000 of secondary antibody (Annexure 2.9) was made from stock in TBST buffer (Annexure 2.7). The membrane was incubated with a secondary antibody for one hour with gentle shaking at 25ºC. After incubation, the membrane was washed thrice with TBST buffer.

2.2.11.9.5. Visualization

Chemiluminescent Substrate (Cat No: WBKLS0500, Merck Millipore, Germany) for horseradish peroxidase (HRP) enzyme was utilized for visualization. Hydrogen peroxidase and Luminol were mixed in a 1:1 ratio. The membrane was allowed to incubate with the substrate in dark conditions for 5 minutes at 25ºC. After incubation,

the membrane was visualized by using a chemiluminescence system (ECL® PLUS; GE Healthcare).

2.2.11.10. Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the separation of purified proteins based on their size. Then nitrocellulose membrane was used to transfer the separated proteins onto it. After successful transferring, the nitrocellulose membrane was incubated with primary antibody and then with secondary antibody under optimized conditions to detect the presence of target protein. For western blotting, protein samples from transgenic spinach plant tissues were used. Wild-type plants were used as negative controls.

2.2.11.10.1. SDS PAGE

The apparatus was cleaned and assembled properly. Two gels were prepared i.e., 18% resolving gel and 5% stacking gel. 18% Resolving gel (Annexure 2.10) was first prepared and was poured between the two glass plates. 1ml isopropanol was poured above the resolving gel to linearize the gel surface. The gel was allowed to set completely. Then 5% stacking gel (Annexure 2.11) was prepared. Isopropanol was removed completely before the stacking gel was poured above the resolving gel. The gel was allowed to set before the comb was immediately inserted.

2.2.11.10.2. Preparation and loading of protein samples

The protein samples were prepared by using a 4X sample buffer (Annexure 2.12). A total of 30µl of protein sample was loaded in each well of the gel for SDAS-PAGE. Each sample was composed of purified protein, loading buffer, and bromophenol blue. Purified protein from two transgenic lines was loaded in two lanes with negative control. Before loading each protein sample was heated at 95ºC in a water bath for 10 minutes. In one well of gel, 4µl of prestained protein ladder (Cat No. 26616, Thermo Scientific, USA) was also loaded.

2.2.11.10.3. Electrophoresis

After the gel was set, the assembly was placed in the gel tank. The tank was filled with running buffer (Annexure 2.13). Before loading the sample in the wells, the comb was removed and the wells were washed with a running buffer. Following sample loading, the lid of the gel tank was closed and positive and negative electrodes of the gel tank were connected to the power supply. Initially the gel was run for 20 minutes at 80 volts

and then the voltage was increased to 110 volts for 90 minutes or till the gel is fully run. After the gel has run, it was subjected to both Coomassie staining and western blotting.

2.2.11.10.4. Transfer of protein

Semi-dry method was used for the protein transfer. A semi-dry blotting apparatus was used for this purpose. For transfer of one gel, twelve Whatman's filter paper cut equal to the size of the separating gel. These filter papers and nitrocellulose membrane was soaked in transfer buffer (Annexure 2.14). Sandwich was prepared by placing six filter papers, nitrocellulose membrane, separating gel and the remaining six filter papers from positive terminal (anode) to negative terminal (cathode) as shown in figure 2.7. Use a roller to remove air bubbles as it would affect the transfer efficiency. The terminals of transfer apparatus were connected a power supply and voltage was adjusted to 10 volts for 45 minutes.

2.2.11.10.5. Blocking

To avoid non-specific binding of primary antibody blocking is done. Blocking solution (Annexure 2.6) is used for this purpose. The membrane is dipped in blocking solution for 60 minutes with continuous shaking, at room temperature. After an hour, the blocking solution is removed and the membrane is washed thrice with TBS-T wash buffer (Annexure 2.7) with continuous agitation.

2.2.11.10.6. Treatment with primary antibody

Primary antibody used was anti-His-tag antibody. 1:10,000 (Annexure 2.8) working dilution was prepared in TBS-T (Annexure 2.8) from stock. Membrane was placed in primary antibody overnight at 4°C**.** After the treatment, it was washed thrice using wash buffer (Annexure 2.7).

2.2.11.10.7. Treatment with secondary antibody

House-radish peroxidase (HRP) conjugated goat anti-mouse IgG was used as the secondary antibody. The membrane was incubated with secondary antibody (Annexure 2.9) for 1-2 hours with constant shaking at room temperature. Washing with wash buffer (Annexure 2.7) was done after treatment with secondary antibody.

2.2.11.10.8. Visualization

For visualization, Chemiluminescent substrate (Cat No: WBKLS0500, Merck Milipore, Germany) was used. Equal volumes of Luminol and hydrogen peroxide were mixed. The membrane was treated with the substrate for 2 minutes in dark. After incubation, the blot was visualized using gel documentation system (Alpha View SA Version 3.4.0.0).

2.2.11.12. ELISA

Indirect ELISA was done for checking the protein concentration within the samples. A 96 well plate was used for carrying out indirect ELISA. Samples were prepared by using ELISA extraction buffer (Annexure 2.15). The plate was incubated at 37°C for an hour after the samples were loaded into separate wells. After an hour, it was washed thrice with wash buffer (Annexure 2.15). Each well received 200µl of blocking solution (Annexure 2.6). The plate was once more incubated for an hour at 37°C.The blocking solution was removed and the wells were washed thrice using wash buffer (Annexure 2.7). Each well received 50µl of primary antibody (Annexure 2.8), and the plate was then incubated at 37 °C for an hour. Secondary antibody (Annexure 2.9) was added to each well after the wells had been washed three times with wash buffer (Annexure 2.7). The plate was incubated at 37°C for 1 hour, and then washed to remove unbound antibody using wash buffer (Annexure 2.7). Each well was filled with an enzyme-specific substrate called TMB (Cat No. A3840, Applichem, Germany), and the plate was then incubated once more at room temperature. 20–30 minutes. Later, when the blue color developed, 0.1N H2SO4 (stop solution) was added, and absorbance was measured at 595nm.

Annexures

Annexure 2.1: Solutions composition for plasmid isolation

Solutions	Components	Concentration
Solution I	Glucose Tris EDTA	50mM 25mM, pH 08 10mM, pH 08
Solution II	SDS NaOH	01% 0.2N
Solution III	Sodium acetate	3M, pH 4.8
Phenol-chloroform	Phenol Chloroform	25ml 25ml
70% ethanol	Ethanol Distilled water	70ml 30ml

T.E buffer	Tris HCl (pH 8) EDTA	01M 0.5M

Annexure 2.2: Solutions for DNA isolation from plants

Chemicals	Composition	Concentrations
CTAB buffer	CTAB Tris-HCl EDTA NaCl PVP	2% 100mM 20mM 1400mM 1%
Phenol-Chloroform-Isoamyl	Phenol Chloroform Isoamyl alcohol	25ml 24ml 01ml
70% Ethanol	Ethanol Water	70ml 30ml
T.E buffer	Tris HCl (pH 8) EDTA	01M 0.5M

Annexure 2.3: Solution for Agarose Gel Electrophoresis

TBE buffer recipe 10X (pH 8.0)	
Components	**Concentration (g/L)**
Tris base	110
Boric Acid	55
EDTA	9.3

1X TBE buffer

Components	**Concentration**
10X TBE buffer	10ml
Distilled water	90ml

Ethidium Bromide (10mg/ml)

Components	**Concentration**
Ethidium Bromide	1g
Distilled water	100ml

Loading dye

Components	**Concentration**
Bromophenol blue	0.25g
Distilled water	100ml

Annexure 2.4: Protein extraction buffer

Protein extraction buffer	
Components	**Concentration**
Sodium chloride	300mM
Ascorbic acid	20mM
Sodium metabisulphite	10mM

Tris (pH 08)	100mM

Annexure 2.5: BSA dilutions for Bradford assay

BSA dilutions		
Concentration (µg/µl)	**BSA (µl)**	**Distilled water (µl)**
0	0	20
0.5	5	15
1.0	10	10
1.5	15	5
2.0	20	0

Annexure 2.6: Blocking buffer

Components	**Concentration per 15ml**
TBST buffer	100ml
BSA	5g

Annexure 2.7: TBST buffer

Components	**Concentration**
Tris HCl	3.08g/l (pH: 7.6)
NaCl	8.775g/l
Tween 20	1ml/l
Distilled water	1L

Annexure 2.8: Dilution of primary antibody

Components	**Concentration**
His tag antibody	3µl
Sodium azide	0.015g
BSA	1.5g
TBST buffer	30ml

Annexure 2.9: Dilution of secondary antibody

Components	**Concentration**
HRP-conjugated goat anti-mouse IgG	3µl
BSA	1.5g
TBST buffer	30ml

Annexure 2.10: Resolving gel

Components	**Concentration**
Distilled water	17.6ml
30% Stock acrylamide solution	35.25ml
4X Resolving tris solution	17.6ml
10% ammonium persulphate	700µl

TEMED	70µl

Annexure 2.11: Stacking gel

Components	**Concentration**
Distilled water	16.8ml
30% Stock acrylamide solution	2.6ml
4X Resolving tris solution	6.4ml
10% ammonium persulphate	178µl
TEMED	26.8µl

Annexure 2.12: 4X Sample buffer

Components	**Concentration**
Glycerol	4ml
2-mercaptoethanol	2ml
SDS	1.2g
4X Stacking tris	5ml
Bromophenol blue	0.03g

Annexure 2.13: Running buffer

Components	**Concentration**
Tris HCl	3g
Glycine	14.4g
SDS	1g
Distilled water	1L

Annexure 2.14: Transfer buffer

Components	**Concentration**
Tris-base	3.028g
Glycine	14.4g
Methanol	200ml
Distilled water	Up to 1L

Annexure 2.15: ELISA extraction buffer

Components	**Concentration (200 ml)**
$MgCl_2$	5mM
NaCl	1M
$CaCl_2$	5mM
HEPES- free acid	20mM (pH 7.40)
MSF	1mM
Triton X-100	0.01%

3. Results

In the current study, the best seed sterilization technique was examined, and the optimum regeneration medium and hygromycin concentration were then adjusted using explants of *Spinacia oleracea. Agrobacterium* infiltration was performed on the leaves of *Spinacia oleracea* for the transient expression of the OmpK gene. Additionally, nuclear transformation mediated by *Agrobacterium* was carried out utilizing *Spinacia oleracea*'s nodal explants. Both conventional PCR and quantitative real-time Polymerase chain reaction (qRT-PCR) were used to confirm the transient and stable transformation. Finally, dot blot, Western blot, and ELISA were done to validate the presence of OmpK protein.

3.1. Seed germination

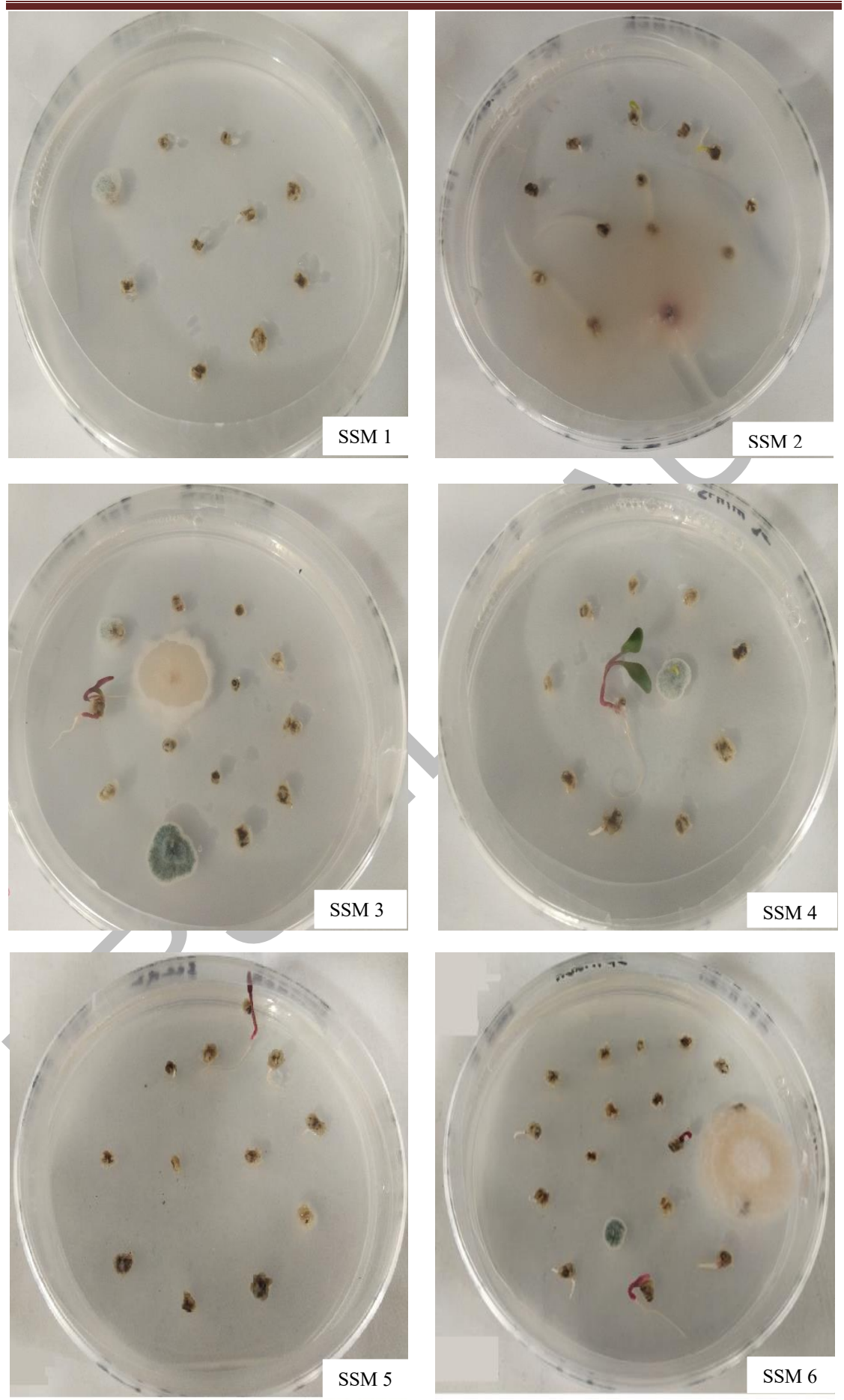
3.1.1. Surface sterilization of spinach seeds

