Antibiotic Optimization And Stable Transformation of Spinach With Outer Membrane Protein K Antigen

By

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CERTIFICATE

This thesis, submitted by **Ms. Hira Kanwal** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

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Dated: **October 19, 2023**

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.

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.

Hira Kanwal

This thesis is dedicated whole-heartedly to

My Loving Parents

For their endless love, affection, support and encouragement.

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LIST OF ABBREVIATIONS

β	Beta
BAP	6-benzylaminopurine
µl	Microlitre
µm	Micrometre
½ MS medium	Half MS Medium
Bp	Base pair
cm	Centimetre
CTAB	Cetyl Trimethyl Ammonium Bromide
d.H_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine tetra acetic acid
GIT	Gastrointestinal tract
HCl	Hydrochloric acid
HEPES	4-2-Hydroxyethylpiperazine-1-ethanesulfonic acid
HRP	Horse radish peroxidase
i.e.	That is
IAA	Indole Acetic Acid
IDs	Infectious Diseases
Kb	Kilo base
L	Litre
LA	Luria-bertani Agar
LB	Left border

LB	Lysogeny broth
LFH	Laminar Flow Hood
M	Molar
MES	N-morpholino ethanesulfonic acid
mg	Milligram
mg/L	Milligram per litre
ml	Millilitre
MS medium	Murashige and Skoog Medium
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
°C	Degree Centigrade
OD	Optical Density
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pH	Power of Hydrogen ions
TBE	Tris Boric Acid EDTA
TBS-T	Tris buffered saline with tween
T-DNA	Transferred DNA
TE	Tris EDTA
Ti	Tumor-inducing
Tm	Melting temperature
TMB	Melting Temperature
TSP	Total Soluble Protein

UV	Ultra violet
Vir	Virulence
WHO	World Health Organization
2,4-D	2,4-Dichlorophenoxyacetic acid

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ABSTRACT

Vibriosis is a bacterial disease that is caused by fish pathogen *Vibrio anguillarum*. This deadly hemorrhagic disease affects numerous fish species that are economically important for aquaculture, causing high rates of morbidity and mortality. To prevent fish, bivalves and crustaceans from vibriosis, development of cost-effective vaccine is required. Outer membrane protein K (pOmpK) is an immunogenic protein of *Vibrio anguillarum* and serves as potential candidate for subunit vaccine development. Plants can be used as affordable bio-factories for expression of vaccine antigens. The present research work aimed to optimize the tissue culture conditions for *Spinacia oleracea* leaf explants and to develop an efficient transformation protocol for *Agrobacterium*-mediated stable transformation of *Spinacia oleracea* with OmpK antigen. Sterilization of spinach seeds with 0.2% mercuric chloride provided good results. Full MS media gave maximum regeneration efficiency for spinach plant. For tissue culture, full MS media supplemented with 1 mg/L BAP and 0.5 mg/L IAA showed the highest callus formation efficiency. Optimization of hygromycin antibiotic was performed for *Spinacia oleracea*; with 20 mg/L being the optimal hygromycin concentration for selection of transformed leaf explants. Successful stable transformation of spinach was carried out with OmpK antigen. Transformed explants were grown on selection media containing suitable concentration of hygromycin. Explants with 3 days co-cultivation time showed 52.94% callus formation efficiency. Transformation was confirmed through PCR using gene specific primers. Transgene expression in plants was analyzed by quantitative real-time PCR (qRT-PCR) in comparison with *β-actin* gene as control. Protein expression was confirmed through Dot Blot, Western blotting and ELISA. Taken together, successful expression of *Vibrio anguillarum* OmpK antigen may facilitate the development of edible and cost-effective subunit vaccine against vibriosis.

Keywords: *Spinacia oleracea*, OmpK antigen, Vibriosis, *Agrobacterium*-mediated stable transformation, PCR, qRT-PCR, Western blotting, Dot blot.

CHAPTER # 01

1. INTRODUCTION

1.1. Fish and its global significance

Aquaculture production has grown into one of the quickly expanding animal food manufacturing industries. It contributes to one half of the fish and shellfish that humans consume (Guo *et al*., 2014). Fish is one of the world's most prominent natural resources. Fish constitute a major proportion of net productivity of marine ecosphere. They are responsible for proper functionality and biodiversity of aquatic biosphere. This aquatic vertebrate carries immense nutritional, financial, social and economic significance. Fish is a crucial source of nourishment for mankind. Fish fulfills 16% of animal protein requirement of world's population (Tidwell *et al*., 2001). It also provides omega-3 fatty acids, iron, zinc, lysine as well as vitamin A, B and D. Fish is a source of livelihood to about 200 million people.

Ecological importance of fish has been observed regarding marine preservation, ecosystem regulation, revival, and marine habitat management (Ormerod, 2003). Marine systems are exposed to certain prominent hazards including habitat deprivation and destitution, introduction of exotic species, contamination, overutilization, global warming and acid rain. Many marine and freshwater fish have been exposed to local and worldwide extinction (Arthington *et al*., 2016).

Microbial diseases cause economic losses in aquaculture. Bacterial infections, fungal diseases and external protozoan parasites are primarily responsible for ailments in fish population (Meyer, 1991). Economical and environmentally friendly aquaculture is critical for the management and prevention of infectious diseases. Prophylactic measures that induce fish's immune response are an effective approach for the maintenance of viable aquaculture (Gudding and Muiswinkel, 2013).

1.2. Infectious Diseases

Diseases that are caused by pathogenic microorganisms such as bacteria, fungi and viruses are called infectious diseases (IDs). Despite significant advancements in field of medicine, IDs remain a prominent cause of morbidity and mortality globally (Kundu *et al*., 2018). Emerging and re-emerging IDs impose serious and significant health concern (Waheed *et al*., 2016).

Immune system is responsible for the detection and defense against microbial pathogens. When pathogen associated molecular patterns (PAMPs) are recognized, signal transduction cascade begins that yield pro-inflammatory and anti-microbial responses (Lee *et al*., 2018). If the immune system is weak and unable to combat the infectious pathogens, then our body suffers from IDs (Kurup and Thomas, 2020).

Vaccination is the most promising measure for the prevention of IDs. Considerable research is in progress for vaccine development against new targets. Clinical trials have been performed with various vaccine candidates that are important for decreased health care expense and life safety (Wagner and Weinberger, 2020).

1.3. Vibriosis

Vibriosis is a highly fatal septicaemia haemorrhagic disease that affects numerous marine and fresh water fish, bivalves and crustaceans (Paillard *et al*., 2004). On the basis of clinical signs, this disease is also referred to as salt-water furunculosis, boil disease, ulcer disease, red pest, red disease, cold pest or eye disease. Major occurrence of vibriosis has been reported in warm water, particularly at high density, salinity and organic load (EI-Son *et al*., 2021). Major factors responsible for this disease include chemical, biological and physical stressors (Austin *et al*., 2007). Vibriosis causes significant economic losses globally. Its morbidity and mortality rates are quite high (Yilmaz *et al*., 2022). Consequently, considerable research has been conducted to explain the virulence mechanism of the causative agent so that quick detection methods and impactful disease-prevention practices can be developed.

1.3.1. *Vibrio anguillarum*

Vibrio anguillarum is responsible for causing vibriosis. It is also called as *Listonella anguillarum* and belongs to the family Vibrionaceae. *V. anguillarum* is a Gram-negative rod bacterium (Noga, 2010). It is a comma-shaped bacterium (figure 1.1) that is halophilic, motile, non-spore forming, polarly flagellated and facultatively anaerobic. *V. anguillarum* grows faster at 25-30°C temperature. Most suitable media for rapid bacterial growth is the one containing 1-2% sodium chloride (NaCl), hence cream-coloured and round-shaped colonies are formed (Frans *et al*., 2011). *V. anguillarum's* genome size is 4.2 Mbp and GC content is 43-46% (Naka *et al*.,

2011). Common characteristic shared by *Vibrio* species is that they contain two circular chromosomes of 3.0 and 1.2 Mbp (Heidelberg *et al*., 2000).

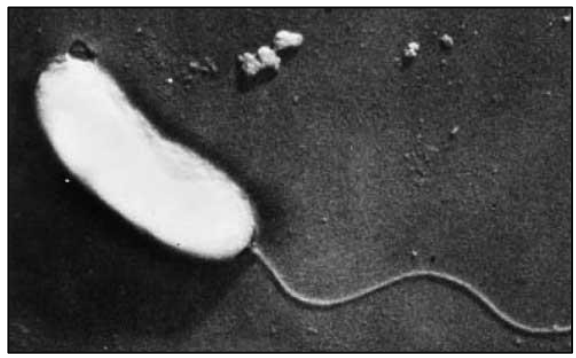


Figure 1.1: Electron micrograph of a *Vibrio anguillarum* indicating the single polar flagellum (Adapted from Actis, 1999).

1.3.2. Susceptible fish species

More than 50 fresh and salt-water fish species, encompassing several economically significant species for the fish farming and mariculture industry are affected by *V. anguillarum*. *V. anguillarum* causes vibriosis in Pacific salmon, Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), striped bass (*Morone americanus*), cod (*Gadus morhua*), Japanese eels (*Anguilla japonica*), European eel (*Anguilla anguilla*), and ayu (*Seriola quinqueradiata*); causing significant economic damage (Toranzo and Barja, 1990). Bivalve molluscs and crustaceans can also be affected (Paillard *et al*., 2004).

1.3.3. Clinical signs of disease

Clinical symptoms of vibriosis include weight loss, lethargy, red spots, dark skin lesions and eye infection that results in opacity, ulceration and exophthalmia. Internally, dilation/bloating of the intestine occurs and it gets filled with transparent, viscous fluid. More severe pathology occurs in posterior gastrointestinal tract (GIT) and rectum than in the anterior parts due to the pH gradient in GIT (Frans *et al*., 2011).

1.3.4. Mode of transmission of *V. anguillarum*

Oral ingestion of *V. anguillarum* via contaminated food or water as well as penetration through skin results in the development of vibriosis in fish. The causative pathogen enters GIT, where low pH considerably hampers its growth. However, research indicates that *V. anguillarum* is capable of surviving the stomach's acidic conditions (Larsen, 1984). It passes through the stomach and employs intestinal mucus as prime nutrient source for adherence, colonization and propagation in the gut (Olsson *et al*., 2008). It is released into lamina propia and consequently moves into the intestinal epithelium via endocytosis. Finally, *V. anguillarum* enters the blood stream, causing septicaemia, blood poisoning, and infection of liver, spleen and kidney (Grisez *et al*., 1996).

1.4. Vaccines against vibriosis

1.4.1. History

Use of antibiotics as common chemotherapeutic agents has resulted in several drawbacks to fish and marine habitat. Antibiotic resistant bacterial strains and antibiotic residues remain in fish muscles (Alderman and Hastings, 1998). Hence its utilization has been prohibited by FDA, so as to prevent the human customers from hazardous health outcomes. Consequently, researchers have focused on the prevention rather than treatment of disease. Vaccination is a safer strategy that has been practiced for 50 years. It boosts the fish immunity and enhances its protection against vibriosis (Yilmaz *et al*., 2022).

1.4.2. Suitable fish vaccines

After *Aeromonas salmonicida* inactivated vaccine has been introduced in 1942, aquatic vaccine production has emerged significantly (Du *et al*., 2022). Aquatic vaccines have been classified on basis of more than one criterion. For instance, on basis of anti-pathogen:these vaccines are grouped as parasitic, viral and bacterial. Several types of fish vaccines include live attenuated vaccines, inactivated vaccines, subunit vaccines, recombinant vaccines, DNA/RNA vaccines, genetically engineered vaccines, virus-like particles-based vaccines, peptide vaccines, plant-based edible vaccines and nano vaccines (Dadar *et al*., 2017).

Several vaccines that have been developed against *Vibrio* species include inactivated bacterins, live attenuated vaccines, subunit vaccines and live vector vaccines (Ji *et al*., 2020). They are comparatively safe and generate effective immune responses that protect the fish from being affected by vibriosis (Toranzo *et al*., 1997).

1.5. Outer Membrane Protein K (OmpK)

Outer membrane protein K is an immunogenic protein present in the outer membrane of *Vibrio anguillarum*. Outer membrane proteins are main part of gram-negative bacteria. They are important for bacteria in colony formation, protection from bile, and pathogenesis (Wang *et al*., 2003). The antigenic determinants expressed on cell membrane are primarily responsible for immunogenicity of outer membrane proteins (Xu *et al*., 2019).

OmpK serves as potential candidate for subunit vaccine development. Antibodies production and enhanced sIgM positive have been stimulated by recombinant OmpK (rOmpK). DNA vaccine expressing *Vibrio anguillarum*'s OmpK has been produced and *in vitro* as well as *in vivo* studies have confirmed its successful induction (Xu *et al*., 2019).

1.6. Plant biopharming

Plant biopharming is also referred to as plant molecular farming. It involves using living system as a host for production of non-natively produced biological pharmaceuticals. The first noted instance of plant biopharming was that the chimeric human growth hormone had been produced via transformed tobacco and sunflower (Barta *et al*., 1986). There are many benefits that are associated with using plants as affordable and cheap biofactories such as low infrastructure costs and easy biomass expansion requisites. These benefits have generated an enormous level of intrigue among researchers in employing suitable crop species for producing edible vaccines (LeBlanc *et al*., 2020).

1.7. Edible vaccines

Production of edible vaccines involves genetic engineering of the plant with gene of interest and subsequently inducing it to synthesize the gene products. Development of edible vaccines has become a common practice for numerous human and animal

diseases. They have proven beneficial for protection against infectious diseases, autoimmune diseases, birth control and cancer therapy (Lal *et al*., 2007). Genetically modified plants have gained acceptance in industrial as well as developing countries. Barley, tobacco, tomato, banana, lettuce, rice, wheat, maize, spinach, alfalfa, soybean, papaya and cucumber have been employed for producing edible vaccines (Khalid *et al*., 2022).

1.7.1. Immunological Mode of Action of Edible Vaccines

Edible vaccines are administered orally, they imitate the way natural infection occurs. Major benefit in comparison with conventional vaccines is that plant-based vaccines are responsible for stimulating mucosal as well as systemic immunity in the body (Koo *et al*., 1999). Direct ingestion of plant parts and bio-encasement shields the antigen from enzymatic degradation as well as from gastric and intestinal secretions. Upon reaching the intestine, cell wall shatters and antigens are liberated. The antigen is taken up by M cells of Peyer patches and gut-associated Lymphoid tissue GALT (Lal *et al*., 2007). The antigen is moved from M cells to macrophages and other Antigen Presenting Cells (APCs), which exhibits it to helper T cells (Th cells). Th cells are responsible for activating B cells that release IgA, IgE, and IgG antibodies and memory responses (figure 1.2). Consequently, neutralization of antigen occurs in the process resembling traditional vaccines (Sharma and Sood, 2010).

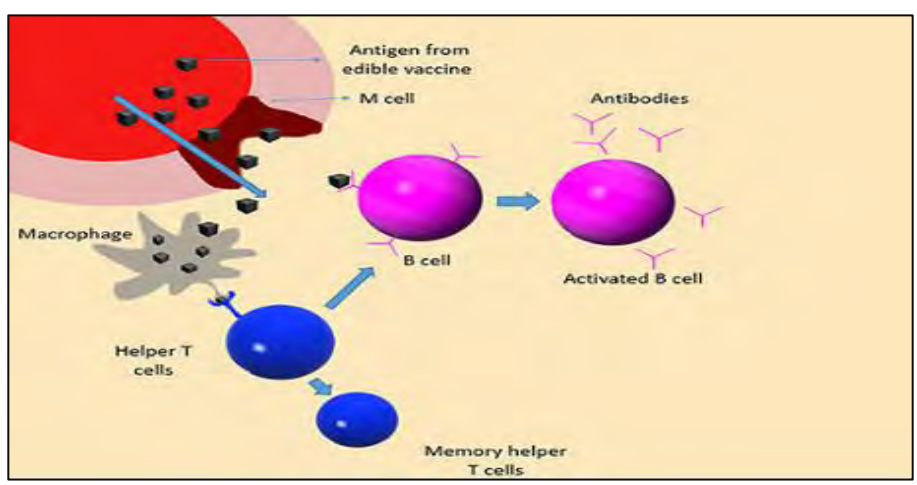


Figure 1.2: Mechanism of action of edible vaccines (Adapted from Gunasekaran and Gothandam, 2020).

1.7.2. Pros of Edible Vaccines

- Edible vaccines carry antigenic subunits and are deprived of pathogenic epitopes hence considered safe (Sharma and Sood, 2010).
- Systemic as well as mucosal immunity is stimulated by them (Koo *et al*., 1999).
- They are processed in simple and economical way in form of juice, powder or sauce.
- There is risk of contamination for vaccines that are prepared in mammalian cells. However, plant-based vaccines don't pose any such risk since plant viruses don't cause infection in humans (Lal *et al*., 2007).
- Oral administration is more convenient than injections and goes well with children as well. Also, syringes employed in traditional vaccination carry the likelihood of environmental contamination and second-hand ailments (Yu, 2008).
- The combination of edible vaccines with numerous antigens can be done for enhanced effectiveness.
- The second generation vaccines permit different antigens to reach M cells at the same time. For example; considerable immune response is generated by trivalent vaccine against cholera, enterotoxigenic *E. coli* (ETEC) and rotavirus (Yu and Langridge, 2001).

1.7.3. Cons of Edible Vaccines

Certain challenges have been associated with the application of edible vaccines.

- Weight and age of patient, size and ripeness of fruit or plant determines the dosage (Yu, 2008). These differences lead to the considerable difference in protein proportions. Ultimately, underdosing that produces less antibodies or overdosing that causes tolerance can result. Hence, maintaining a consistent dosage among fruits, plants and generations is a problem posed by plant-based vaccines (Tripurani *et al*., 2003).
- To check the compatibility of chosen antigen and selected plant type.

- Shelf life of plant is important. Proper storage of the plant in order to prevent infection or ailment via decay (Richter and Kipp, 1999).
- Transgene escape is an issue that needs to be addressed. To prevent the vaccines misadministration, recognition between normal fruit and vaccine fruit is important (Tripurani *et al*., 2003).
- Gene silencing in plant DNA due to excess mRNA formation by retarded plant growth and reduced fruit formation employed for enhancing antigenic protein content (Lal *et al*., 2007).
- Consumption of edible vaccine may be associated with several side effects such as allergic reactions, autoimmune diseases, nervous system toxicity or cytokine-stimulated sickness (Sharma and Sood, 2010).
- People's insight and environmental risks are also to be considered for acceptance of plant-based vaccines.

1.8. Biotechnology and Plants

Biotechnology is regarded among 21st century's integral technologies. Two scientific terms biology and technology make the word "Biotechnology". Hungarian Engineer Karl Erkey coined the term "biotechnology" in 1919. Biotechnology is defined as using living organisms or their products for the benefit of human health and its surroundings (Verma *et al*., 2011). It includes recombinant DNA technology, cloning and genetics, resistant crops and vegetables, higher milk producing animals, and applying microbiology for producing everyday goods such as bread, beer, cheese as well as antibiotics (Ratledge and Kristiansen, 2001).

An important branch of biotechnology is plant biotechnology. Plant biotechnology is also called Green biotechnology (Wiel *et al*., 2016). It is associated with using plants as bio-factories for the production of vaccines, growth hormones, antibodies, subunits, antigens and enzymes via gene manipulation procedures (Sohrab *et al*., 2017). Gene of interest that encodes antigen protein for particular diseases is incorporated into plant genome via several techniques for the production of plant-based vaccines (Laere *et al*., 2016). Bacterial, viral, parasite and immunocontraceptive vaccines have been produced using transgenic plants (Guan e*t al*., 2013).

1.8.1. Plant Tissue Culture

Production of entire plants, organs, tissues or cells is possible through plant tissue culture systems (figure 1.3) under aseptic and regulated environment of temperature, light and humidity in the growth chamber (Ahloowalia *et al*., 2004). In plant tissue culture, plant tissues are removed from plant and grown on culture media. Part of plant that has been excised and transferred to nutrient media is referred to as explant. Several explants include leaf disks, roots, shoot tips, cotyledons, hypocotyls, axillary buds, or zygotic embryos (Kumar and Loh, 2012).

Plant tissue culture is based on Haberlandt's theory of totipotency, which was proposed by Gottlieb Haberlandt in 1902 (Feher, 2019). According to this theory, separated plant cells have got an innate tendency to regenerate into entire plant (Haberlandt, 1902).

A single somatic cell can form somatic embryo via direct somatic embryogenesis and multiple somatic cells form somatic embryo via indirect somatic embryogenesis (Williams and Maheswaran, 1986). In indirect somatic embryogenesis, embryogenic callus is formed when explant is treated with phytohormone auxin. This undifferentiated mass of cells has the tendency of totipotency acquisition (Ikeuchi *et al*., 2013). Exogenous auxin is then removed, so that the level of endogenous auxin increases and somatic embryogenesis is stimulated (Wojcikowska *et al*., 2013). Direct embryogenesis is not marked by callus stage. Single somatic cells undergo mitosis, gain totipotency, and morphologically identifiable somatic embryos are developed (Williams and Maheswaran, 1986).

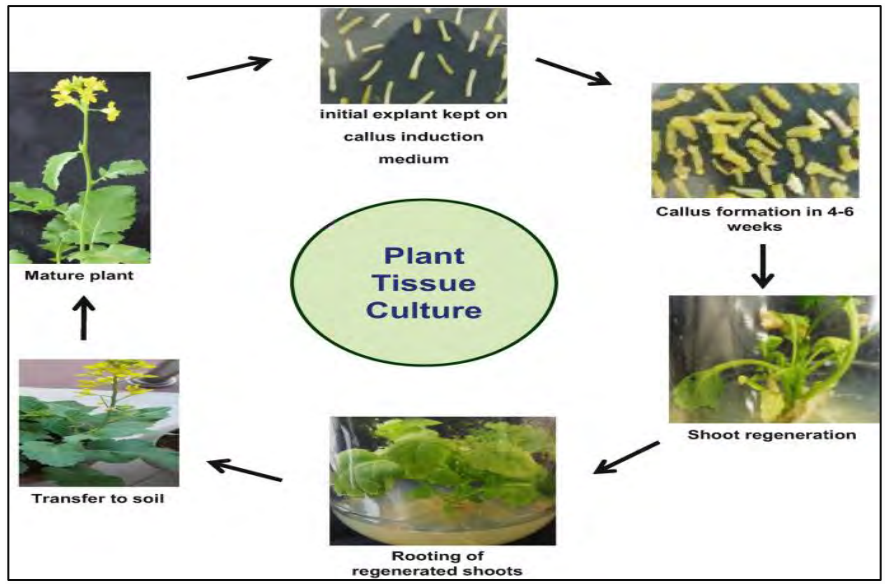


Figure 1.3: Plant tissue culture (Adapted from Gautam *et al*., 2021).

1.8.2. Tissue culture media

When a plant is grown *in vivo*, all the necessary nutrients and elements required for its growth are provided by soil or fertilizers. Similarly plant growth in tissue culture requires several macro- and micronutrients for its growth, metabolism and development (Beyl, 2005). Necessary nutrients, energy and water that is required for the growth of plant or explant is provided by tissue culture systems via basal media. Adding phytohormones either natural plant growth regulators or their synthetic forms at various phases of plant growth or development inculcate a positive impact on plant maturation (Hussain *et al*., 2012).

The most widely employed basal media include Murashige and Skoog media (MS) and modified MS media (MMS), Gamborg's B5 medium and B5 modifications, Woody Plant Medium (WPM), and Driver and Kuniyuki Woody plant medium (DKW). Carbohydrates such as sucrose and gelling agents such as agar, agarose, gellan gum or calcium alginate are added to plant tissue culture media. The most extensively employed basal media is MS media. It is a good regeneration media that can be used for dicots as well as monocots (Phillips and Garda, 2019).

1.8.3. Plant Growth Regulators

Plant growth regulators are also referred to as plant growth substances or plant bioregulators. They are naturally occurring compounds or their synthetic forms that influence various developmental and metabolic responses in higher plants. Their formation occurs at several cytological and morphological sites. They effect florescence, fruit formation, maturation, fruit drop, defoliation or quality characteristics (Rademacher, 2015). Classical plant hormones include auxins, cytokinins, gibberellins, abscisic acid and ethylene. Several discovered phytohormones include jasmonates, brassinosteroids, salicylates, turgorins etc. (Gaspar *et al*., 1996).

1.9. Plant Transformation

Plant transformation involves the introduction of exogenous genetic material into plant cell, thereby altering the genetic makeup of plant. It is a significant research practice in plant biotechnology and pragmatic approach for cultivar refinement. There are proven procedures for stably integrating new genes into the nuclear genomes of more than 120 varied plant species (Birch, 1997).

Plant genetic transformation allows the agriculturally or horticulturally beneficial genes to be introduced directly into suitable plants resulting in the production of new and genetically engineered crops. The transmitted gene is called transgene and genetically modified organisms that are formed by victorious gene transfer are called transgenics (Babaoglu *et al*., 2000).

The integration and expression of various extrinsic genes into plants was initially narrated for tobacco in 1984 (DeBlock *et al*., 1984; Horsch *et al*., 1984; Paszkowski *et al*., 1984). Since then, this approach has expanded to 35 families of plant species (Keshavareddy *et al*., 2018).

1.9.1. Methods of Plant Transformation

There are various methods of plant transformation for edible vaccine production (figure 1.4). Two fundamental methods of gene transfer include:

- Direct gene delivery method
- Indirect gene delivery method

Direct gene delivery method involves direct introduction of DNA or RNA into plant cell via physical and chemical procedures. Electroporation, protoplast transformation, microinjection, silicon carbide fibre- or whisker-mediated transformation and particle bombardment (biolistics) are methods used for direct gene delivery (Ozyigit *et al*., 2021; Ozyigit, 2020).