The experiments of the current study were carried out by utilizing seeds of *Spinacia oleracea*. Twenty-four different methods were used for surface sterilization of *Spinacia oleracea* seeds. After sterilization seeds were grown on Full MS media. Each batch consisted of three Petri plates. A total of three batches were set up separately and observed. It was observed that by altering the method of seed sterilization, the germination efficiency of seeds was also affected. The growth efficiency of seeds was calculated after two weeks of inoculation on full MS media (figure 3.1). During this experiment, different perimeters were under consideration, either bacterial contamination or fungal contamination. A detailed comparison between the germination efficiency of seeds sterilized with different methods is tabulated in table 3.1. A graphical representation of twenty-four different seed sterilization methods is presented in Figure 3.2. The seed sterilization method 14 shows highly significant seed germination efficiency as compared to the other methods. The data was statistically analyzed by statistix 10 which displays the tendencies of all different used methods for seed germination. After statistical analysis, the SSM 13 second best and SSM 15 third best sterilization method after SSM 14.

Table 3.1: Effect of sterilization method on the seed germination efficiency

Seed Sterilization Methods (SSM)	Chemical Used	Time of treatment	Germination Efficiency calculated after 2 weeks			
			Total number of seeds	Germinated seeds	Germination efficiency (%)	Contamination
SSM 1	70 % ethanol, 20% bleach	5 mins, 20 mins	30	0	0 ± 0^{k}	Fungal contamination
SSM 2		10 mins, 25 mins	39	9	24 ± 1.708^{g}	Bacterial contamination
SSM 3		15 mins, 30 mins	42	5	12 ± 0.578^{i}	Fungal contamination
,SSM 4		20 mins, 35 mins	33	6	18 ± 1^{h}	Fungal contamination
SSM 5	70 % ethanol, 30% bleach	5 mins, 20 mins	36	6	17 ± 1^{h}	No contamination
SSM 6		10 mins, 25 mins	51	15	29 ± 1^{f}	Fungal contamination
SSM 7		15mins, 30 mins	57	15	26 ± 1^{fg}	No contamination
SSM 8		20 mins, 35mins	60	21	35 ± 4.359^{e}	No contamination
SSM 9	70 % ethanol, 40% bleach	5 mins, 20 mins	45	15	33 ± 1^{e}	No contamination
SSM 10		10 mins, 25 mins	45	3	7 ± 0^{j}	No contamination
SSM 11		15 mins, 30 mins	36	3	8 ± 0^{j}	Fungal contamination
SSM 12		20 mins, 35 mins	36	6	17 ± 1^{i}	No contamination

SSM 13	0.2% mercuric chloride	1 min	36	24	67±3.464[b]	No contamination
SSM 14		3 mins	36	33	92±8.544[a]	No contamination
SSM 15		6 mins	36	21	58±4.359[c]	No contamination
SSM 16		9 mins	36	18	50±1.732[d]	No contamination
SSM 17		12 mins	45	9	20±1[h]	No contamination
SSM 18	0.5% mercuric chloride	40 secs	42	21	50±4.359[d]	Fungal contamination
SSM 19		1 min	45	9	20±1[h]	Fungal contamination
SSM 20		3 mins	45	9	20±1[h]	No contamination
SSM 21		6 mins	57	0	0±0[k]	Fungal contamination
SSM 22		9 mins	36	0	0±0[k]	No contamination
SMM 23	70% ethanol, 3% sodium hypochlorite +0.1% tween 20	30 secs, 2 hours	57	0	0±0[k]	Bacterial contamination
SSM 24	Distill water, 5% sodium hypochlorite +1% tween 20, 70% ethanol	5 mins, 20 mins, 20 mins	48	0	0±0[k]	Bacterial contamination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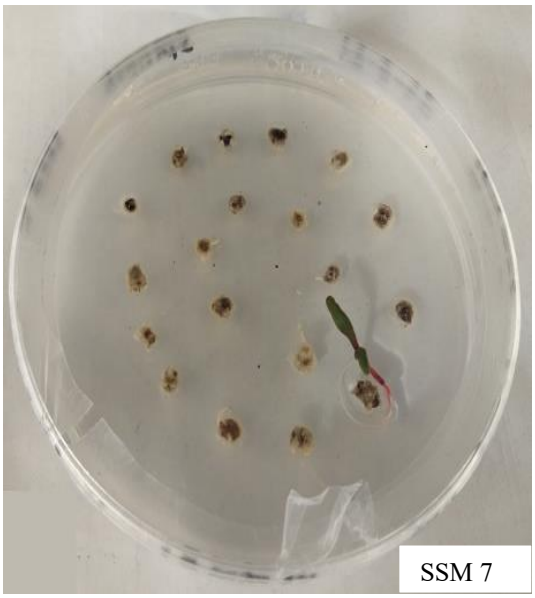
SSM 2
SSM 3
SSM 4
SSM 5
SSM 6



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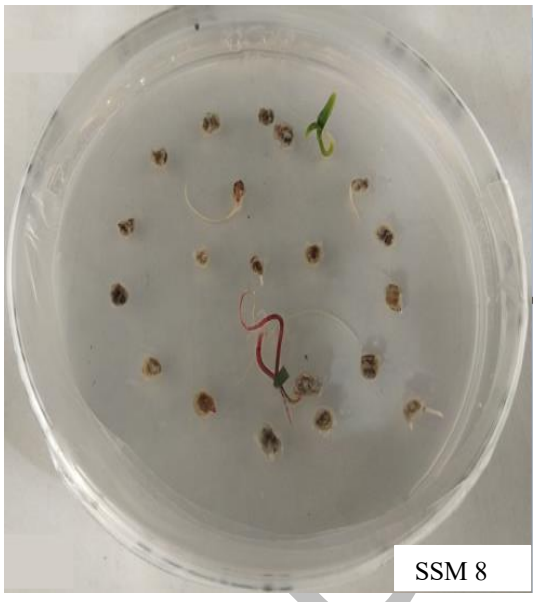

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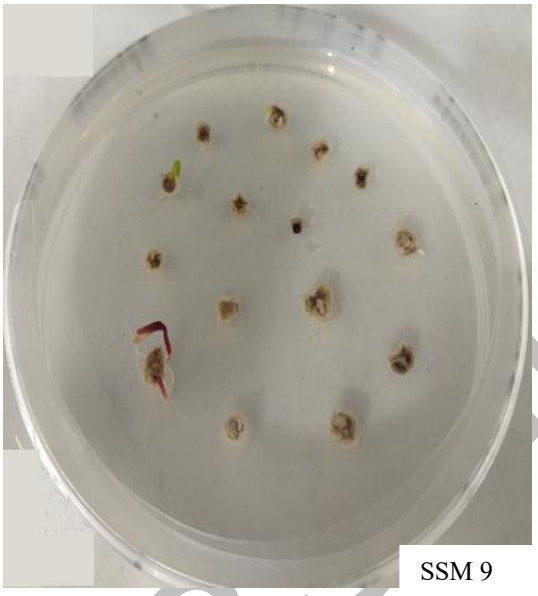

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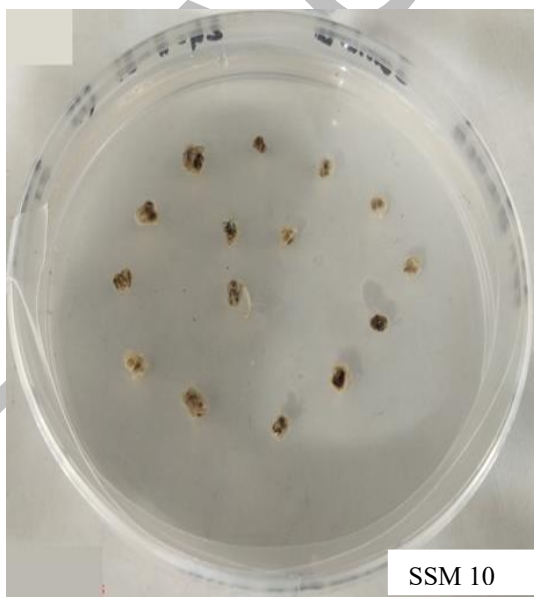
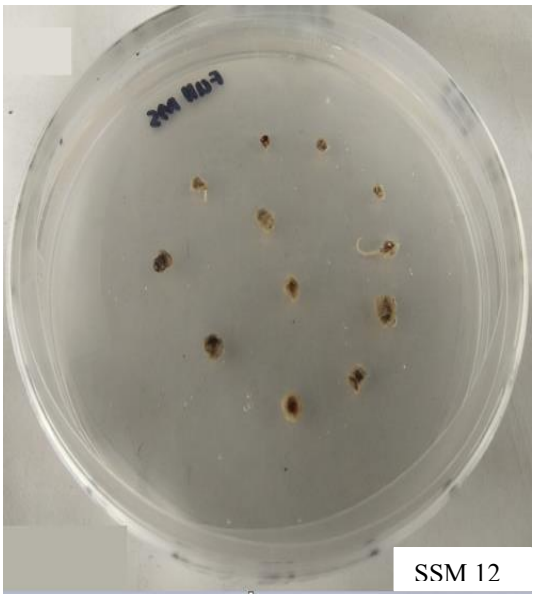