Biolistic method is also referred to as gene gun or micro-projectile bombardment method. In this transformation strategy, gold or tungsten particles are coated with desired DNA or RNA. High pressure of helium is generated in the gene gun which is responsible for the penetration of DNA coated gold particles into the plant cells (Kurup and Thomas, 2020). Chloroplast transformation can be performed via gene gun. In nuclear transformation, desired gene is integrated into plant nuclear genome. While in chloroplast transformation, gene insertion happens in plastome (Guillermo *et al*., 2022). Vaccines for canine parvovirus, tetanus, rotavirus, cholera, plaque, anthrax and Lyme disease have been developed via biolistics method (Khalid *et al*., 2022).

Indirect gene delivery method involves the use of vector for transfer of gene of interest into plant cell. *Agrobacterium tumefaciens* is employed as a vector for *Agrobacterium*-mediated stable as well as transient nuclear transformation (Hwang *et al*., 2017).

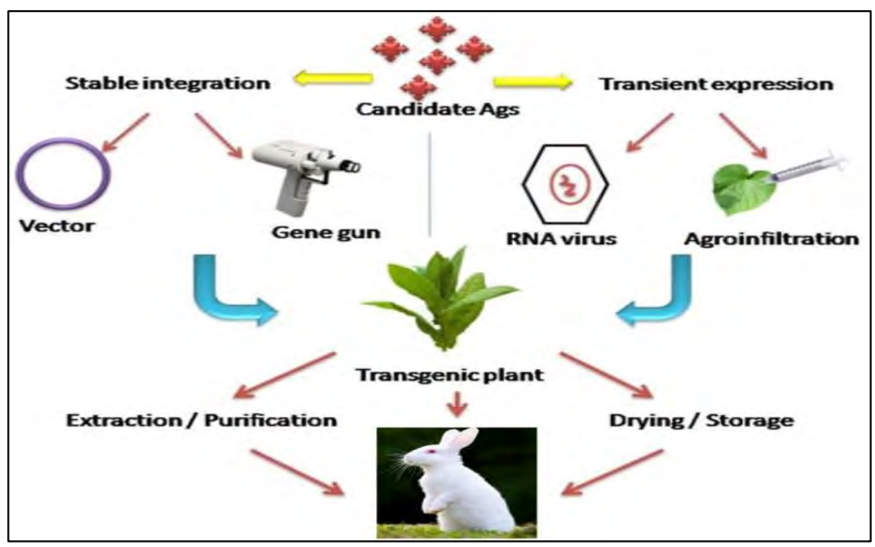


Figure 1.4: Different methods for edible vaccine production (Adapted from Khalid *et al*., 2022).

1.9.1.1. Stable Nuclear Transformation

Agrobacterium-mediated transformation finds extensive applications in plant research (Iwakawa *et al*., 2021). *Agrobacterium tumefaciens* is employed as a vector that carries our gene of interest and appropriate plant is treated with the bacterium in the laboratory (figure 1.5). Consequently, desired gene is integrated into plant nuclear DNA. The word "stable" here represents that the integrated gene of interest is transmitted over the generations. Transgenic seeds can be utilized for mass production of target protein (Davies, 2010). With this simple technique, upto 150 Kb segments of DNA can be transferred to plant genomic DNA. Transformation efficiency is quite high and insertion of T-DNA happens precisely. Numerous dicotyledonous and monocotyledonous plants have been transformed via this method (Kumar *et al*., 2004).

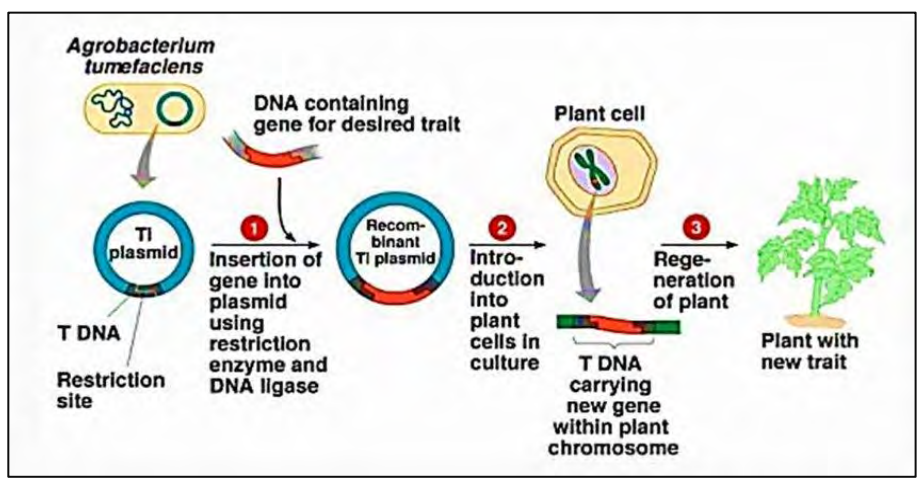


Figure 1.5: *Agrobacterium*-mediated plant transformation (Adapted from Sabu *et al*., 2021).

1.9.1.1.1. *Agrobacterium Tumefaciens*

Agrobacterium tumefaciens is a common gram-negative soil bacterium that causes crown gall disease in plants (Anand *et al*., 2008). *Agrobacterium* pathogenesis is a distinctive and an extremely designed procedure that involves gene transfer between bacterium and plant kingdom. It involves two fundamental components; transformation and tumorigenesis. Firstly, there is transmission of tumorigenic DNA into plant genome. As a result, plant cell metabolism is changed, uncontrolled cell proliferation occurs and certain nutritious substances are produced that give *A.*

tumefaciens an extra benefit. (Escobar and Dandekar, 2003). The oncogenic trait possessed by *A. tumefaciens* is conferred by tumor-inducing (Ti)-plasmid (Holsters *et al*., 1978). During infection, *A. tumefaciens* attaches to its host and a portion of its Ti plasmid is donated to plant cell. The transmitted segment of Ti-plasmid is called transferred or T-DNA that gets stably merged into plant nuclear genome (Hooykaas and Beijersbergen, 1994).

1.9.1.1.2. Ti plasmid in *Agrobacterium tumefaciens*

Ti plasmid is an extrachromosomal DNA present in disease-causing species of *Agrobacterium* such as *A. tumefaciens*, *A. rhizogenes*, *A. rubi* and *A. vitis* responsible for their tumorigenic characteristic. Ti plasmid is a natural genetic engineer (Nester, 2015). Its tendency to genetically transform the host plants has been exploited by researchers to introduce gene of interest into plants.

Two fundamental constituents that are present on Ti plasmid include T (transferred)-DNA and virulence (vir) region. Primary function of *vir* genes is to deliver T-DNA into plant cells. Their induction is caused due to phenolic compounds that are released at wound site in plants. Seven different loci present on vir region include *virA*, *virB*, *virC*, *virD*, *virE*, *virF*, *virG*. VirA and virG are principal regulator proteins that are responsible for regulating other *vir* genes expression (Hwang *et al*., 2017).

1.9.1.1.3. T-DNA

T-DNA is flanked by 25 base pair direct repeat sequences, called T-DNA right and left borders. Two kinds of genes present in wild-type T-DNA include oncogenes and opine producing genes. Following T-DNA expression in plant, cells divide uncontrollably (Gaudin *et al*., 1994). Substantial quantities of indole-3-acetic acid (IAA) and cytokinin (CK) are formed, thereby promoting unregulated plant growth, as a result crown gall tumors are formed (Subramoni *et al*., 2014). Opines serve as carbon and nitrogen sources for *A. tumefaciens* (Savka *et al*., 2002).

Genes needed for transfer and integration of T-DNA are not encoded by T-DNA itself. However, T-DNA left and right borders are responsible for this task (Zambryski *et al*., 1982). Thus, we can replace whole wild-type T-DNA sequence

with our desired gene of interest for its transfer and integration into the plant genome (Potrykus *et al*., 1998).

1.9.1.1.4. Signal recognition in *Agrobacterium tumefaciens*

Agrobacterium tumefaciens recognize the chemical signals and phenolic compounds that are released by plants and consequently induces the expression of virulence genes. Acetosyringone and hydroxyacetosyringone have been found to be important activators of *A. tumefaciens* virulence and pathogenesis (Stachel *et al*., 1985 ; Hess *et al*., 1991).

1.9.1.1.5. T-DNA processing

Vir region of Ti plasmid is primarily responsible for mediating T-DNA processing. *Vir* genes-encoded products are expressed only when there is a wounded site in host plant. This regulation of gene expression is maintained due to two regulator proteins virA and virG. In response to the phenolic signals released at the site of injury, autophosphorylation of virA occurs. virA phosphorylates virG, hence the transcription of *vir* genes is induced. VirD1/D2 detects T-DNA and performs its endonucleolytic cleavage (Zupan and Zambryski, 1995). A single stranded copy of DNA called T-strand is formed that is released from Ti plasmid. VirD1 is liberated while virD2 remains covalently bound at T-strand 5' end (Tzfira *et al*., 2003).

1.9.1.1.6. T-DNA migration

Transfer of T-DNA from *A. tumefaciens* into plant cell occurs as a T-strand-virD2 complex. Single stranded DNA binding protein virE2 is also migrated into plant cell during infection/pathogenesis. C-terminal Nuclear Localisation Signal (NLS) present on virD2 and virE2 mediates the delivery of these proteins and T-strands into the nucleus of plant cell (Dombek and Ream, 1997).

1.9.1.1.7. T-DNA integration

Integration of T-DNA into plant nuclear genome occurs via illegitimate recombination. Ω domain of virD2 is responsible for integrating T-DNA into plant nuclear genome. VirD2 prevents the right border of T-DNA from any sort of degradation while virE2 protects the integrity of left border (Mysore *et al*., 1998).

1.9.1.1.8. T-DNA expression

Once T-DNA has integrated into the host cell genome, there are two possibilities. Either it is expressed within host cell or it is not expressed at all (Primrose and Twyman, 2006).

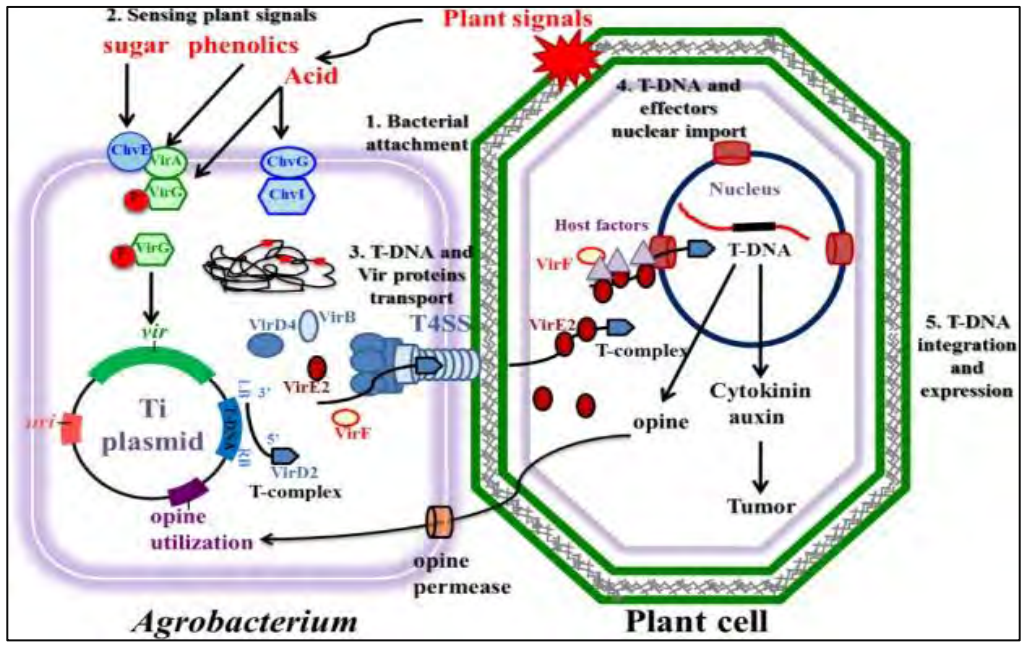


Figure 1.6: Major steps of *Agrobacterium tumefaciens*-mediated plant transformation (Adapted from Hwang *et al*., 2017).

(1) *Agrobacterium tumefaciens* attaches to the plant cell. (2) *Agrobacterium tumefaciens* senses plant signals, these signals are transduced and regulation of *Agrobacterium* virulence genes occur. (3) T-DNA and virulence proteins are transported from *Agrobacterium* into plant cell. (4) T-DNA and effector proteins are imported into nucleus of plant cell. (5) Finally, T-DNA is integrated and expressed in plant genome.