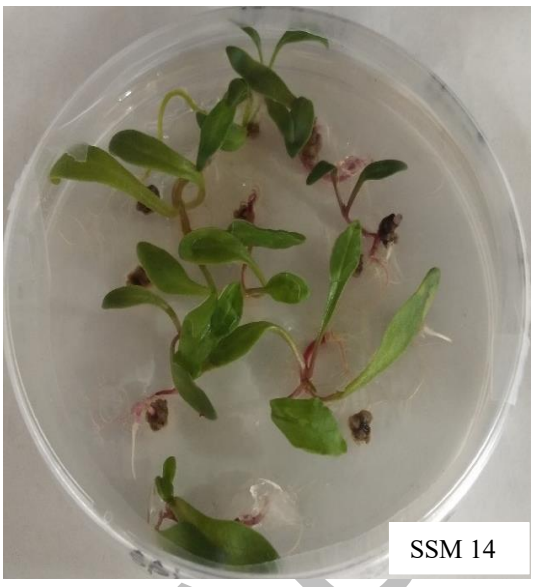
SSM 10

SSM 11

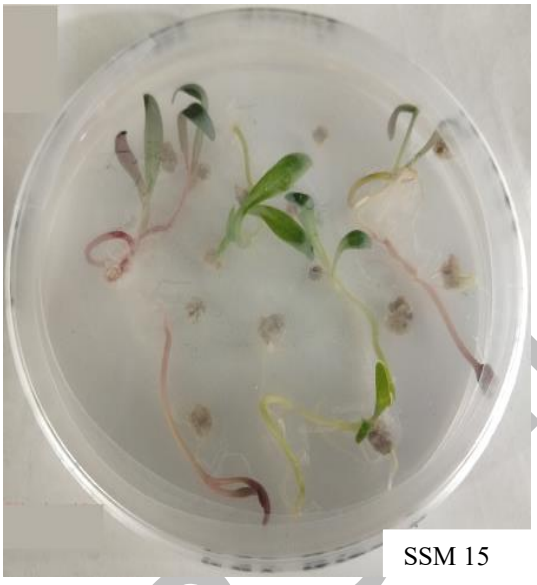


SSM 12

SSM 13

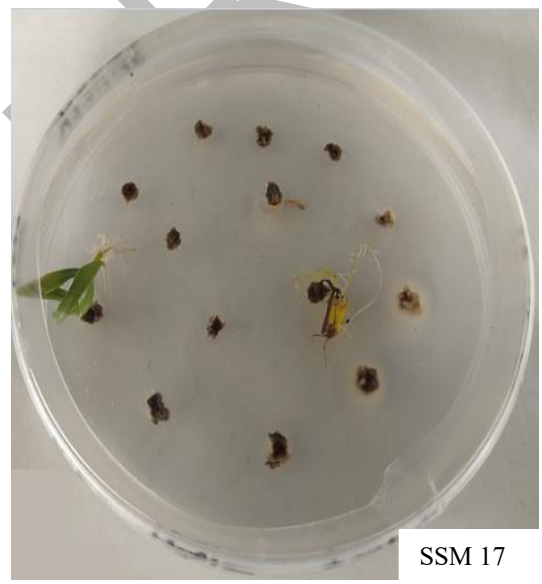


SSM 14

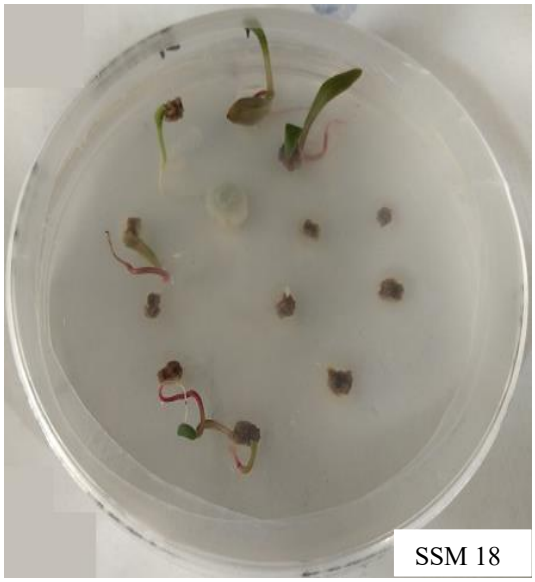


SSM 15

SSM 16



SSM 17



SSM 18

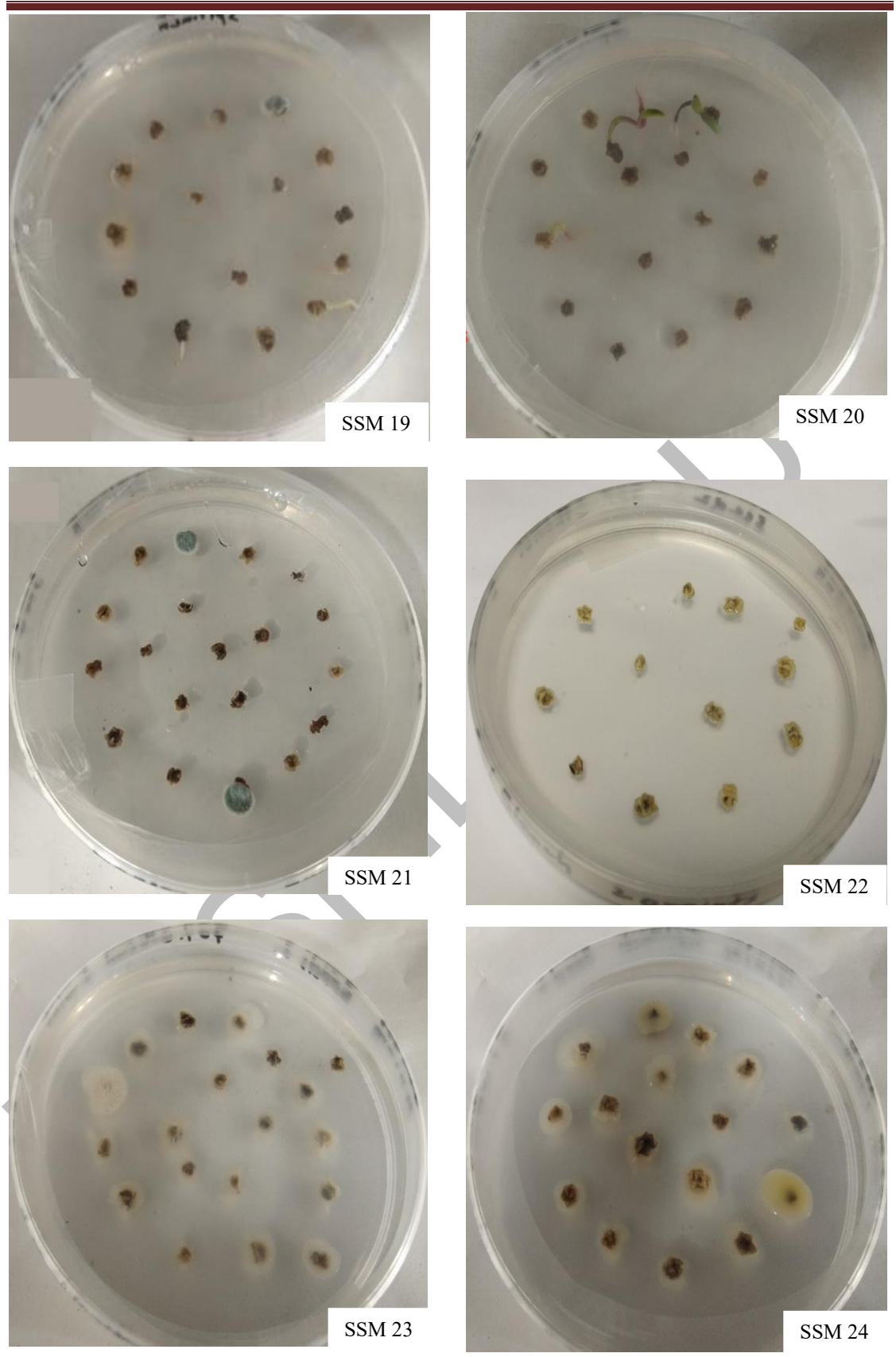


Figure 3.1: Different seed sterilization methods (SSM 1 to SSM 24) of spinach. Seeds were sterilized using different chemicals, analyzed after two weeks of inoculation on full MS media. After two weeks of observation SSM 14 gave the best results.

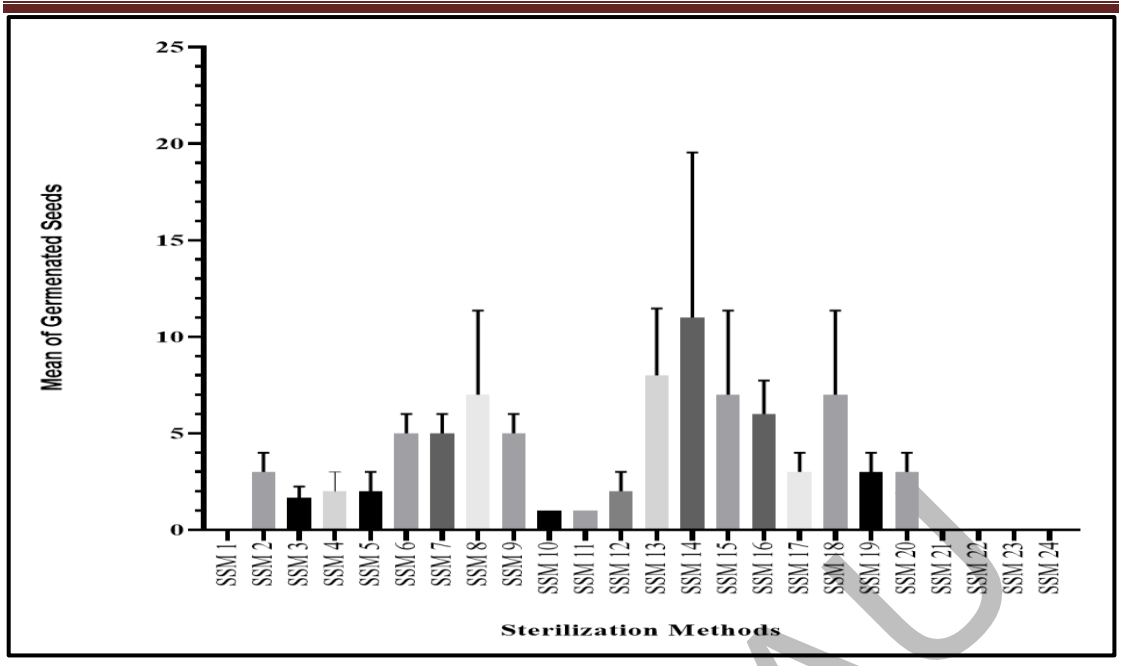


Figure 3.2: Graphical representation of spinach seed germination efficiency. Germination efficiency of spinach seeds using different sterilization methods. The graph represents data from three batches. SSM 14 gave the best results.

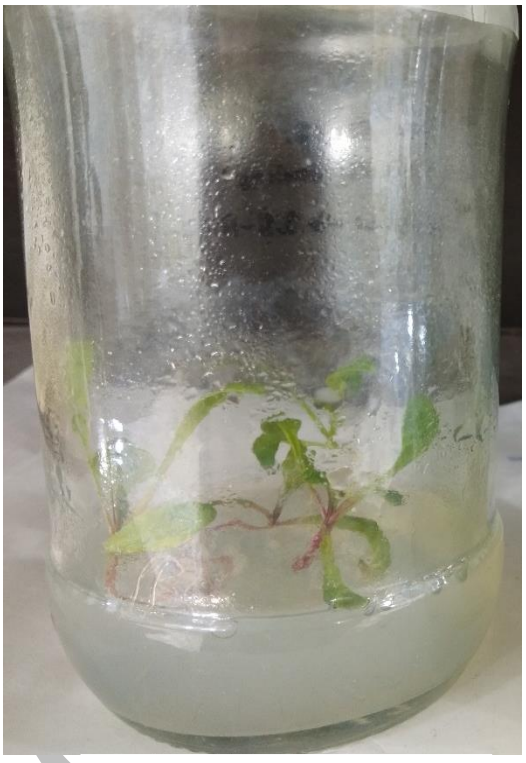
3.1.2. Germination media efficiency of spinach seeds

Sterilized seeds were divided into two groups- the first group was inoculated in ½ MS while the second was inoculated in full MS media within the sterile conditions of LFH. A total of 30 seeds were used. 15 out of total seeds were inoculated on half MS and the other 15 on full MS. The experiment was done in triplicates such that each jar consisted of 5 seeds only (5x3=15). From the results, it was observed that seeds on full MS media had higher germination efficiency than those on ½ MS media figure 3.3. Germination efficiency was calculated for both groups of seeds presented in table 3.2. Forty seven percent efficiency was observed on the half MS media and ninety-three percent efficiency was observed on the full MS media. Thus, for later experiments, spinach seeds were inoculated only on full MS media due to high germination efficiency. The graphical representation of spinach seeds germination on half and full MS depicted in figure 3.4.

Table 3.2: Germination efficiency of *Spinacia oleracea* on different MS strengths

Sr.no.	MS Strength	Total seeds inoculated	Seeds germinated	Germination efficiency (%)
1.	½ MS	15	7	47±1
2.	Full MS	15	14	93.3±2.082

(A) 0 day on ½ MS



(B) After 21 days on ½ MS

(C) 0 day on Full MS

(D) After 21 days on Full MS

Figure 3.3: Spinach seeds on different MS strengths**.** Seeds on ½ MS and full MS media. (A). Day 0 on ½ MS (B). Day 21 on ½ MS (C). Day 0 on full MS (D). Day 21 on full MS. Spinach seeds on Full MS media gave maximum germination.

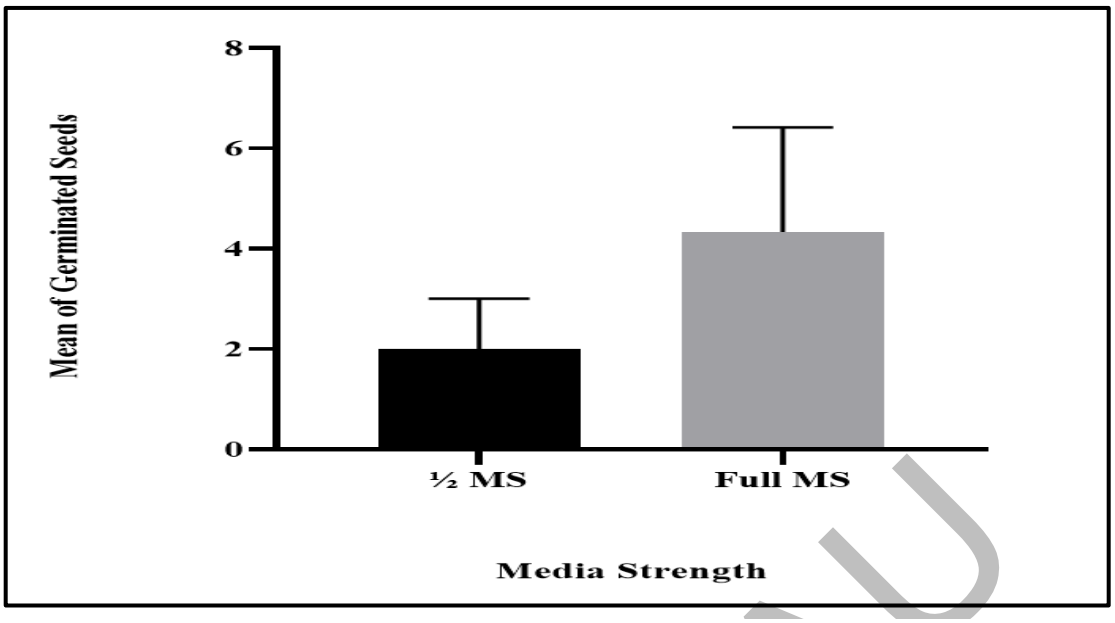


Figure 3.4: Graphical representation of seeds germination on both MS strengths. The data is from three batches (n=3).

3.2. Plant Regeneration

3.2.1. Regeneration in spinach explants

Experiments were done to analyze the best media for the regeneration of nodal explants. MS media with a combination of plant growth regulators was used. Explants were shifted to twenty-two different media (table 3.3). All these media were different from one another based on the MS strength and hormones that were used. The regeneration efficiency of nodal explants of *Spinacia oleracea* in different media is tabulated in table 3.3. After inoculation of nodal explant on MS media, they were observed for four weeks. The experimental results showed that, spinach regeneration media 15 (SRM 15) was the best regeneration media for the development of nodal explants depicted in figure 3.5.

In SRM 15, the callogenesis efficiency was 100%, which means the callus was formed from all the nodal explants. After one month, shooting was started from the callus of the nodal explants. The graphical representation for each media is provided in figure 3.6. The graph also represented that, SRM 15 was the best shooting or regeneration media for further experiments in this research. The SSM 15 shows highly significant regeneration efficiency as compared to the other media. The data was statistically analyzed by statistix 10 which displays the tendencies of all different spinach regeneration media used for shoot germination. After statistical analysis, the SRM 3 second best and SRM 12 third best regeneration media after SSM 15.

Table 3.3: Regeneration efficiency of nodal explants of spinach

Sr. No.	Spinach Regeneration Media (SRM)	Regeneration Efficiency calculated after 2 weeks				
		Total no of nodes inoculated	No. of nodes regenerated	No. of callus formed	Regeneration efficiency (%)	Callogenesis efficiency (%)
1	SRM 1	14	9	10	64 ± 1^{c}	71 ± 1.527^{b}
2	SRM 2	14	6	7	50 ± 1^{e}	50 ± 1.155^{e}
3	SRM 3	14	9	10	71 ± 1^{b}	71 ± 1.155^{b}
4	SRM4	14	5	6	43 ± 0.578^{f}	43 ± 1^{f}
5	SRM 5	14	6	8	43 ± 1^{f}	57 ± 1.155^{d}
6	SRM 6	14	0	0	0 ± 0^{j}	0 ± 0^{i}
7	SRM 7	14	0	0	0 ± 0^{j}	0 ± 0^{i}
8	SRM 8	14	0	0	0 ± 0^{j}	0 ± 0^{i}
9	SRM 9	14	5	7	36 ± 1.155^{g}	50 ± 1.527^{e}
10	SRM 10	14	8	9	57 ± 0.578^{d}	64 ± 1^{c}
11	SRM 11	14	5	6	36 ± 0.578^{g}	43 ± 1^{f}
12	SRM 12	14	9	10	64 ± 1^{c}	71 ± 1.527^{b}
13	SRM 13	14	6	7	43 ± 1^{f}	50 ± 0.578^{e}
14	SRM 14	14	3	8	21 ± 0^{i}	57 ± 0.578^{d}
15	SRM 15	14	14	14	100 ± 2.062^{a}	100 ± 2.082^{a}
16	SRM 16	14	3	4	21 ± 0^{i}	29 ± 0.578^{h}
17	SRM 17	14	0	0	0 ± 0^{j}	0 ± 0^{i}
18	SRM 18	14	0	0	0 ± 0^{j}	0 ± 0^{i}
19	SRM 19	14	4	5	29 ± 0.578^{h}	36 ± 1.155^{g}
20	SRM 20	14	4	5	29 ± 0.578^{h}	36 ± 0.578^{g}
21	SRM 21	14	8	9	57 ± 0.578^{d}	64 ± 1^{c}
22	SRM 22	14	7	8	50 ± 1.155^{e}	57 ± 1.527^{d}

SRM 1

SRM 2

SRM 3

SRM 4

SRM5

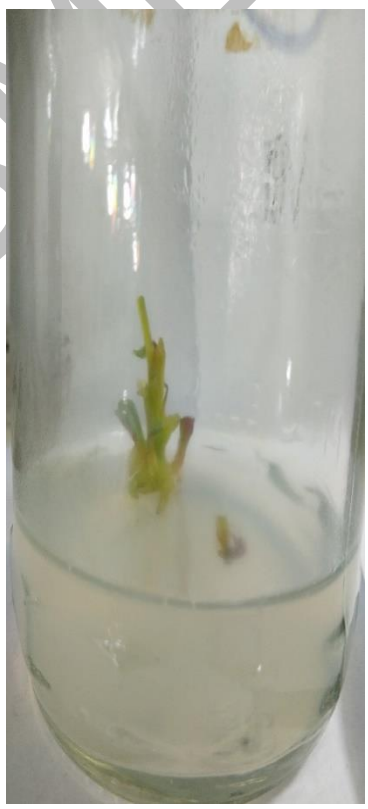
SRM 6

SRM 7

SRM 8

SRM 9

SRM 10



SRM 11

SRM 12

SRM 13

SRM 14

SRM 15

SRM 16

SRM 17

SRM 18

SRM 19

SRM 20

SRM 21

SRM 22

Figure 3.5: Spinach regeneration media (SRM 1 to SRM 22). Nodal explants were inoculated on twenty two different media, after two weeks of observation Spinach Regeneration Media 15 (SRM 15) showed the best results.

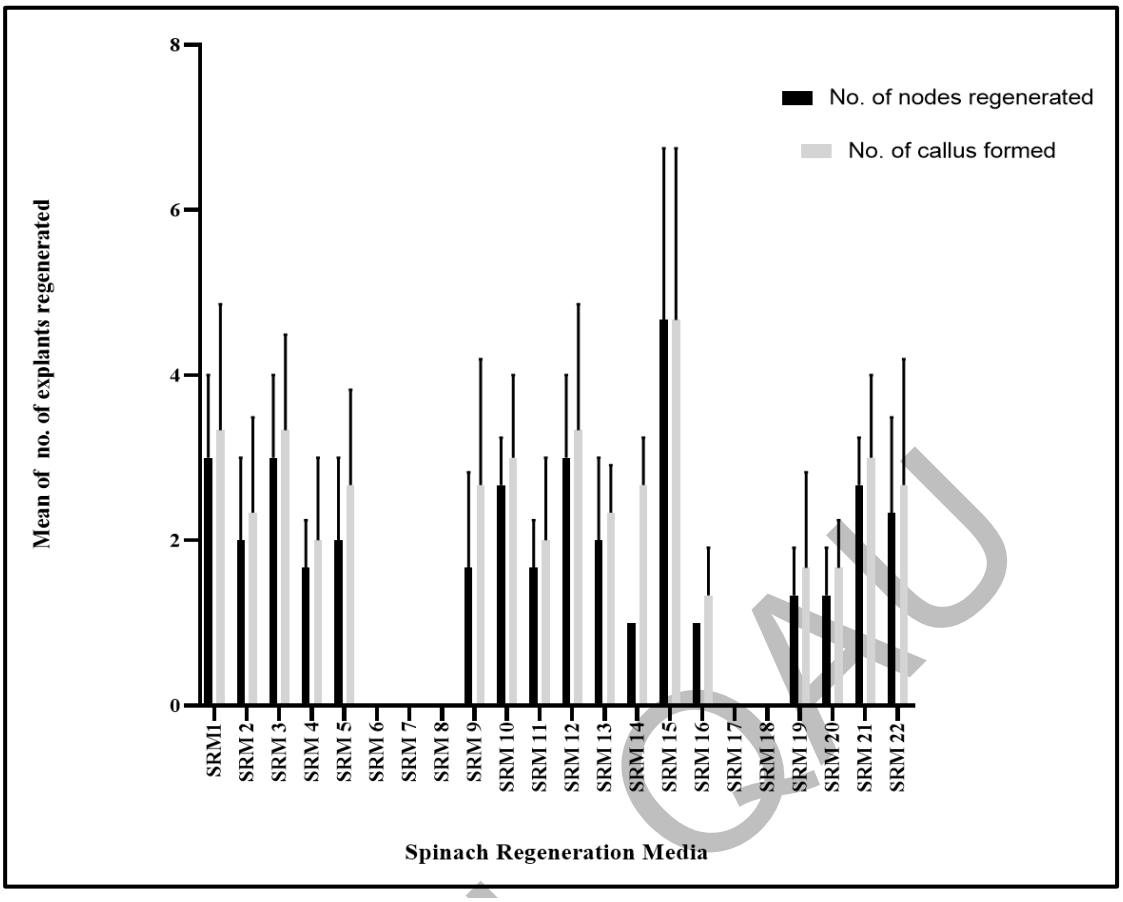


Figure 3.6: Graphical representation of percentage efficiencies of *Spinacia oleracea* explants on different regeneration media. The data shown here is from three batches (n=3). Regeneration Media 15 (SRM 15) showed the best results.

3.3 Effect of Hygromycin concentration on survival of explants

Wild-type explants were prepared and shifted to different concentrations of hygromycin to check its effect on these explants. The concentration of hygromycin that bleaches out wild type explants was used later for selection of transformed explants.

3.3.1 Effect of Hygromycin concentration on *Spinacia oleracea* explants

Optimal concentration of hygromycin was determined for selection of transgenic explants. *Spinacia oleracea* explants were inoculated on Spinach regeneration media 15 (SRM 15) with different concentrations of hygromycin i.e. 0 mg/l, 5 mg/l, 10 mg/l, 15 mg/l, 20 mg/l and 25 mg/l and results of following 3-4 weeks were recorded. It was noticed that with the increase in concentration of hygromycin the regeneration ability decreases and more explants tend to bleach out. Figure 3.7 shows the effect of different concentration of hygromycin on spinach explants with the results tabulated in table 3.4. Graphical representation of these results is given in figure 3.8. 10mg/l hygromycin were selected for transformation.

Table 3.4: Effect of hygromycin on nodal explants

Sr. No.	Concentration of hygromycin (mg/l)	Total no. of nodal explants	Explants regenerated	Explants bleached	Percentage of explants regenerated (%)	Percentage of bleached explants (%)
1.	0	15	15	0	100±1	0±0
2.	5	15	12	0	80±1	0±0
3.	10	15	5	14	33.3±0.578	93.3±2.082
4.	15	15	5	3	33.3±0.578	20±0
5.	20	15	4	4	27±0.578	27±0.578
6.	25	15	3	5	20±0.578	33.3±0.578