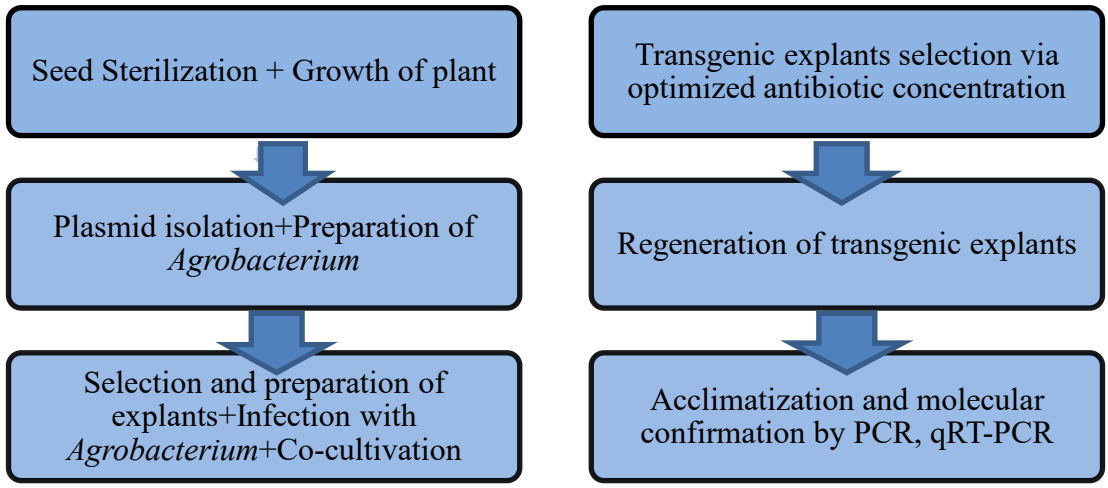


Figure 1.7: Schematic representation of *Agrobacterium*-mediated stable transformation.

1.9.1.1.9. Pros and Cons of Stable Transformation

- The desired traits are passed onto the succeeding generations.
- There is no requirement of expensive instruments in this technique.
- Many genetically engineered plants can be produced via single *Agrobacterium* transformed leaf disk (Meyers *et al*., 2010).
- Single binary vector can be used with various plant species capable of *Agrobacterium*-mediated transformation (Lee and Gelvin, 2008).
- Translation of nuclear transgene produces only 1-2% of total soluble protein (Staub *et al*., 2000).
- Position effects and RNA silencing may occur since T-DNA is integrated at random positions (Fischer *et al*., 2008).

1.9.1.2. Transient transformation

In *Agrobacterium*-mediated transient transformation, the transgene is not integrated into plant genome. As soon as the transgene is moved into the cell, its transcription can begin. Maximum levels of chimeric protein can be obtained from plant tissues about 1-2 weeks after gene delivery (Chen *et al*., 2011). Effective transient expression requires that bacterial suspensions are successfully perforated into intercellular air spaces of plant tissue. It gives results remarkably faster in

comparison with stable *Agrobacterium*-mediated transformation (Gonzalez *et al*., 2023).

Successful *Agrobacterium*-mediated transient transformation has been reported in diverse range of plant species including Arabidopsis, sunflower, tobacco, rose, Medicago, petunia and Antirrhinum, maize, lettuce, aspen and switchgrass (Lizamore and Winefield, 2015).

1.9.1.2.1. Agroinfiltration

Agroinfiltration is a rapid and an extremely efficient technique. It is responsible for mediating transformation and expression of transient transgene in plants (Simmons *et al*., 2009). It has emerged as a standard practice for quickly analyzing the gene expression and function *in vivo*. Three fundamental steps for agroinfiltration include growing the plant, preparing the *A. tumefaciens* culture and infiltration (Leuzinger *et al*., 2013).

Infiltration of *Agrobacterium* suspension can be performed via two methods: vacuum infiltration and syringe infiltration. More extensively used procedure is syringe infiltration. *Agrobacterium* is introduced into plant leaves using needleless syringe (Santi *et al*., 2008). As soon as the intercellular spaces of leaf get the *Agrobacterium* mixture, darkening of light green color occurs which is an indication of successful infiltration. It is a simple strategy that does not require any costly equipment. Infiltration of whole leaf with one target genetic construct or different regions of single leaf with multiple DNA constructs can be performed (Leuzinger *et al*., 2013).

Due to numerous advantages that syringe infiltration offers, it is being employed for transient gene expression in many applications including studies of plant-pathogen interactions, protein-protein interactions, abiotic stresses, protein localization and function, as well as plant gene functional analysis with transient silencing assay (Vaghchhipawala *et al*., 2011).

1.9.1.2.2. Pros and cons of Transient Transformation

- It is a simple and an efficient technique that is less labor-intensive and more economical.

- Time is considerably reduced for the production of chimeric vaccines (Habibi *et al*., 2014).
- There is no requirement of tissue culture (Habibi-Pirkoohi *et al*., 2021).
- It is impracticable for various organs like roots, woody tissues and waxy leaves.

1.10. *Spinacia oleracea*

Spinach (*Spinacia oleracea*) is a dark green leafy vegetable. This great medicinal plant, regarded as power food is rich in crucial nutrients. It is deemed as one of nature's most perfect foods (Metha and Belemkar, 2014). Spinach is a dioecious species that can give rise to either male or female flowers, carrying 2n=12 chromosomes. Its cultivation occurs in spring as well as autumn. The term spinach has been derived from Persian word ispanai, which means "green hand" (Naseer *et al*., 2019). Edible leaves including spinach, mustard, dandelions, turnip tops and beets tops are usually regarded as "greens". Spinach is frequently consumed in form of salads, cooked vegetable or mixed with cooked meat and different vegetables (Morelock and Correll, 2008).

1.10.1. Taxonomic classification of spinach

Spinacia oleracea is an edible plant that is the member of flowering plant family Amaranthaceae (Cai *et al*., 2017). The family comprises about 160 genera and 2400 species (Villalba *et al*., 2014). This annual plant was regarded to be present in family Chenopodiaceae, however in 2003 it was added in family Amaranthaceae. Spinach is contained in subfamily Chenopodiaceae.

Table 1.1: Taxonomic classification of *Spinacia oleracea.*

Kingdom	Plantae
Phylum	Angiosperms
Order	Caryophyllales
Family	Amaranthaceae
Genus	*Spinacia*
Species	*S. oleracea*

1.10.2. Morphology

This leafy green flowering plant grows to a height of upto 30 centimetres. Spinach has alternate, plain, oval to trigonal, either smooth or curled leaves. Leaves are 2-30 cm in length, 1-15 cm in width. Larger leaves are present at the bottom of plant while small leaves are found at the peduncle. Its unnoticeable flowers are yellowish green in colour about 3-4 mm in diameter. They mature and form little rigid dry bulging, seed carrying fruit bunch that is 5-10 mm (Jiraungkoorskul, 2016). Requirements for optimal growth for spinach include cool weather, fertile soil and milder temperature.

1.10.3. Types of Spinach

There are three main varieties of spinach (figure 1.8) savoy, semi-savoy and flat-leafed (Hu *et al*., 2007). They are further divided into cultivars or sub-cultivars.

- **Savoy**

More productive type is the savoy spinach that handles the cold finer than other two varieties. Its leaves are wrinkled in shape. Cultivars include Bloomsdale and Regiment.

- **Semi-savoy**

Semi-savoy is fine and productive variety of spinach. Its leaves are slightly crinkled. Best thing about it is that it grows in spring, summer and fall. Indian Summer, Tyee and Catalina are some of the cultivars of semi-savoy spinach.

- **Flat-leafed**

Flat-leafed or smooth-leafed spinach is named so since it has got a plane surface. Space spinach and Red Carnival are significant cultivars of flat-leafed spinach.

Figure 1.8: Types of *Spinacia oleracea*. (A) Savoy. (B) Semi-savoy. (C) Flat-leafed spinach.

1.10.4. Centre of origin and world distribution of Spinach

The origin of spinach is not exactly known. It is presumed that it was first found in Iran, former Persia, about 2000 years ago (Rubatzky and Yamaguchi, 1997). It is assumed that expansion of spinach occurred late in history since no evidence about it has been found in Greek and Roman cultures. The oldest record hint about spinach has been established from 4th century AD in Mesopotamia. The distribution of spinach from native land to various geological regions is undetermined to a great extent. Expansion in Muslim territories is perhaps responsible for the spread of spinach to the West (Sneep, 1983). Recent proof indicates that Moors inaugurated spinach in Europe via Iberian Peninsula. Spinach had been cultivated in Moorish Spain since the 11th century. The foremost paleoethnobotanical proof came from Pyrenees mountain range (Hallavant and Ruas, 2014). How it spreaded into Central and Eastern Asia is even slightly defined. It has been reported that introduction of spinach into China through Nepal occurred in the 7th century (Laufer, 1919). However, it is still unambiguous how it was initiated in Nepal (Ribera *et al*., 2021).

Spinach is closely related to *Spinacia tetrandra* and *Spinacia turkestanica*. It has been proposed that one or both of them may be predecessors of wild spinach. But phylogenetics shows that *S. oleracea* is more genetically closer to *S. turkestanica* instead of *S. tetrandra*. Therefore, the most probable ancestor of spinach is *S. turkestanica* (Ribera *et al*., 2021). Currently, there has been expansion in populations of wild spinach since Centre for Genetic Resources Netherlands had arranged two accumulating expeditions.

Spinach is highly liked by Arabs, they consider it as the queen of vegetables. Cultivation of spinach requires low salinity soil and can occur in various climates. High salinity in soil or irrigation water reduces the spinach harvest. Spinach is fondly eaten as salads and soups. At industrial scale, noodles, steamed buns and mixed meatballs of spinach are formed (Zhou *et al*., 2023).

Chinese regarded spinach as Persian herb (Rashid *et al*., 2020). Major manufacturers of this economically significant crop include California, Texas, Arkansas, Oklahoma, Maryland, Virginia, New Jersey and Colorado (Correll *et al*., 1994). In Pakistan, cultivation of spinach occurs at 8820 hectares area, producing 108, 725 tons spinach.

1.11. Choice of *S. oleracea*

Iron rich leafy vegetable *Spinacia oleracea* is prioritized for the production of plant-based edible vaccine. Spinach is rich in vitamin A and other important nutrients. It can be consumed in cooked as well as raw form. It can be grown easily and its cultivation occurs in all three seasons; spring, summer and fall. Vaccine development for HIV using HIV-1 Tat protein as potential vaccine candidate and for anthrax using spinach is headway (Naik, 2022).

1.12. Aims and Objectives

The present research was conducted in order to express *OmpK* gene into *Spinacia oleracea* via *Agrobacterium*-mediated stable nuclear transformation in an attempt to develop plant-based edible vaccine against Vibriosis disease caused by *Vibrio anguillarum*. The objectives of this research work are mentioned below:

- To optimize hygromycin concentration for leaf explants of *Spinacia oleracea* in order to carry out its stable nuclear transformation.
- To carry out *Agrobacterium*-mediated stable nuclear transformation of *Spinacia oleracea*.
- To validate the integration of *OmpK* transgene in *Spinacia oleracea* via various molecular biology techniques such as PCR and qRT-PCR.
- To examine the OmpK protein expression using different techniques like Dot blot, Western blotting and ELISA.

CHAPTER # 02

2. MATERIALS AND METHODS

The present research work was conducted in Plant Biotechnology Laboratory, Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. Aim of this study was to optimize the hygromycin concentration for wild type *Spinacia oleracea* leaves and to develop an efficient protocol for *Agrobacterium*-mediated stable transformation of *Spinacia oleracea* with plasmid containing *OmpK* gene from *Vibrio anguillarum*. The integration and expression of *OmpK* gene was confirmed using polymerase chain reaction (PCR) and various other techniques of molecular biology in transformed plants.

2.1. Materials

2.1.1. Laboratory Instruments

Each laboratory instrument was used after getting proper information of each and every instrument. Information about various instruments that were used for carrying out this research work is mentioned in table 2.1.

Table 2.1: Laboratory Instruments

Appliances	**Company**
Autoclave	Yamato, USA
Weigh 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

Various glass and plastic wares were used for conducting the research. They were purchased from several different manufacturing and supplying companies. Plastic wares such as pipette tips, eppendorfs, PCR tubes etc. were discarded after one time use. While the glass wares such as magenta boxes, jars, flasks, beakers, measuring cylinders, petri

plates were re-used. After use, they were washed using commercial liquid detergent and dipped in bleach overnight. Then the apparatus was dried in hot oven at 150°C. The apparatus was wrapped properly in aluminium foil and autoclaved at 121°C, 15 Pound per square inch (Psi) for 20 minutes. The autoclaved apparatus was kept in incubator at 37°C. Information on various glass and plastic wares is given in table 2.2.

Table 2.2: Consumables

Consumables	**Company**
Glassware	Pyrex, USA
1.5/2.0 ml Eppendorf tubes	Axygen Scientific, USA
15/50 ml Falcon tubes	Corning®, USA
PCR tubes	ThermoScientific, USA
Petri plates	Pyrex, USA
Gloves	Qualtex, Malaysia
Nitrile gloves	Gen-X, Malaysia
Magenta boxes	Pakistan
Pipette tips	Extra Gene, USA
Parafilm PM-96	Bemis, USA
Surgical blades	XINDA, China
Syringe filter (0.2 µm)	Sartorius, Germany
Microtitre plates	Costar, USA
Whatman filter paper	GE Healthcare, UK

2.1.3. Chemicals and Reagents

Table 2.3: Chemicals and Reagents

Chemicals	**Company**
Agar (Bacteriological grade)	Bioworld, Dublin, Ireland
Ammonium Persulphate (APS)	AnalaR^{TM}, England
Ammonium sulfate $(NH_4)_2SO4$)	GPR, BDH laboratory supplies, UK
Agar Plant TC	Phytotech lab, USA
Bovine Serum Albumin (BSA)	MP Biomedical, USA
Boric Acid	AnalaR^{TM}, England
Bromophenol Blue	Sigma-Aldrich, USA
Beta-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 Deodecyl Sulfate (SDS)	Phyto Technology 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
Sulfuric Acid	Sigma-Aldrich, USA
Tris base	Invitrogen, USA
Tris-(hydroxymethyl) aminomethane	Phyto Technology Laboratories, USA

Triton X-100	AnalaR^{TM}, England
Tween-20	Sigma-Aldrich, USA

2.1.4. Ready-to-use Laboratory Kits and Reagents

Table 2.4: Ready-to-use Laboratory Kits and Reagents

Reagents	**Company**
Anti-His Antibody	Abcam, USA
Chemiluminiscence 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'- tetramethylbenzidine (TMB)	Thermofisher, USA
Taq DNA Polymerase (5U/ µl)	Thermofisher, USA

2.1.5. Primers

Table 2.5: Primers

Primers	**Sequence**
OmpK-nuclear forward primer	**5'**GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGTAAATCACTTTTAGCTCTAGGCC **3'**
OmpK-nuclear reverse primer	**5'**GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTGATGGTGATGGTGATG **3'**
OmpK-internal forward primer	**5'**CTAAGCAACCCAAGCAGTGACAAAG **3'**
OmpK-internal reverse primer	**5'**CCTTCATCTTTCAGACCATAAACATCTTTGTAGC **3'**

2.2. Methods

2.2.1. Lab precautionary measures

Sterilized environment is the first and foremost necessity of all the experimental procedures that were performed especially during seed sterilization, seed germination, plant tissue culture, inoculation of bacterial colonies, streaking of bacterial colonies on Luria Agar plates and plant transformation experiments. Failure to maintain aseptic conditions lead to bacterial and fungal contamination in media and the wastage of time and money resources. The best effort was done to maintain aseptic conditions during performance of all laboratory procedures.

Following safety and precautionary measures were followed during lab work:

- Lab coat, gloves and mask were worn during lab work.
- Apparatus and media used were autoclaved for 20 minutes at 121°C and 15 Psi.
- Distilled water was used for the preparation of each solution.

- Surface sterilization of Laminar Flow Hood (LFH) was performed by swabbing with 70% ethanol and then exposing it to ultraviolet (UV) radiations for 20 minutes before starting work. All the materials were exposed to UV light within LFH except thermosensitive reagents including hormones, antibiotics, chemicals, seeds, plants and bacterial cultures.
- The procedures of seed sterilization, tissue culture, bacterial inoculation, streaking of bacterial colonies and plant transformation were done within LFH that ensured perfect aseptic environment.
- Hands were properly sprayed with 70% ethanol before working in LFH.
- After working, hands were washed with soap or disinfected using sanitizer.

2.2.2. Sterilization of Laminar Flow Hood (LFH)

Sterile working conditions were maintained within LFH which contains High Efficiency Particulate Absorption (HEPA) filter. Before working, LFH was swabbed properly with 70% ethanol (alternative: spirit). To ensure complete surface-sterilization, UV was turned on for 20-30 minutes. The apparatus required for work was sprayed with 70% ethanol (alternative: spirit) and was exposed to UV radiations for 20-30 minutes. After 30 minutes, UV was turned off and hands were sprayed with 70% ethanol (alternative: spirit) before starting work within sterile environment of LFH. Before using instruments like forceps and scalpels, their proper sterilization was performed by dipping them in 70% ethanol (alternative: spirit) and flamed till red-hot.

2.2.3. Different types of culture media preparation

Different media were used for various purposes. Half Murashige and Skoog media (1/2 MS), Full Murashige and Skoog media (full MS), Luria-bertani broth (LB), Luria Agar (LA) were used for seed germination, plant tissue culture, bacterial inoculation and bacterial streaking.

2.2.3.1. Plant culture media preparation

2.2.3.1.1. Murashige and Skoog Media (MS Media)

Full and half MS media (Annexure 2.1) were used for plant tissue culture and seed germination. For its preparation, calculated quantities of MS and sucrose were weighed using the weigh balance and dissolved in required amount of distilled water in reagent bottle. pH was adjusted from 5.75-5.85 using the pH meter using 0.1 N HCl and NaOH solutions. Finally, calculated amount of agar was added. The media was autoclaved at 121°C, 15Psi for 20 minutes. The media was poured in jars (50 ml each) within sterile environment of LFH. Jars and plates were wrapped with parafilms and stored in the growth chamber at 25±2°C.

2.2.3.2. Bacterial culture media

Luria Broth (LB) media was used for bacterial inoculation and Luria Agar (LA) media was used for streaking of bacterial cultures.

2.2.3.2.1. Luria Broth Media

Luria broth was used for bacterial inoculation. For its preparation, calculated amount of LB (Annexure 2.1) was weighed using weigh balance and dissolved in desired amount of distilled water in reagent bottle. The media was autoclaved at 121°C, 15 Psi for 20 minutes. For inoculation, LB media was placed in UV for 15-20 minutes. LB was poured in autoclaved flask along with specific quantities of kanamycin and rifampicin, and bacterial colony was added. The flask was sealed with aluminium foil and placed in shaking incubator.

2.2.3.2.2. Luria Agar Media

LA media (Annexure 2.1) were used for the streaking of bacterial colonies. For its preparation, calculated amounts of LB and bacteriological grade agar were weighed using the weigh balance and dissolved in required amount of distilled water in reagent bottle. The media was autoclaved at 121°C, 15 Psi for 20 minutes. In the sterile environment of LFH, antibiotics kanamycin and rifampicin were added in the slightly warm media. The

media was poured in the petri plates (25 ml). After proper solidification, the plates were wrapped with parafilms and kept in the growth room at 25±2°C.

2.2.4. Preparation of stock solutions

The composition of various hormones and antibiotics used in this research study is mentioned in table 2.6.

Table 2.6: Composition of stock solutions of hormones and antibiotics

Hormones

Stock solutions	Concentration
BAP	1mg/ml
IAA	1mg/ml
Kinetin	2mg/ml
2,4-D	1mg/ml

Antibiotics

Stock solutions	Concentration
Hygromycin	50 mg/ml
Kanamycin	50 mg/ml
Rifampicin	20 mg/ml
Acetosyringone	100 mM
Cefotaxime	250 mg/ml

2.2.4.1. Stock solution preparation of plant hormones

2.2.4.1.1. Indole Acetic Acid (IAA)

For the preparation of 1 mg/ml stock solution of IAA, 50 mg IAA was weighed using weigh balance. IAA was added in 50 ml falcon tube within sterile conditions of LFH. 2ml of 1 M NaOH solution was added to dissolve the IAA powder. Volume was raised to 50 ml with autoclaved distilled water. The stock solution was added in beaker and filter sterilized using 0.2 µm syringe filter. 1.2 ml aliquots were prepared in eppendorf tubes. They were stored at -20°C.

2.2.4.1.2. Benzylaminopurine (BAP)

In order to prepare 1 mg/ml stock solution of BAP, 50 mg BAP was weighed. The powder was added in 50 ml falcon tube within the sterile conditions of LFH. 2 ml autoclaved distilled water along with few NaOH pellets were added. It was shaken for several minutes until BAP was dissolved completely. The volume was raised to 50 ml using autoclaved distilled water. The stock solution was filter sterilized using 0.2 µm syringe filter. 1.2ml aliquots were prepared in eppendorfs and wrapped in parafilms. They were stored at -20°C.

2.2.4.1.3. Kinetin

For the preparation of 2 mg/ml stock solution of kinetin, 100 mg kinetin powder was weighed. The powder was added in 50 ml falcon tube within the sterile conditions of LFH. 20 ml autoclaved distilled water was added. It was shaken for several minutes until kinetin was dissolved completely. The volume was raised to 50 ml using autoclaved distilled water. The stock solution was filter sterilized using 0.2 µm syringe filter. 1.2ml aliquots were prepared in eppendorfs and wrapped in parafilms. They were stored at -20°C.

2.2.4.1.4. 2,4-Dichlorophenoxyacetic acid (2,4-D)

In order to prepare 1 mg/ml stock solution of 2,4-D, 50 mg 2,4-D was weighed. The powder was added in 50 ml falcon tube within the sterile conditions of LFH. 5 ml autoclaved distilled water along with few NaOH pellets were added. It was shaken for

several minutes until 2,4-D was dissolved completely. The volume was raised to 50 ml using autoclaved distilled water. The stock solution was filter sterilized using 0.2 µm syringe filter. 1.2ml aliquots were prepared in eppendorfs and wrapped in parafilms. They were stored at -20°C.

2.2.4.2. Stock solution preparation of antibiotics

2.2.4.2.1. Hygromycin

For the preparation of 50 mg/ml stock solution of hygromycin, 500 mg hygromycin powder was weighed. It was added to 15 ml falcon tube within LFH. 10 ml autoclaved distilled water was added. The falcon tube was shaken continuously for few minutes in order to dissolve the powder completely. The stock solution was filter sterilized using 0.2 µm syringe filter. 1.2 ml aliquots were prepared in eppendorfs and sealed in parafilms. They were stored at -20°C.

2.2.4.2.2. Kanamycin

In order to prepare 50 mg/ml stock solution of kanamycin, 500 mg kanamycin powder was weighed. Kanamycin was added in 15 ml falcon tube within LFH. 10 ml autoclavd distilled water was added. The falcon tube was shaken for several minutes in order to dissolve the kanamycin completely. Stock solution was filter sterilized using 0.2 µm syringe filter. 1.2ml aliquots were prepared in eppendorf tubes and wrapped in parafilms. They were stored at -20°C.

2.2.4.2.3. Rifampicin

For the preparation of 20mg/ml stock solution of rifampicin, 200 mg rifampicin was weighed and dissolved in 10 ml dimethyl sulfoxide (DMSO) within LFH. Rifampicin was dissolved in DMSO since it is insoluble in distilled water. It was shaken for several minutes in order to dissolve the powder completely. The stock solution was filter sterilized using 0.2 µm syringe filter. 1.2 ml aliquots were prepared. The aliquots were stored at -20°C.

2.2.4.2.4. Cefotaxime

In order to prepare 250mg/ml stock solution of cefotaxime, 1g cefotaxime powder was added in 4 ml autoclaved distilled water within LFH. It was shaken for several minutes. Upon complete dissolution, the solution was filter sterilized using 0.2 µm syringe filter. 1.2 ml aliquots were prepared in eppendorfs. They were stored at -20°C.

2.2.4.2.5. Acetosyringone

In order to prepare 100mM stock solution of acetosyringone, 196.2 mg acetosyringone was weighed. It was added to 15 ml falcon tube within LFH and dissolved in 10 ml DMSO through continuous shaking. Upon complete dissolution, the solution was filter sterilized using 0.2 µm syringe filter. 1.2 ml aliquots were created in eppendorfs. They were stored at -20°C.

2.2.5. Plant Material

In this study, spinach seeds were used. The seeds were purchased from Awan seed store, Rawalpindi, Pakistan. The seeds were stored in 15 ml falcon tube with parafilm and kept in warm, dry place at room temperature.

2.2.5.1. Spinach seeds sterilization

Spinach seeds were sterilized within sterile conditions of LFH. The sterilization was performed to minimize the risk of fungal as well as bacterial contamination and to enhance the seed germination efficiency. Spinach seeds were sterilized with 0.2% mercuric chloride ($HgCl_2$) for three minutes. After treatment with $HgCl_2$, seeds were washed thrice with autoclaved distilled water. Sterilized seeds were dried on autoclaved filter paper.

2.2.5.2. Germination of spinach seeds

Full MS media (Annexure 2.1) was used for the germination of spinach seeds. After preparation, the media was autoclaved and poured in jars. Before inoculating seeds, the apparatus was placed in UV light within sterile conditions of LFH for 15-20 minutes. The

seeds were inoculated in media jars (5-6 seeds in each jar). The jars were sealed with parafilms and stored in growth chamber at 25±2°C in 16/8 hours light/dark cycle.

2.2.5.3. Preparation of explants

Explant is a small fragment of node and leaf that was used for plant regeneration through tissue culturing. In this study, leaf explants were tissue cultured for regeneration and transformation.

2.2.5.4. Preparation of leaf explants

2-3 weeks old, *in vitro* grown plants were used for the preparation of leaf explants. Sterile forceps and scalpel was used for cutting the leaves and slicing them into 1.5 to 2 cm pieces.