0mg/l Hygromycin

After two weeks

After one month

5mg/l Hygromycin

After two weeks

After one month

10mg/l Hygromycin

After two weeks

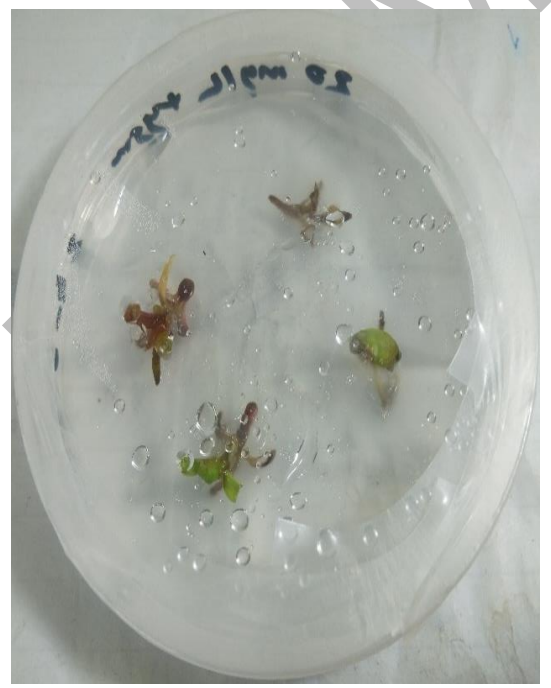
After one month

15mg/l Hygromycin

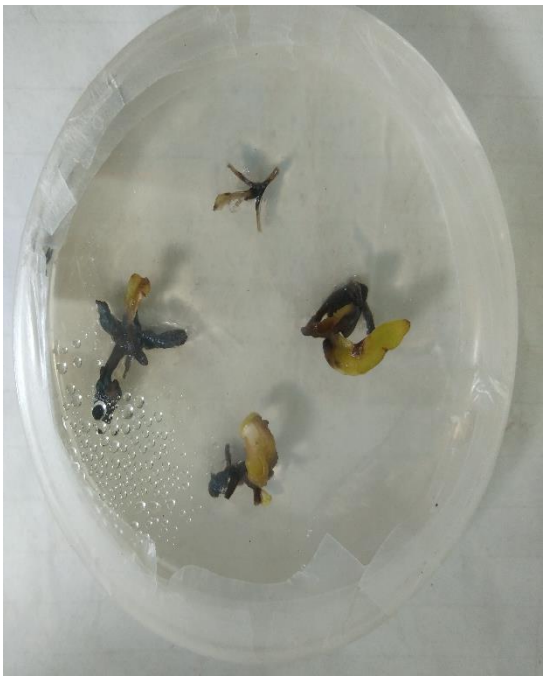
After two weeks

After one month

20mg/l Hygromycin



After Two weeks



After one month

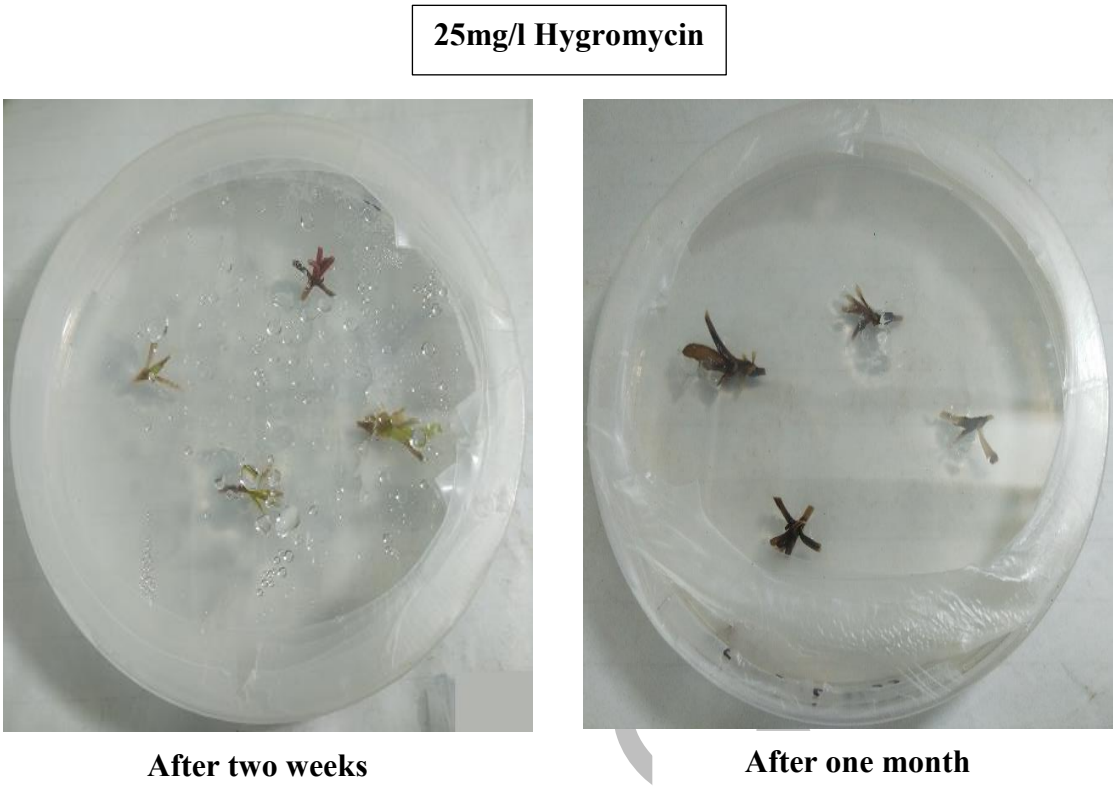


Figure 3.7: Effect of different hygromycin concentrations on nodal explants of *Spinacia oleracea*.

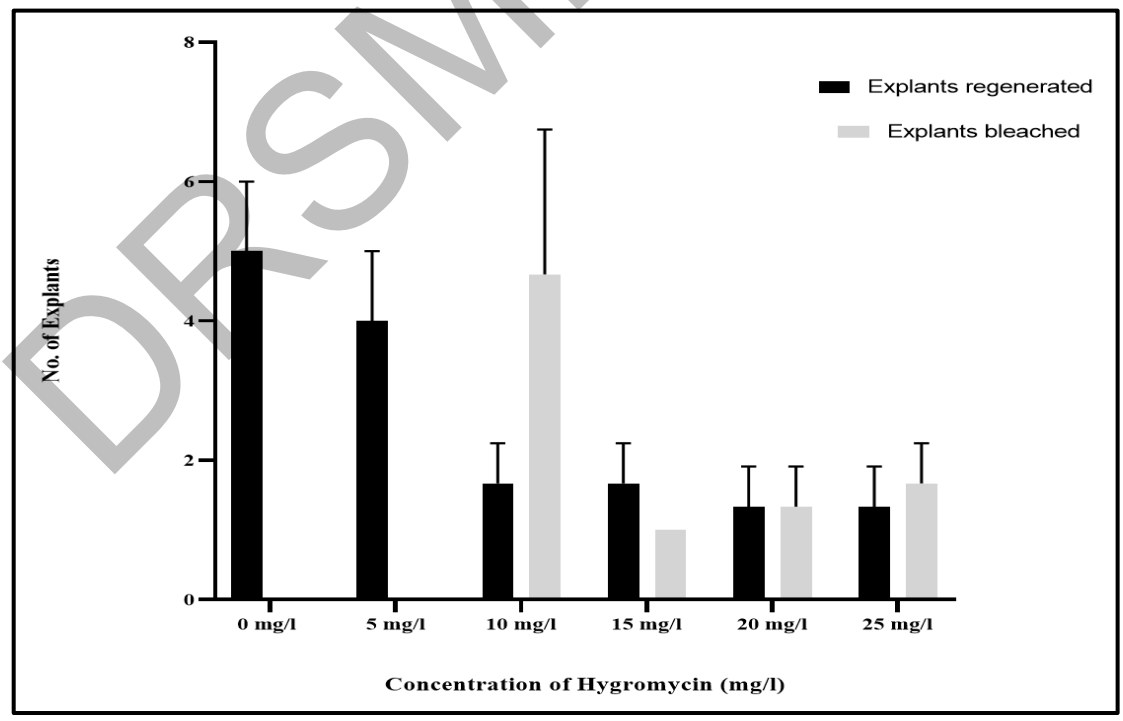


Figure 3.8: Graphical representation of hygromycin concentration effect on regeneration of explants. The graph represents data from three batches (n=3). 10mg/l hygromycin were selected for transformation.

3.4. PCR confirmation of plasmid containing OmpK gene

Sambrook and Rusell (2001) plasmid isolation protocol was used to isolate plasmid. Presence of OmpK gene in plasmid was confirmed by conventional PCR by using specific primers for amplification of the transgene. OmpK forward and reverse primers were used for the confirmation of OmpK gene in the plasmid. Gel results showed the presence of transgene in the isolated DNA samples (figure 3.9). Through OmpK primers transgene integration was confirmed.

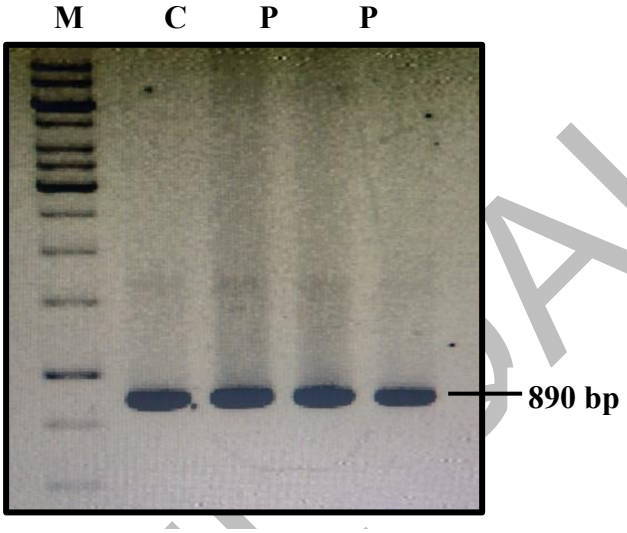


Figure 3.9: PCR confirmation of plasmid showing 890 bp fragment OmpK gene. 1Kb Marker (M), Positive control (C), Plasmids (P1, P2, P3).

3.5. *Agrobacterium*-mediated transformation

3.5.1. *Agrobacterium*-mediated Stable transformation of *Spinacia oleracea*

Nodes of *Spinacia oleracea* were used for transformation due to their greater ability to regenerate. Explants were prepared by cutting them into smaller pieces. *Agrobacterium tumefaciens* with pGWB5 having OmpK gene was used to infect these nodal explants. After infection, nodes were shifted to co-cultivation media before transferring them to antibiotic selection media.

3.5.1.1 Optimization of infection time

In this experiment nodes were divided into two groups. Both groups were infected with *Agrobacterium*. Some of the nodes were treated with bacteria culture for 8 minutes whereas the other group was treated for 10 minutes. The explants were observed for 7 days (figure 3.10). It was noticed that with the increase in the infection time it gets more difficult to get rid of excess bacteria and the regeneration efficiency of nodes also reduces. Thus, 8 minutes was considered as an optimum infection time for

transformation of nodal explants. Data regarding the infection time is provided in table 3.5 and graphically represented in figure 3.11.

Table 3.5: Effect of infection time on regeneration of explants

Infection time	Total no. of nodes	Nodes regenerated	Regeneration efficiency (%)
8 minutes	5	3	60±1
10 minutes	5	0	0±0

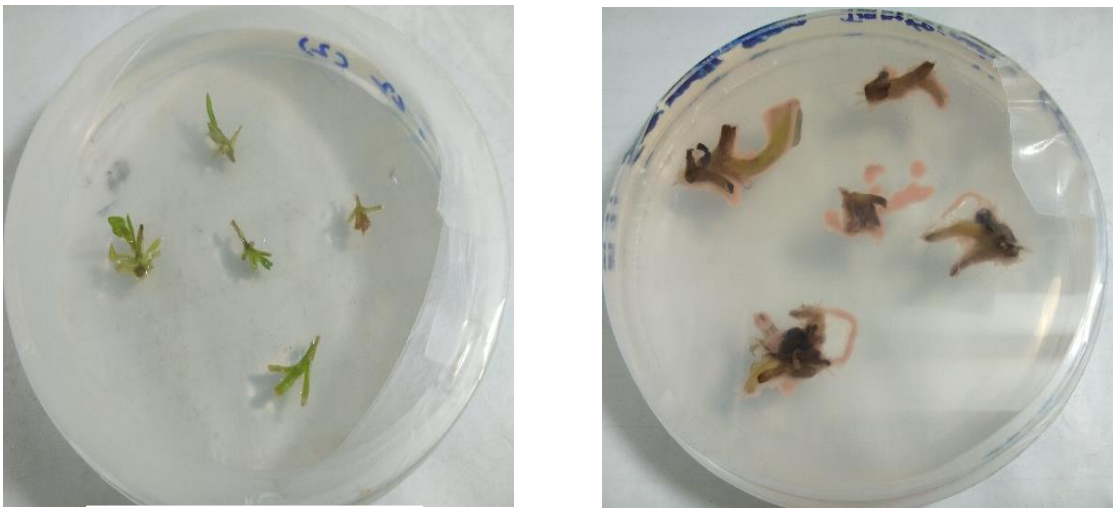


Figure 3.10: Nodal Explants were observed after 7 days observation, after infection on co-cultivation media. Explants were treated with *Agrobacterium tumefaciens* culture for different time periods.

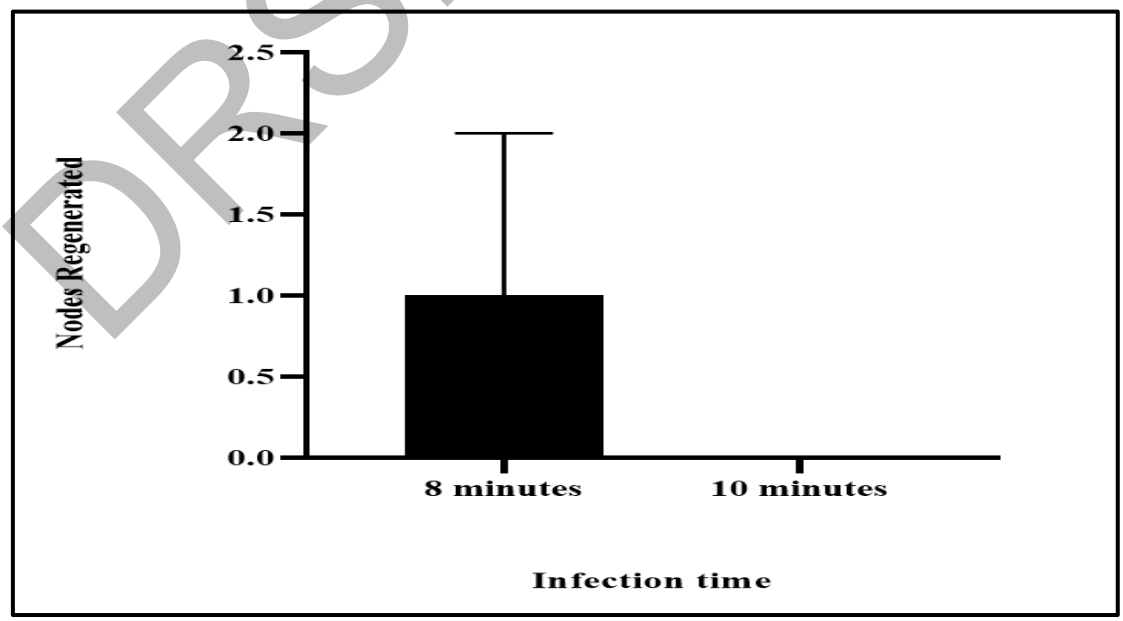


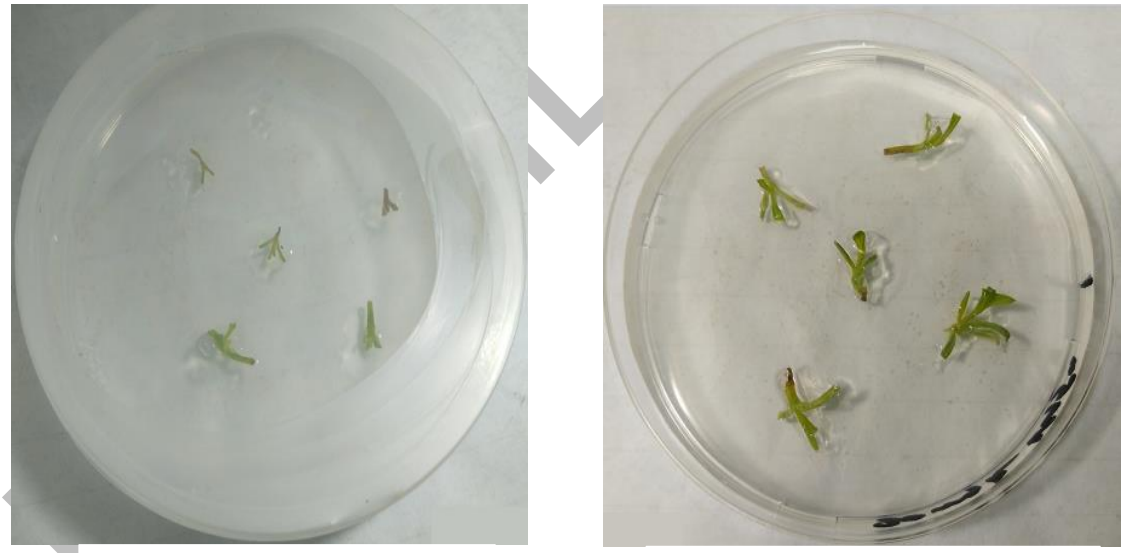
Figure 3.11: Graphical representation of effect of infection time on regeneration efficiency of explants. 8 minutes was considered as an optimum infection time for transformation of nodal explants.

3.5.1.2. Optimization of Co-cultivation time

After infection, explants were shifted on to co-cultivation media and were kept in dark for 2 and 3 days. After the respective co-cultivation time, the explants were observed. It was noticed that with the increase in the co-cultivation time increased regeneration efficiency in nodes were observed. A higher regeneration potential was observed in explants being kept for 3 days on co-cultivation media than the ones with co-cultivation time of 2 days as shown in figure 3.12. The results for optimization of co-cultivation time are tabulated in table 3.6 with graphical representation provided in figure 3.13.

Table 3.6: Effect of co-cultivation time on regeneration efficiency of explant

Co-cultivation time	Total no. of nodes	Nodes regenerated	Regeneration efficiency (%)
2 days	5	0	0±0
3 days	5	3	60±1



2 days co-cultivation time **3 days co-cultivation time**

Figure 3.12: Explants regeneration efficiencies after 2 days of co-cultivation time and 3 days.

From the results obtained, it was concluded that with the infection time of only 8 minutes and co-cultivation time of 3 days maximum transformation efficiency in nodal explants was observed. Thus, these optimum conditions were used to transform nodal explants of *Spinacia oleracea.*

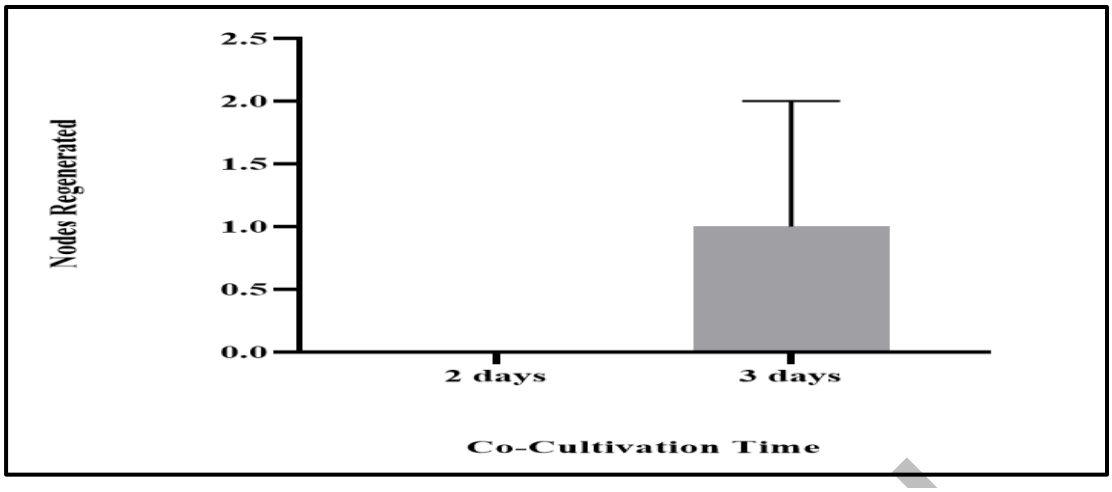


Figure 3.13: Graphical representation of Explants regeneration efficiencies after 2 days of co-cultivation time and 3 days. A higher regeneration potential was observed in explants being kept for 3 days on co-cultivation media.

3.5.1.3. Optimization of selection media

After co-cultivation plants were shifted to selection media containing 10mg/l hygromycin (figure 3.14).

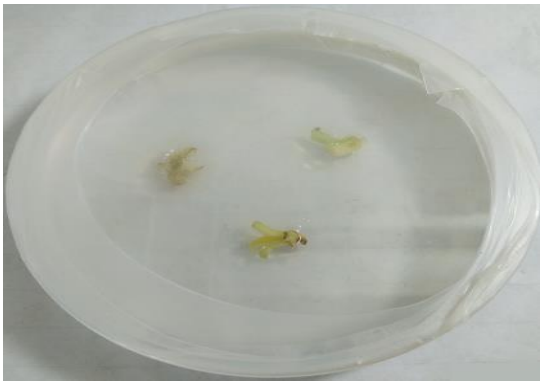
(A) Explants on selection media after co-cultivation

Batch 1

Batch 2

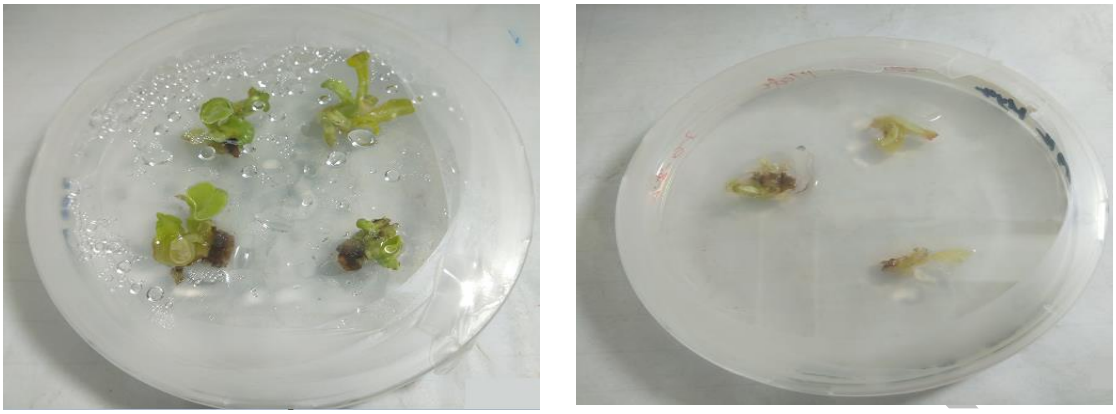
(B) Explants on selection plates after day 15

Batch 1



Batch 2

(C) Explants on selection plates after day 21



Batch 1 **Batch 2**

Figure 3.14: Selection of nodes on hygromycin.

3.5.1.4. PCR confirmation of transgene

Before PCR confirmation, extracted DNA was checked through agarose gel electrophoresis (figure 3.15). OmpK forward and reverse primers specific for our gene of interest (OmpK) were used. Figure 3.16 shows the gel image with amplified bands of 890 bp, which confirms the presence of OmpK transgene. Resultantly, successful transformation of *Spinacia oleracea* plant was confirmed.

M **N1** **N2**

Figure 3.15: Confirmation of DNA isolated from plant samples.1 kb marker (M), Sample 1 (N1), Sample 2 (N2)

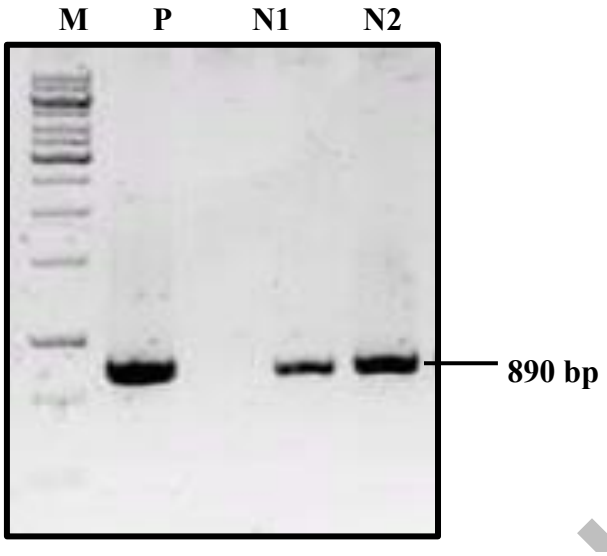


Figure 3.16: Confirmation of transgene. 1 kb marker (M), positive control (P), negative control (N), sample 1 (N1), sample 2 (N2).

3.5.1.5. Calculation of OmpK copy number in transgenic *Spinacia oleracea* by qRT-PCR

Copy number of all transformed samples was calculated by following the protocol established by Schmittgen and Livak (2008). qRT-PCR was used to calculate the copy number for the transgene (OmpK) in PCR positive samples. An endogenous gene (β actin) was also used. Cq values of transgene and the endogenous gene (table 3.7) were used to plot the standard curve which provided the correlation coefficient values for both genes, respectively. SQ values were used to calculate rline which ultimately provided us with the copy number of transgene by using the formula

δr line=rline[(δsq trans/Sqtrans)^2+(δSQend/Sqend)^2)]^1/2

This provided the correlation coefficient values for OmpK transgene and β actin. All these values were used to calculate the copy number for OmpK gene which was equal 3.78.

Table 3.7: Average CQ values of transgene vs endogenous gene

Sample	Average CQ values for OmpK transgene	Average CQ value for β actin
OmpK-1	26.7662409	29.82290853
OmpK 1:10	33.4414343	31.82694862
OmpK 1:100	34.1174909	32.77485378
OmpK 1:1000	34.7384188	33.31442407

3.5.1.6. Dot Blot

Before western blotting Dot blot was done to check the presence of protein within the samples. Specific antibodies were used to detect OmpK protein. Both wild-type and samples were spotted on the blot. Unlike wild-type positive results were observed for both samples (figure 3.17). This confirmed the presence of transgenic protein in our samples.

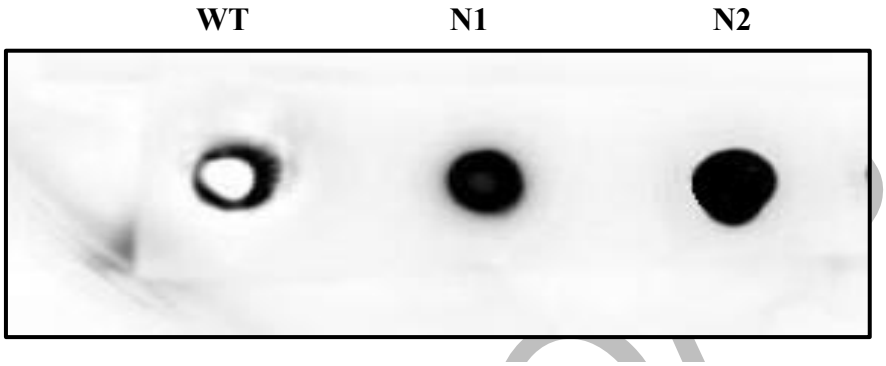


Figure 3.17: Confirmation of presence of OmpK protein in transformed samples. Wild type (WT), sample 1 (N1), sample 2 (N2).

3.5.1.7. ELISA

Indirect ELISA was used to confirm the presence of OmpK antigen within our transgenic samples. Total soluble protein extracted from transgenic plant was used. The data presented in figure 3.18 shows an increased absorbance of transgenic samples compared to the wild-type one. Among the two samples, N2 shows highest absorbance which means that it contains highest amount of antigen.