2.2.5.5. Regeneration of leaf explants

For spinach leaf explants, two regeneration media were used for optimization purpose. Each regeneration media composed of full MS media (Annexure 2.1) along with hormones. The composition of regeneration media used is given below.

- **Media I:** It comprised of full MS media along with hormones BAP (1 mg/L) and IAA (0.5 mg/L).
- **Media II:** It comprised of full MS media along with hormones Kinetin (2 mg/L) and 2,4-D (0.5 mg/L).

2.2.6. Optimization of hygromycin concentration for spinach leaf explants

Optimization of hygromycin concentration for spinach leaf explants was performed to determine the optimum amount of hygromycin that is required for spinach leaf explants regeneration and transformation. Different concentrations of hygromycin (15,20,25, and 30 mg/L) were added into regeneration media and it was poured into petri plates within LFH. Spinach leaf explants were cut and placed on the regeneration media. Plates were wrapped with parafilm and kept in growth room for observation. Sub-culturing was done every 2 weeks. Optimization was performed in replicates, with each replicate containing 4-5 leaf explants.

2.2.7. Preparation of media for transformation

Three types of media were prepared for stable transformation of *Spinacia oleracea*. Detail of these media is given below.

2.2.7.1. Preparation of co-cultivation media

For the preparation of co-cultivation media (Annexure 2.3), 200 µM acetosyringone and hormones (optimized concentrations) were added to the slightly warm autoclaved full MS media in the reagent bottle within LFH. The media was poured into petri plates and it was allowed to solidify. Upon solidification, petri plates were sealed with parafilm and kept in growth room at 25±2°C until further use.

2.2.7.2. Preparation of washing media

Liquid ½ MS (Annexure 2.1) was used as the washing media. It was prepared by adding the calculated amounts of MS and sucrose in distilled water within reagent bottle. pH was adjusted within 5.75-5.85 range.. The media was autoclaved and stored in growth room at 25±2°C. 400 mg/L cefotaxime was added to it before use.

2.2.7.3. Preparation of selection media

Selection media (Annexure 2.3) was prepared by adding hygromycin (20 mg/L) and cefotaxime (250 mg/L) and hormones to full MS media. Media was poured in petri plates. Upon complete solidification of the media, petri plates were wrapped with parafilm and stored in growth room at 25±2°C until further use.

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

In this bacterial transformation, *Agrobacterium tumefaciens* was transformed with binary vector pGWB5 carrying our gene of interest i.e. *OmpK.*

2.2.8.1. Preparation of *Agrobacterium tumefaciens* growth culture

Agrobacterium strain GV3101 was streaked on LA plates containing 50 mg/L kanamycin and 50 mg/L rifampicin. It was kept in growth chamber for 2-3 days so that the bacterial colonies appear. Inoculating loop was used to pick the single bacterial colony from plate and was inoculated in flask containing LB medium as well as 50 mg/L rifampicin and 50

mg/L kanamycin. Flask was then placed in the shaking incubator so that the bacterial growth appears. Value of OD_{600} was measured using spectrophotometer (Thermo Scientific Multiskan GO).

2.2.8.2. Electrocompetent cells preparation

For this study, electrocompetent cells of *Agrobacterium* strain GV3101 were prepared. For the preparation of electrocompetent cells, we transferred the bacterial inoculum (in LB media) into 50 ml falcon tube. Centrifugation of inoculated LB was performed at 2400 rpm for 20 minutes at 4°C. To the pellet, 40 ml chilled distilled water was added in order to dissolve it. Again, centrifugation was performed at 2400 rpm for 20 minutes at 4°C and the pellet was dissolved in 20 ml chilled distilled water. Centrifugation was done at 2400 rpm for 20 minutes at 4°C and 10 ml chilled distilled water was added to the pellet. Finally, centrifugation was performed and pellet was dissolved in 500 µl ice-cold 10% glycerol (10 ml glycerol: 90 ml distilled water). After gentle mixing, 60 µl aliquots were formed and stored at -70°C.

2.2.8.3. Electroporation

We transformed the electrocompetent cells with plasmid (7 µl) through electroporation using the Electroporator (BioRad, USA) in accordance with the protocol that was mentioned in the manual. After thawing electrocompetent cells on ice, 7 µl of plasmid was added to 50 µl of these cells and the sample was shifted to the cuvette. The conditions for electroporation were maintained as mentioned in the manufacturer's manual. Electric shock of 1.8 kilovolts (KV) was given by pressing the PULSE button on the electroporator. 400 µl LB media (Annexure 2.1) was added to the cuvette. The sample was placed into new eppendorf tube and incubated at 37°C for three hours. 30 µl of this sample was used for streaking the LA plates containing specific antibiotics and 100 µl of the same sample was used for inoculating LB containing corresponding specific antibiotics. The streaked plates and inoculated LB were stored overnight at 28°C.

Table 2.7: Antibiotics for the selection of transformed *Agrobacterium tumefaciens*

Antibiotics	Stock concentration	Working concentration
Kanamycin	50 mg/ml	50 mg/L
Rifampicin	20 mg/ml	50 mg/L

2.2.8.4. Binary vector pGWB5 containing *OmpK*

Agrobacterium tumefaciens strain GV3101 was transformed with binary vector pGWB5 carrying gene of interest *OmpK*. The transformed *Agrobacterium tumefaciens* was used for transformation of *Spinacia oleracea*. T-DNA region of pGWB5 binary vector consisted of our gene of interest i.e. *OmpK* along with 35S promoter and NOS terminator.

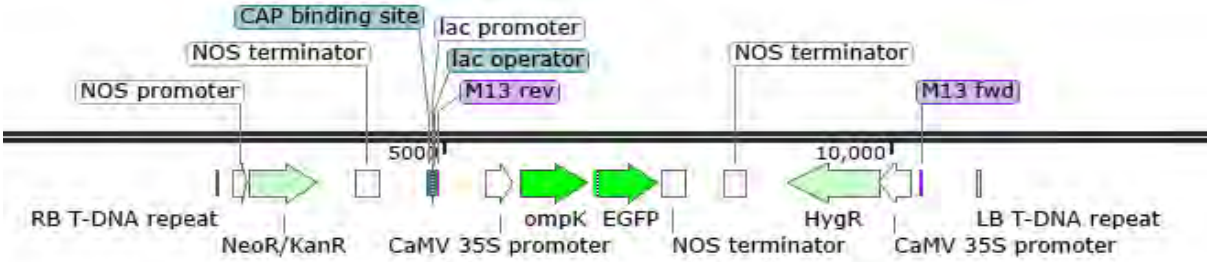


Figure 2.1: T-DNA Region of pGWB5 binary vector. **RB:** Right border; **NOS promoter:** Nopaline synthase promoter; **NeoR/KanR:** aminoglycoside phosphotransferase from Tn5; **CAP binding site:** *E.coli* catabolite activator protein; **lac promoter:** promoter for *E.coli* lac operon; **lac operator:** lac repressor encoded by lacI; **M13 rev:** common sequencing primer, one of multiple similar variants; **CamV 35S promoter:** strong constitutive promoter from cauliflower mosaic virus; **OmpK:** *OmpK* gene; **EGFP:** original enhanced GFP; **NOS terminator:** Nopaline synthase terminator; **HygR:** aminoglycoside transferase from *E. coli*; **M13 fwd:** common sequencing primer, one of multiple similar variants; **LB:** Left Border.

2.2.9. Second transformation: Transgenic *Spinacia oleracea*

Stable nuclear transformation method was used for the transformation of *Spinacia oleracea*.

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

2.2.9.1.1. Co-Cultivation

Agrobacterium tumefaciens strain GV3101 containing the binary vector pGWB5 was inoculated in flask containing 50 ml LB (Annexure 2.1). 50 mg/L rifampicin and 50 mg/L kanamycin were present in LB media for selection. The inoculated LB was kept in growth room and growth appeared within 2-3 days. OD_{600} was maintained at 0.6-0.8. The inoculated LB was shifted to 50 ml falcon tube and centrifuged at 4000 rpm for 20 minutes at room temperature. The supernatant was discarded and pellet was dissolved in 50 ml liquid ½ MS within LFH. Meanwhile, the leaf explants were cut and mixed with pellet and liquid ½ MS (Annexure 2.1). 200 µM acetosyringone was added to the infection media. Total infection time was 8 minutes. The media was discarded and explants were dried on filter paper. The explants were added to the co-cultivation plates. The plates were sealed with parafilm and stored in growth chamber at 25±2°C in dark for different time periods i.e. 2 and 3 days. Wild-type explants (control) was also transferred to co-cultivation media. Whole work was done in replicates. Following formula was used for calculating regeneration efficiency and callus formation efficiency of explants.

Regeneration efficiency (%) = Number of shoots regenerated/Total number of explants inoculated×100

Callus formation efficiency (%) = Number of callus produced from explants/Total number of explants inoculated×100

2.2.9.1.2. Selection

After the co-cultivation time period of 2 and 3 days, washing of the explants was performed. Washing of leaf explants was performed thrice (5 minutes each) with liquid ½ MS containing 400 mg/L cefotaxime. Finally, washing was done with autoclaved distilled water for five minutes. The explants were allowed to dry on autoclaved filter

paper and were shifted to selection plates containing 20 mg/L hygromycin and 250 mg/L cefotaxime. Each plate received 5-6 leaf explants. The plates were wrapped with parafilm and stored in growth chamber for observation.

2.2.10. Techniques of molecular biology to verify transformation

2.2.10.1. Plasmid isolation from transformed *Agrobacterium tumefaciens*

Plasmid isolation was performed in accordance with protocol reported by Rusell and Sambrook (2001). Inoculated LB (OD_{600} 0.6-0.8) was transferred to 50 ml falcon tube and centrifuged at 4°C, 14000 rpm for 10 minutes. The supernatant was discarded and 400 µl of solution I (Annexure 2.2) was added to the pellet. After vortexing, the mixture was shifted to two eppendorfs and 400 µl solution II (Annexure 2.2) was added to the pellet. After incubation of 5 minutes at -20°C, 200 µl solution III (Annexure 2.2) was added. It was incubated for 5 minutes at -20°C. The centrifugation was done at 4°C, 14000 rpm for 5 minutes. 600 µl of supernatant was collected in freshly autoclaved eppendorf tube and equal volume of phenol-chloroform (Annexure 2.2) was added. Aqueous and organic layers were formed. 600 µl of upper aqueous layer was collected and 600 µl of chilled isopropanol was added. It was incubated at -20°C for 1 hour or overnight. The sample was then centrifuged at 4°C, 14000 rpm for 5 minutes. Supernatant was discarded and 1 ml 70% ethanol was added to the pellet. Centrifugation was done for 2 minutes at 14000 rpm. Supernatant was discarded and pellet was allowed to dry. 20 µl TE buffer was added to the pellet and stored at -20°C.

2.2.10.2. Isolation of plant genomic DNA

Cetyltrimethyl ammonium bromide (CTAB) method was used for DNA extraction from plants (reported by Murray and Thompson, 1980). DNA was extracted from wild-type and transgenic plants. Temperature of water bath was pre-adjusted at 65°C. Before use, CTAB (Annexure 2.4) was placed in water bath at 65° C for 30-60 minutes. Leaf explants were ground into fine powder in mortar with liquid nitrogen. The powdered sample was transferred to freshly autoclaved eppendorf tube. 700-900 µl CTAB was added to sample in eppendorf. The homogenate was vortexed and placed in water bath

for 60 minutes at 65°C. 600 µl of phenol-chloroform-isoamyl (Annexure 2.4) was added to each sample. Centrifugation was performed at 4°C, 14000 rpm for 15 minutes. Supernatant was collected and equal volume of chilled isopropanol was added. The sample was incubated at -20°C for 3 hours or overnight. The sample was centrifuged at 4°C, 14000 rpm for 5 minutes. Supernatant was discarded and 1ml 70% ethanol (Annexure 2.4) was added to the pellet. Centrifugation was done for 2 min at 4°C and 14000 rpm. Supernatant was discarded and T.E. buffer (Annexure 2.4) was added to the pellet about 30 µl or according to the size of pellet. It was stored at -20°C.

2.2.10.3. Micro-volume quantification of DNA concentration

Colibri microvolume spectrophotometer was used for measuring the concentration of DNA isolated from spinach, employing a highly sensitive approach called NanoDrop micro-volume quantification (Desjardins and Conklin, 2010). Spectrophotometer's top and bottom panels were wiped using 2-3 µl de-ionized H_2O. 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 using dry, clean lab wipes free of fur. Nucleic Acid application was selected. 1 µl of TE buffer (Annexure 2.4) was added onto the lower optical panel for obtaining the blank reading. Once the blank measurement was taken, both the optical panels were cleaned using lab wipe. For the quantification of DNA samples, right constant was selected. 1 µl of DNA sample was poured onto the lower optical panel. "Measure" option was selected. The concentration as well as purity ratio of DNA was automatically calculated by the software.

2.2.10.4. Polymerase Chain Reaction (PCR)

To confirm the successful integration and transformation of *OmpK* gene into the nuclear genome of *Spinacea oleracea*, PCR was performed. 25 µl volume of master mix was prepared for 1X PCR. Positive (*OmpK* containing plasmid) and negative (wild-type plant) controls were used. The expected size of amplicon was 500 bp using the internal primers for *OmpK* gene and 890 bp using *OmpK* nuclear forward and reverse primers.

***OmpK* Nuclear Forward Primer:**

5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGTAAATCACTTTTAGCT CTAGGCC **3'**

***OmpK* Nuclear Reverse Primer:**

5'GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTGATGGTGATGGTGATG **3'**

***OmpK* Internal Forward Primer:**

5'CTAAGCAACCCAAGCAGTGACAAAG **3'**

***OmpK* Internal Reverse Primer:**

5'CCTTCATCTTTCAGACCATAAACATCTTTGTAGC **3'**

Table 2.8: For 1X PCR, composition of master mix

Reagents	**Concentration (µl)**
PCR water	18
Taq buffer	2.5
$MgCl_2$ (25mM)	1.5
dNTPs	0.5
Forward primer	0.5
Reverse primer	0.5
Taq Polymerase	0.5
Template (DNA)	01

Table 2.9: For PCR, standard conditions

Steps	Temp. (°C)	Time (min:sec)
Initial denaturation	95	05:00
Final denaturation	95	00:45
Annealing	66	01:00
Initial extension	72	02:00
Final extension	72	10:00
Hold	04	infinity

2.2.10.5. Agarose Gel Electrophoresis and Imaging

Agarose gel electrophoresis was used to confirm the PCR results. PCR product of transgenic as well as wild-type samples were loaded into the wells. Before loading, 7 µl PCR product was mixed with 3 µl loading dye (Annexure 2.5). 1 kb gene ruler (Cat No: SM0314, Thermo Scientific, USA) (Figure 2.2) was used for checking the size of DNA sample. Each sample was carefully loaded into distinct wells. Following sample loading, lid of gel tank was closed, power source was attached, and electrophoresis was carried out for 60 minutes, 110 volts and 500 milli amperes. DNA bands were seen under UV light using gel documentation system.

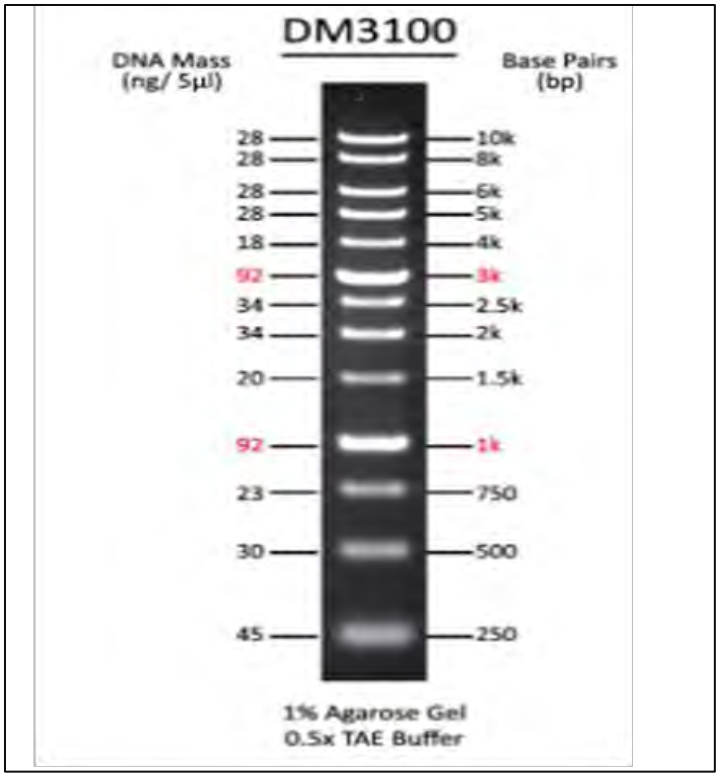


Figure 2.2: 1 kb DNA ladder for agarose gel electrophoresis (Taken from www. Gentechbio.com)

2.2.10.6. Quantitative Real Time (qRT)PCR

After successful transformation, qRT-PCR was used to check the expression level of transgene within plant cell in accordance with protocol described by Wen *et al*. (2012). qRT-PCR was performed using MyGo Pro Real time PCR (Stokesly Middlesbrough, UK). DNA samples of both wild-type and transgenic were used. 1:10 and 1:100 dilutions were prepared for each DNA sample. A fluorescent dye, SYBR Green (Cat No: K0221 ThermoScientific, USA) was used in this technique. Mastermix was prepared as mentioned in the Table 2.10. Total reaction mixture was 10 µl. 9.5 µl reaction mixture was added to autoclaved RT-PCR tubes and 0.5 µl sample was added. *OmpK* internal primers were used in the technique whose sequence is mentioned below:

***OmpK* internal forward primer:**

5' CTAAGCAACCCAAGCAGTGACAAAG **3'**

***OmpK* internal reverse primer:**

5' CCTTCATCTTTCAGACCATAAACATCTTTGTAGC **3'**

Table 2.10: For qRT-PCR, composition of reaction mixture

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

Table 2.11: Conditions for qRT-PCR

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

2.2.10.7. Protein extraction

Plant tissue was sliced into 100 mg small pieces and ground into fine powder in mortar using liquid nitrogen. To this powder, 500 µl of protein extraction buffer (Annexure 2.6) was added. The mixture was mixed properly and shifted to freshly autoclaved eppendorf tube. Centrifugation was done at 20,000g, 4°C for 10 minutes. Supernatant was collected in fresh eppendorf tube and pellet was discarded. Total Soluble Protein (TSP) was centrifuged once again at 20,000g, 4°C for 10 minutes in order to remove the cellular debris. Supernatant was collected and shifted to a fresh eppendorf tube and was stored at -20°C.

2.2.10.8. Bradford Assay (Protein Quantification)

After protein extraction, Bradford assay was performed to determine the concentration of Total Soluble Protein (TSP). Bovine Serum Albumin (BSA) was used as a standard to plot the standard curve. 2mg/ml stock solution of BSA was prepared in order to prepare five serial dilutions of BSA (Annexure 2.7). Protein extraction buffer was used as a blank. We loaded 20 µl of BSA, sample and protein extraction buffer (Annexure 2.6) was used as a blank. 20 µl of blank, BSA dilutions and samples were loaded into separate wells of 96 well plate. After it, 200 µl of Bradford's reagent was added in each well containing sample. The plate was incubated in dark at 37°C for 30-60 minutes. Absorbance was measured at 595 nm using spectrophotometer. Standard curve was plot using the absorbance values of BSA dilutions. The standard curve was used to calculate the concentration of each protein sample using the TREND formula on Excel sheet.

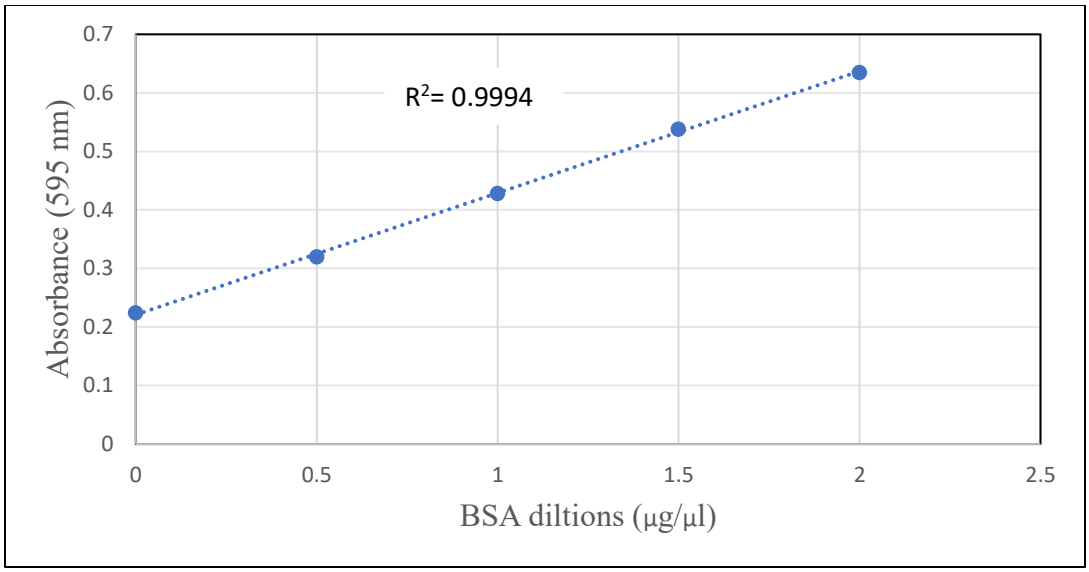


Fig 2.3: BSA Standard curve for protein quantification.

2.2.10.9. Dot blot assay

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 specific antibody. Dot blot assay was performed for detection and identification of protein. Various steps such as blotting, blocking, treatment with primary antibody, treatment with secondary antibody and visualization were performed in accordance with standard protocol mentioned by biotechne® R and S system.

2.2.10.9.1. Blotting

Nitrocellulose membrane (NCM) was used for blotting the protein samples. Protective coverings of NCM were removed with the help of forceps and 20 µl of wild as well as transgenic protein sample was transferred to the NCM. The blotted sites were marked with pencil. The membrane was incubated for one hour at 25°C.

2.2.10.9.2. Blocking

20 ml Blocking buffer (Annexure 2.8) was loaded onto the membrane. The membrane was incubated for 30-60 minutes at 25°C with gentle shaking. After incubation, the

blocking buffer was discarded. The membrane was washed with TBS-T wash buffer (Annexure 2.9) three times for 10 minutes in order to avoid excess BSA binding.

2.2.10.9.3. Primary antibody treatment

His tag antibody (Abcam, USA) was used as the primary antibody. Working dilution 1:10,000 of primary antibody (Annexure 2.10) was made from the stock in TBS-T. The membrane was incubated overnight at 4°C. After incubation, the membrane was washed thrice with TBS-T (Annexure 2.9).

2.2.10.9.4. Secondary Antibody treatment

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Abcam, USA) was used as the secondary antibody. Working dilution 1:10,000 of secondary antibody (Annexure 2.11) was made from stock in TBS-T buffer. Incubation was done for 1.5-2 hours with gentle shaking at 25°C. After incubation, the membrane was washed thrice with TBS-T buffer (Annexure 2.9) for 10 minutes.

2.2.10.9.5. Visualization

Chemiluminescent substrate (Cat No: WBKLS0500, Merck Millipore, Germany) for horseradish peroxidase (HRP) enzyme was used for visualization. Hydrogen peroxidase and Luminol were mixed in 1:1 ratio. The membrane was allowed to incubate with the substrate in dark conditions for 5 minutes at 25°C. After incubation, the membrane was visualized using chemiluminescence system (ECL® PLUS; GS Healthcare).

2.2.10.10. Western blotting

In order to seperate the purified protein on the basis of their size, Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was performed. For transfer of separated proteins, Nitrocellulose membrane (NCM) was used. After successful transfer, NCM was incubated with primary and secondary antibody under optimized conditions for detection of target protein. Protein samples from transformed spinach tissues were used for western blotting. Wild- type plants were used as negative controls.

2.2.10.10.1. SDS-PAGE

18% resolving gel and 5% stacking gel was prepared. 18% resolving gel was prepared and poured between two glass plates. 1 ml isopropanol was poured over the resolving gel (Annexure 2.12) for linearizing the gel surface. The gel was allowed to set properly. 5% stacking gel (Annexure 2.13) was prepared. Isopropanol was removed completely before pouring the stacking gel over resolving gel. After the gel was properly set, the comb was inserted so that wells are formed.

2.2.10.10.2. Preparation and loading of protein samples

4X sample buffer (Annexure 2.14) was used for preparation of protein samples. 4 µl sample buffer and 25 µl protein were mixed in eppendorf tubes. Before loading, the protein samples were heated at 90°C for 10 minutes in order to denature the proteins. Samples were loaded into respective wells of polyacrylamide gel. In one well, 4 µl pre-stained PAGE ruler (Cat No. 26616, ThermoScientific, USA) was loaded.

2.2.10.10.3. Electrophoresis

After the gel was properly set, the assembly was placed in gel tank. Gel tank was filled with running buffer (Annexure 2.15). Comb was removed and wells were washed with running buffer before loading the sample in the wells. Samples were loaded, lid of gel tank was closed and positive and negative electrodes of gel tank were connected to power supply. 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 was run, it was subjected to western blotting.

2.2.10.10.4. Transfer of protein

Semi-dry method was used for transfer of protein. A semi-dry blotting apparatus was used for this purpose. For transfer of one gel, eight Whatman's filter papers were cut equal to the size of separating gel. The filter papers and NCM were soaked in 1X transfer buffer (Annexure 2.16). Sandwich was prepared by placing four filter papers, NCM, separating gel, four filter papers (bottom to top) from positive terminal (anode) to negative terminal (cathode). Roller was used to remove any air bubbles present as it

would affect the transfer efficiency. Terminals of transfer apparatus were connected to power supply and voltage was adjusted at 12 volts for 90 minutes.