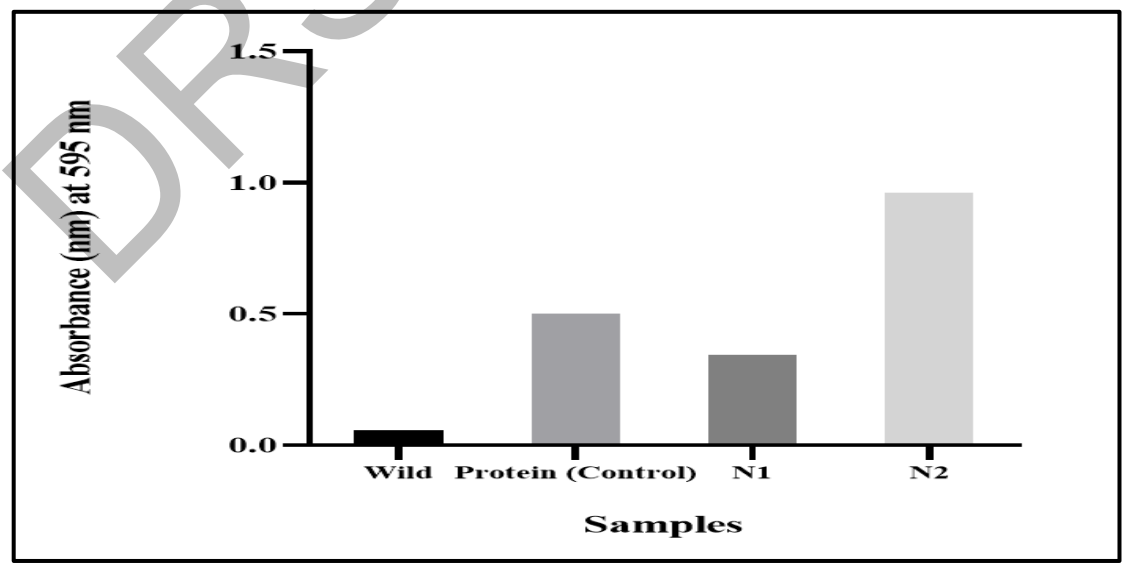


Figure 3.18: Graphical representation of ELISA of wild-type sample, positive protein control and transgenic spinach samples. N1 and N2 are two transgenic nodal samples.

3.5.2. Agroinfiltration of spinach leaves

Leaves of *Spinacia oleracea* were in filtered with *Agrobacterium* containing binary vector pGWB5 having OmpK.

3.5.2.1. Leaves collection

Leaves were infiltrated by protocol mentioned by Sparkes *et al*. (2006). They were picked at different days i.e., on 1st, 2nd, 3rd and 4th day of infiltration and were used for PCR confirmation and for gene expression analysis.

3.5.2.2. PCR analysis

DNA was extracted from the leaves by following protocol given by Murray and Thompson (1980), with few modifications. Bands on agarose gel confirmed the successful isolation of DNA (Figure 3.19). Presence of transgene (OmpK) was confirmed by conventional PCR.

Specific primers were used for confirmation of transgene in our leaves samples. Figure 3.20 shows a gel image with 890 bp bands of amplified PCR product of controls vs transgenic samples. The expected bands were observed on all four days of leaves samples. This confirmed the presence of transgene in the infiltrated leaves (Figure 3.20).

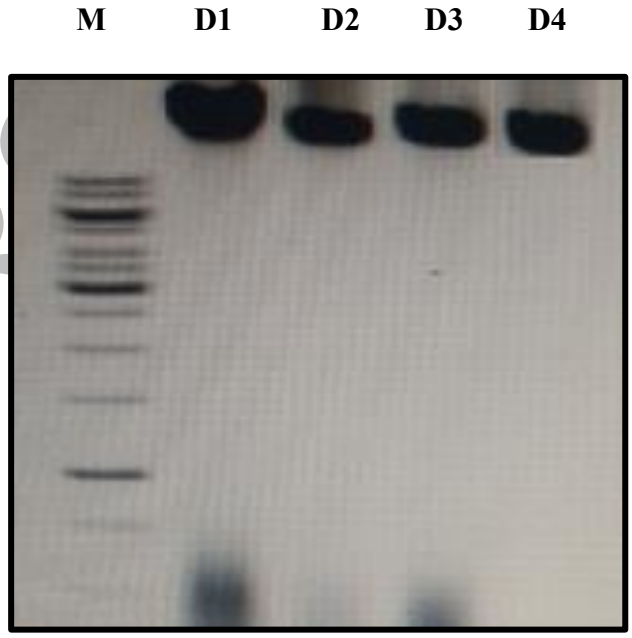


Figure 3.19: Gel image of isolated DNA samples by CTAB method. DNA isolated from infiltrated leaves samples of spinach picked at different days. 1 kb marker (M), leaves collected on day 1 of infiltration (D1), leaves collected on day 2 of infiltration (D2), leaves collected on day 3 of infiltration (D3), leaves collected on day 4 of infiltration (D4).

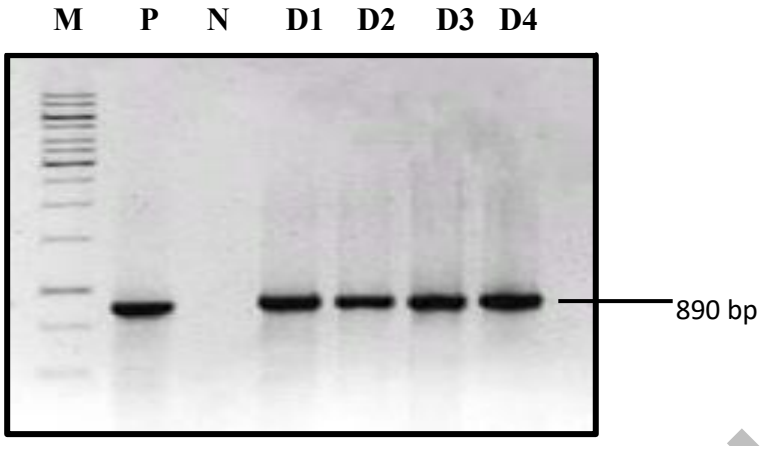


Figure 3.20: Confirmation of transgene in infiltrated leaves samples of *Spinacia oleracea.* 1 kb marker (M), positive control (P), negative control (N), leaves collected on day 1 of infiltration (D1), leaves collected on day 2 of infiltration (D2), leaves collected on day 3 of infiltration (D3), leaves collected on day 4 of infiltration (D4).

3.5.2.3. Dot Blot analysis

For detection of OmpK protein Dot blot analysis was done. Wild-type and sample were spotted on blot. Gel documentation system was used to analyze blot. After treatment with antigen specific antibodies, blot was analyzed. It was noticed that unlike wild-type sample, chemiluminescence was observed for the *Spinacia oleracea* leaves samples (figure 3.21). All days samples of leaves gave positive dot blot.

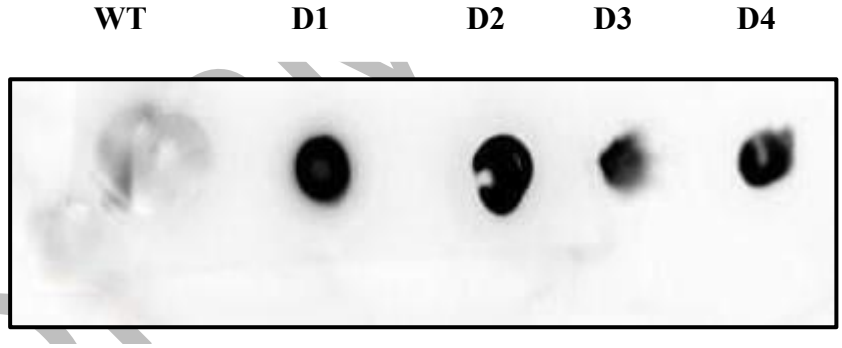


Figure 3.21: Dot blot analysis. Wild-type (WT), leaves collected on day 1 of infiltration (D1), leaves collected on day 2 of infiltration (D2), leaves collected on day 3 of infiltration (D3), leaves collected on day 4 of infiltration (D4). All four days samples of leaves gave positive dot blot.

3.5.2.4. Western blotting

Western blotting was used to check the expression of OmpK transgene. Monomeric form of OmpK with size equal to 30.21 kDa. In our research, dimeric form of OmpK was detected in second and third day samples as shown in figure 3.23. By using specific antibodies, protein was confirmed in the *Spinacia oleracea* leaves sample with no band observed for wild-type sample (figure 3.22). This detection of 61 kDa Ompk protein

confirmed the successful transient transformation of spinach leaves. Samples from day 2 and day 3 had transgenic OmpK protein.

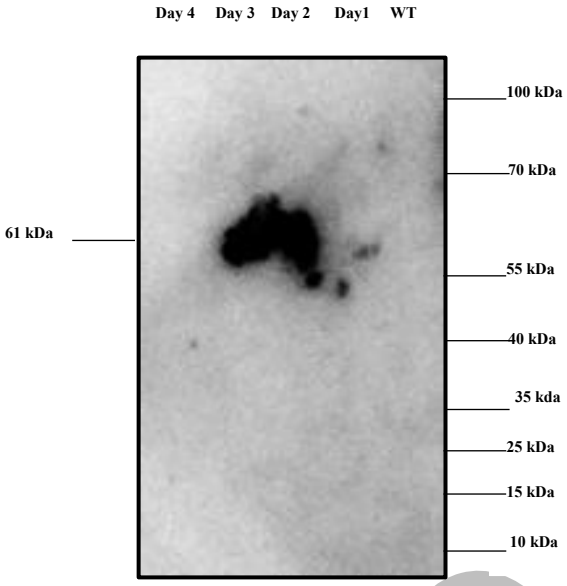


Figure 3.22: Western Blot analysis. Wild-type (WT), leaves collected on day 1 of infiltration (D1), leaves collected on day 2 of infiltration (D2), leaves collected on day 3 of infiltration (D3), leaves collected on day 4 of infiltration (D4). OmpK protein (dimer 61 kDa) was present in day 2nd and day 3rd leaves samples.

3.5.2.5. ELISA

To confirm the presence of OmpK antigen within our transgenic samples indirect ELISA was used. Total protein extract from all four days transgenic samples was used. The data presented in figure 3.23 shows an increased absorbance of transgenic samples compared to the wild-type one. Samples from day 2 and day 3 have transgenic OmpK protein.

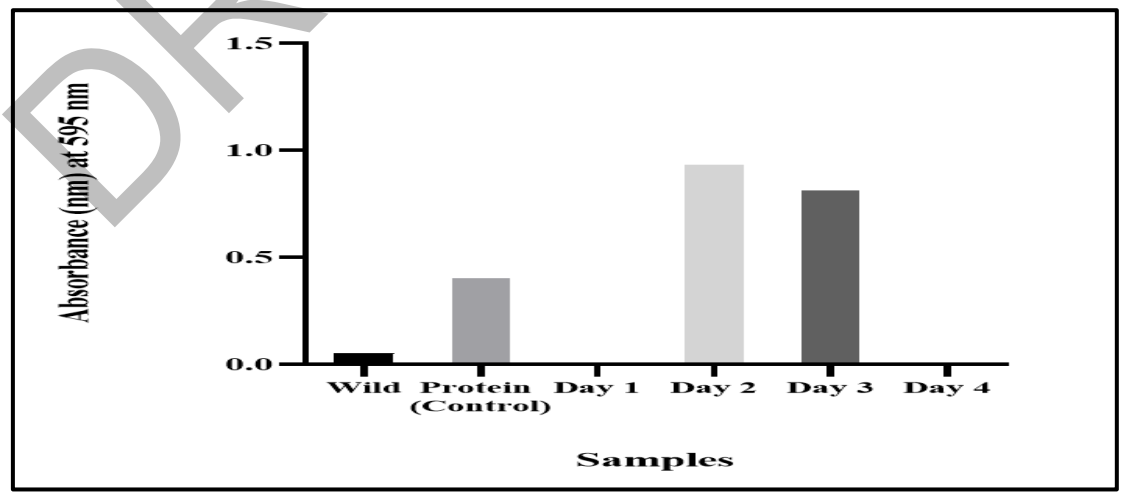


Figure 3.23: Graphical representation of ELISA of wild-type, positive protein control and agroinfiltrated spinach leaves samples. D1, D2, D3, and D4 are transgenic leaves samples. OmpK protein was present in day 2nd and day 3rd leaves samples.

4. Discussion

4.1. Expression of anti-vibriosis vaccine antigens in plants

Vibrio anguillarum is the causative agent of vibriosis, a fatal haemorrhagic septicaemic condition that affects fish, bivalves, and crustaceans in both freshwater and marine environments. Vibriosis is one of the most prevalent and harmful infections in marine aquaculture, causing considerable economic losses and fish species deaths in both freshwater and saltwater around the world. Compared to all other sectors that raise animals for food, aquaculture is expanding more quickly than any other, yet diseases constitute a significant obstacle. The disease "Vibriosis" has a negative impact on aquaculture farms, thus farmers must choose the most effective treatment with consideration (Frans *et al*., 2011).

To eradicate vibriosis, an innovative, secure, and more stable vaccine must be created. The creation of subunit vaccines, which can stimulate an immune response in a protective manner, represents a more successful and promising technique (Kurup & Thomas, 2020). OmpK, one of the multiple antigenic proteins, is the most noticeable antigen of *Vibrio anguillarum* with the ability to trigger a protective immune response, making it an ideal antigen candidate for the creation of a vibriosis vaccine (Maiti *et al*., 2020).

The manufacture of vaccines using a plant-based system has many benefits over traditional techniques. Edible plants can provide biotechnological solutions for the creation of extremely safe, quick, high-yield, and affordable vaccinations. Plant-derived edible vaccines have many important characteristics, such as low health concerns, the minimal need for a cooling chain, great scalability, and cost-effective downstream processing (Cardi *et al*., 2010; Saba *et al*., 2019). The goal of the present study was to investigate the feasibility of developing an edible vibriosis vaccine generated from plants. In order to do this, an edible plant *Spinacia oleracea* was transformed with OmpK gene through *Agrobacterium*-mediated transformation.

4.2. *Spinacia oleracea* as a model plant for the expression of foreign proteins

The important leafy vegetable *Spinacia oleracea* can be grown all over the world. Its genome has been extensively studied. Spinach is unique among plant bioreactors in that it has a shorter production cycle and can be used as an expression system to produce foreign proteins or vaccines quickly and affordably. The expression of foreign proteins

in spinach plants can be promoted under highly favorable conditions thanks to the genetic engineering of spinach, a relatively well-established method. In spinach, post-transcriptional alteration of an exogenous gene's expression product can be accomplished successfully, safely, and affordably (Mane *et al*., 2015; Xu *et al*., 2017).

4.3. Optimization of various parameters for the growth, seed sterilization, tissue culture, and nuclear transformation of *Spinacia oleracea*

In the current research study, we optimized the best seeds sterilization method, best regeneration media, and optimum hygromycin concentration for the selection of transformed nodal explants of spinach. Tissue culture, breeding, and genetic engineering of plants have great importance to improve and add new characteristics in existing cultivars of plants. Being an important food crop, spinach can be regenerated and transformed by using a reliable and effective protocol.

4.3.1. Optimization of Seeds sterilization protocol

Sterilization of the seeds is a very important and necessary process. The effectiveness of seeds to germinate may be impacted by improper sterilization. The surface-sterilization of seeds may be accomplished using a variety of disinfectants, according to research. These include ethanol, mercuric chloride ($HgCl_2$), bromine water, hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOCl)/calcium hypochlorite ($Ca(ClO)_2$), and silver nitrate ($AgNO_3$). Various sterilizing agents including ethanol, commercial bleach (NaOCl)), Tween 20, hydrogen peroxide, sodium dodecyl (SDS), and mercuric chloride are utilized either separately or in combination during sterilization procedures (Bakhsh *et al*., 2016). Commercial bleach is observed as a potent microbe killer and commonly used as a surface disinfectant of seeds for in vitro culture. It is usually utilized in combination with a 70% ethanol that is also examined as a good seed sterilizing agent for in vitro culture of plants (Rifna *et al*., 2019). The type of disinfectant and the kind of seeds being utilized determine the concentration and exposure time. Strong chemicals should not be used on sensitive seeds because they will damage the seed. Therefore, choosing a sterilizing agent is crucial. Although ethanol is a potent sterilizer, it is also poisonous to plants (Lindsey *et al*., 2017).

In this study, spinach seeds were subjected to several disinfectants for varying lengths of time. After two weeks, the germination efficiencies of spinach seeds were calculated, and it was shown that seed sterilization method 14 (SSM 14), produced the maximum germination efficiency.

4.3.2. Optimization of tissue culture

Explants' ability to regenerate in vitro depends on a variety of parameters. These include the quantity and quality of light, humidity, nutrients, phytohormone concentration, ambient temperature, and pH conditions. Proper callus formation and shoot development could happen successfully under the influence of favorable conditions and the right levels of plant growth regulators. Combinations of plant growth regulators were utilized to assess how they affected plant growth. To examine their impacts on shoot development and callus formation, various hormones including BAP, IAA, NAA, thiamine HCl, myoinositol, zeatin, and kinetin were combined.

Only nodal explants were used in this research. Explants of spinach were inoculated into 22 different mediums with various hormone concentrations. On spinach regeneration media 15 (SRM 15) containing BAP and IAA, the nodal explants removed from the spinach plant were inoculated. Results indicate that 100% regeneration efficiency was seen after two weeks of inoculation on SRM 15 media.

4.3.2. Optimization of hygromycin concentration

In order to find the ideal concentration of hygromycin for the selection of transformed nodal explants of spinach, different concentrations of hygromycin, including 0, 5, 10, 15, 20, and 25 mg/l, were added to spinach regeneration media 15 (SRM 15). At 10mg/l of hygromycin, no callus production or shoot regeneration was seen in the spinach nodal explants, which were entirely chlorotic. As a result, the effective concentration of hygromycin for spinach nuclear transformation was chosen to be 10 mg/l. In previous work, 50 mg/l of kanamycin was used to successfully convert *Spinacia oleracea* (Chin *et al*., 2009). In our investigation, utilizing a 10mg/l hygromycin yielded the best outcomes when compared to the earlier study.

4.4. *Agrobacterium*-mediated stable transformation of *Spinacia oleracea*

The *Agrobacterium*-mediated transformation efficiency is influenced by a variety of variables. These variables include the bacterial inoculum density, infection time, and co-cultivation period. The ideal transformation conditions for *Spinacia oleracea* are described in this study. A crucial element is the choice of O.D. It was discovered that O.D between 0.6 and 0.8 was useful for transformation. O.D values greater than 0.8 have a negative impact on the explants and decrease the effectiveness of *Agrobacterium*'s infection. At $O.D_{600}$ 0.6, the best transformation results were attained.

The infection time is another element that influences the effectiveness of transformation. It was found that explants with an infection lasting only 8 minutes had a higher capacity for regeneration that was 60%. On the other hand, explants that received 10 minutes of treatment time showed 0% regeneration efficiency. The effectiveness of *Agrobacterium*'s transformation and the explants' capacity for regeneration are both impacted by the co-cultivation time. Explants with a 3-day co-cultivation period demonstrated a higher rate of regeneration than those with a 2-day co-cultivation period. It was determined that more time enhances nodes' capacity for regeneration. Similar results were shown by Naderi *et al*., (2012).

The pre-conditioning of explants on regeneration media is a crucial factor that affects the transformation efficiency before infection. The regeneration ability of explants is improved and good transformation outcomes are produced by pre-culturing for two to three days. Explants treated with this method prior to infection and co-cultivation with *Agrobacterium tumefaciens* have also been shown to boost the effectiveness of transformation in *Cajanus cajan* and *Vigna unguiculata* (Geetha *et al*., 1999).

In our investigation, nodal explants were treated with an *Agrobacterium* expressing the OmpK gene for 8 minutes, co-cultivated on SRM 15 media, kept in the dark for 72 hours (3 days), and selected using SRM 15 media with 10mg/l hygromycin. *Agrobacterium tumefaciens* growth was also controlled by the addition of 200 mg/L cefotaxime to the selection media. *Agrobacterium* growth was reported to be controlled at 200-300 mg/l by certain other groups (Mehmood, 2016).

Several molecular approaches, including PCR, Dot Blot analysis, qRT-PCR, and ELISA, were used to confirm the incorporation of the transgene. Using the Kang *et al*., (2012) methodology, qRT-PCR was carried out to determine the copy number. It was found that although the transgenic DNA samples produced positive results, the wild-type sample gave no results. The results showed the copy number for OmpK gene which was equal 3.78.

Dot blot analyses was used for the protein analysis. Contrary to transgenic samples, the wild-type sample did not exhibit any chemiluminescence signal when the dot blot was seen. Stably transformed transgenic explants showed promising results.

To further verify the protein content of our samples, ELISA was also conducted. The appearance of the OmpK antigen in our extracted protein samples was confirmed by the change in color that occurred with the addition of TMB substrate. The N2 transgenic

sample was found to have a greater absorbance at 595 nm than the N1 sample, indicating that the former had more integrated protein.

4.5. *Agrobacterium*-mediated transient transformation of *Spinacia oleracea*

Establishing an effective *Agrobacterium*-mediated transient transformation system requires the identification and adjustment of parameters controlling T-DNA delivery. Different factors, such as the strain of *Agrobacterium tumefaciens* used, the genotype of the plant, the expression vector, the O.D. of the bacterial inoculum, the concentration of acetosyringone used during infection, the co-cultivation time, and the amount of infiltrated media, affect the efficiency of transient transformation, as previously reported (Cao *et al*., 2017).

Different strains of agrobacteria can be employed to infect a host. Some of these strains have been found to be significantly more virulent than others (Heidari-Japelaghi *et al*., 2020). In our investigation, we infected spinach leaves with the *Agrobacterium tumefaciens* GV3101 strain, which offered strong expression of OmpK.

Acetosyringone concentrations also alters *Agrobacterium* infection. A naturally occurring phenolic substance called acetosyringone draws bacteria to the location of the wound. Phenolic signals facilitate the transfer of T-DNA into the plant cell and increase the transformation efficiency by inducing pathogenic agrobacterium genes (McCullen & Binns, 2006). Transformation efficiency is improved by incorporating 1M acetosyringone into the infiltration media.

The inoculum density, or O.D., of the bacterial culture, is one of the most crucial elements in transformation. According to reports, the process of agroinfiltration was influenced by *Agrobacterium* density, and every rise in O.D600 of 1.0 had a negative impact on transformation phenomena (Heidari-Japelaghi *et al*., 2020). The final O.D600 of the *Agrobacterium* inoculum used in our current study, for infiltration, was equal to 0.1. The study by Sparkes *et al*., (2006) also made reference to this inoculum density.

The transgene only expresses itself briefly and for a short period of time in a transient expression system. In the case of agroinfiltration in tobacco leaves, the expression only requires a few days, according to Sparkes *et al*., (2006). Additionally, it has been suggested that the post-transcriptional silencing of the gene was connected to the transgene's shorter period of expression. However, co-infection of the transgene with

the viral silencing suppressor gene can increase the transgene's levels of expression (Shamloul *et al*., 2014). *Spinacia oleracea* (leaves) were infiltrated and co-cultivated for 4 days in our study. From the first day of infiltration until the fourth day, all four days showed the maximum expression of our transgene.

Several molecular biology methods, including PCR, Dot blot, western blotting, and ELISA, were used to confirm the transgene's expression. The OmpK gene's incorporation into the genomic DNA of the plant was examined using PCR. It was necessary to use a certain primer pair that would result in an amplified product of 890 bp. As a control, a wild-type sample was employed. In all transgenic plant samples taken from the first to the fourth day of infiltration, an amplified product of 890 bp was found. Samples collected on from day1 to day 4 yielded results for DNA isolation, whereas samples of the wild type yielded no results. Thus, samples from all 4 days were used for qRT-PCR and protein analysis.

Dot blotting and western blotting are two methods for protein analysis. Both of these methods allow verification of transgene incorporation. Total soluble protein was isolated from both wild-type and samples from day 1 to day 4th. Samples of leaves from days 1 to 4 showed positive results in dot blot, but in the case of western blot just two samples from day 2nd and day 3rd showed a dimeric form of OmpK protein had 61 kDa band, which is a confirmation of the transgene OmpK. The dimer formed due to the presence of six histidine molecules in the protein sequence.

OmpK antigenic protein expression was also verified by ELISA. In contrast to the blank and wild-type sample, protein extracts from all days of samples, just two samples from day 2nd and day 3rd changed color when TMB substrate was added.

4.6. Conclusion and Future Perspectives

We have optimized multiple variables required for *Agrobacterium*-mediated transformation of *Spinacia oleracea.*

- Treatment with 0.2% $HgCl_2$ for 3 minutes could be used for sterilization of spinach seeds.
- Spinach seeds germinated more efficiently on full MS media.
- Full MS containing BAP 5mg/l and IAA 0.5mg/l might be used for the regeneration of spinach explants (nodal).

- Parameters like hygromycin concentration, co-cultivation period, infection time and bacterial density were optimized for stable transformation of *Spinacia oleracea.*
- In case of *Agrobacterium*-mediated transient transformation of spinach, factors like O.D of bacterial inoculum and co-cultivation time was optimized.

The current study provides optimized conditions for stable and transient expression of OmpK transgene in *Spinacia oleracea.* Maximum expression of transgene was observed when nodal explants of spinach were co-cultivated for 3 days after 8 minutes of infection time. On the other hand, highest expression of OmpK in transiently transformed spinach leaves were observed in leaves picked from day 2rd and day 3rd of infiltration. Taken together, in future, this data could facilitate the development of a cost-effective antigen-based subunit vaccine against vibriosis, which may have the potential to be delivered orally. Also, our results provide conditions necessary for successful transformation of *Spinacia oleracea* through agroinfiltration that might be used for many other applications.

5. References

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