2.2.10.10.5. Blocking

Blocking is done to prevent non-specific binding of primary antibody. Blocking solution (Annexure 2.6) was used for this purpose. The membrane was kept in blocking solution for 60 minutes with continuous shaking at room temperature. After one hour, the blocking solution was removed and the blot was washed thrice with TBS-T wash buffer (Annexure 2.9).

2.2.10.10.6. Treatment with primary antibody

Anti-His-tag primary antibody was used. 1:10,000 (Annexure 2.10) working dilution of primary antibody was prepared in TBS-T. Membrane was kept in primary antibody overnight at 4°C. After treatment, washing was done thrice with TBS-T (Annexure 2.9).

2.2.10.10.7. Treatment with secondary antibody

Secondary antibody used was Horse-radish peroxidase (HRP) conjugated goat anti-mouse IgG. The membrane was incubated with secondary antibody (Annexure 2.11) for 1.5-2 hours with continuous shaking at room temperature. After treatment, membrane was washed three times with TBS-T (Annexure 2.9).

2.2.10.10.8. Visualization

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

2.2.10.11. ELISA

Indirect ELISA was performed to determine the protein concentration within samples. A 96 well plate was used for performing indirect ELISA. ELISA extraction buffer (Annexure 2.17) was used for sample preparation. The samples were loaded into separate

wells and plate was incubated for one hour at 37°C. After an hour, washing was done three times with wash buffer (Annexure 2.9). 200 µl blocking buffer (Annexure 2.8) was added in each well. The plate was incubated at 37°C for one hour. The blocking solution was removed and the plate was washed thrice using wash buffer (Annexure 2.9). 50 µl primary antibody (Annexure 2.10) was added in each well and plate was incubated at 37°C for one hour. The wells were washed thrice with wash buffer (Annexure 2.9) and secondary antibody (Annexure 2.11) was added in each well. The plate was incubated at 37°C for an hour and washed with wash buffer (Annexure 2.9) three times to remove unbound antibody. Enzyme specific substrate, TMB (Cat No. A3840, Applichem, Germany) was added to each well and plate was incubated at room temperature. After 20-30 minutes, when the blue colour developed, 0.1 N H_2SO_4 (stop solution) was added to stop the reaction and absorbance was measured at 595 nm.

Annexures

Annexure 2.1: Chemical composition of plant culture media

S. No.	Media	Components	Concentration (g/L)
1	½ MS Media	MS media Sucrose Agar	2.2g/L 30g/L 0.8%
2	Full MS Media	MS media Sucrose Agar	4.4g/L 30g/L 0.8%
3	Liquid ½ MS Media	MS media Sucrose	2.2g/L 30g/L
4	LB	LB broth	25 g/L
5	LA	Nutrient Agar	25 g/L

Annexure 2.2: Solutions composition for plasmid isolation

Solutions	Components	Concentration
Solution I	Glucose Tris EDTA	50 mM 25 mM, pH 08 10 mM, pH 08
Solution II	SDS NaOH	1 % 0.2 N
Solution III	Sodium Acetate	3M, pH 4.8
Phenol-Chloroform	Phenol Chloroform	25 ml 25 ml
70% Ethanol	Ethanol Distilled water	70 ml 30 ml
T.E. buffer	Tris HCl EDTA	1 M 0.5 M

Annexure 2.3: Medias required for transformation

Media	Components	Concentration (g/L)
Co-cultivation media	Full MS Sucrose Agar Distilled water BAP IAA	4.4 g 30 g 0.8% 1 L 1000 µl 500 µl

	Acetosyringone	200 µM
Selection Media	Full MS Sucrose Agar Distilled water BAP IAA Hygromycin Cefotaxime	4.4 g 30 g 0.8 % 1 L 1000 µl 500 µl 400 µl 1000 µl

Annexure 2.4: Solutions for DNA isolation from plants

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

Annexure 2.5: Solutions for Agarose Gel Electrophoresis

TBE Buffer recipe 10X (Ph 8.0)

Components	**Concentration (g/L)**
Tris base	107.8
Boric Acid	55.05
EDTA	7.3

1X TBE Buffer

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

Ethidium Bromide (10mg/ml)

Components	Concentration
Ethidium Bromide	1 g
Distilled water	100 ml

Loading dye

Components	Concentration
Bromophenol blue	0.25 g
Distilled water	100 ml

Annexure 2.6: Protein extraction buffer

Protein Extraction Buffer

Components	Concentration
Sodium Chloride	300 mM
Ascorbic Acid	20 mM
Sodium metabisulfite	10 mM
Tris (pH 08)	100 mM

Annexure 2.7: BSA dilutions for Bradford Assay

BSA dilutions

Concentration	Chemicals	Distilled water
0	0	20
0.5	5	15
1.0	10	10
1.5	15	5
2.0	20	0

Annexure 2.8: Blocking Buffer

Components	Concentration
BSA	5 g
TBS-T	100 ml

Annexure 2.9: Wash buffer **(**TBS-T)

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

Annexure 2.10: Dilution for primary Antibody

Components	Concentration
His tag Antibody	3 µl
Sodium Azide	0.015 g
BSA	1.5 g
TBS-T buffer	30 ml

Annexure 2.11: Dilution for Secondary Antibody

Components	Concentration
HRP-conjugated goat anti-mouse IgG	3 µl
BSA	1.5 g
TBS-T Buffer	30 ml

Annexure 2.12: Resolving gel

Components	Concentration
Distilled water	8.35 ml
30% Stock Acrylamide solution	6.7 ml
4X Resolving Tris solution	5 ml
10% ammonium persulfate	400 µl
TEMED	37 µl

Annexure 2.13: Stacking gel

Components	Concentration
Distilled water	6.76 ml
30% Stock Acrylamide solution	2 ml
0.5 M stacking buffer	3 ml
10% SDS	0.12 ml
10% ammonium persulfate	0.12 ml
TEMED	25 µl

Annexure 2.14: 4X Sample buffer

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

Annexure 2.15: Running buffer

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

Annexure 2.16: Transfer buffer

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

Annexure 2.17: ELISA Extraction buffer

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

CHAPTER # 03

3. RESULTS

In this study, suitable hygromycin concentration for leaf explants of *Spinacia oleracea* was optimized. An effective protocol for *Agrobacterium*-mediated stable transformation of leaf explants of *Spinacia oleracea* was developed. Molecular biology techniques including PCR (Polymerase Chain Reaction), qRT-PCR (Quantitative Real time-Polymerase Chain Reaction) were used for the confirmation of *OmpK* gene integration into *Spinacia oleracea*. Dot blot, Western blotting and ELISA were performed for detection of OmpK protein.

3.1. Seed Germination

3.1.1. Plant Material and Seed Sterilization

In the present research, experiments were performed using plants germinated from *Spinacia oleracea* seeds.

3.1.2. Sterilization of *Spinacia oleracea* seeds

Spinach seeds were surface sterilized by treating them with 0.2% mercuric chloride ($HgCl_2$) for 3 minutes. After washing thrice with distilled water, seeds were inoculated on full MS media. The germination efficiency was calculated to be 100% as shown in figure 3.1.

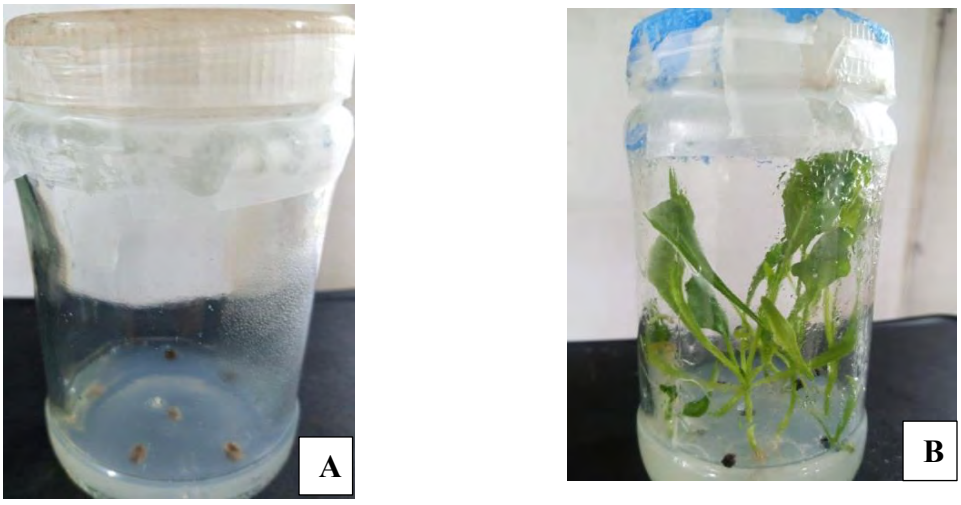


Figure 3.1: Spinach seed sterilization method. Seeds sterilized using 0.2 % $HgCl_2$. (A) At Day 1 of seed sterilization. (B) At Day 21 of seed sterilization.

3.2. Callogenesis of *Spinacia oleracea*

Experiments were done to analyze the best media for callus formation of leaf explants. Full MS media along with combination of plant growth regulators was used. Explants were shifted to two different media (Table 3.1). The media were different from one another based on the hormones that were used. After inoculation, they were observed for four weeks. It was observed that media I containing 1 mg/L BAP and 0.5 mg/L IAA was effective for development of explants (Figure 3.2). Callus formation efficiency of leaf explants of *Spinacia oleracea* in two media is given in table 3.1. Graphical representation for each media is provided in figure 3.3.

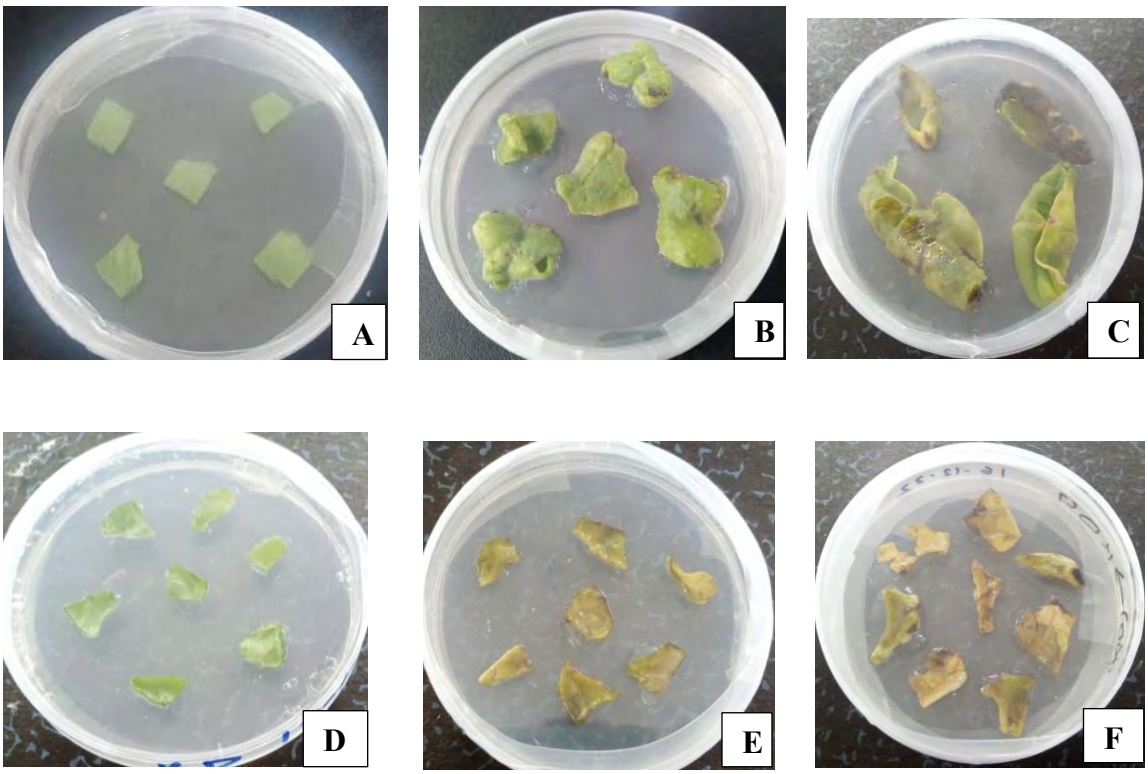


Figure 3.2: Media for callus formation of spinach leaf explants. (A) Day 1 on media I. (B) Day 15 on media I. (C). Day 30 on media I. (D) Day 1 on media II. (E) Day 15 on media II (E) Day 30 on media II.

Table 3.1: Callus formation efficiency of spinach leaf explants.

S. No.	Media	Total no. of leaves inoculated	No. of callus formed	Callogenesis efficiency
1	Media I	15	10	66.67%
2	Media II	21	2	9.5%

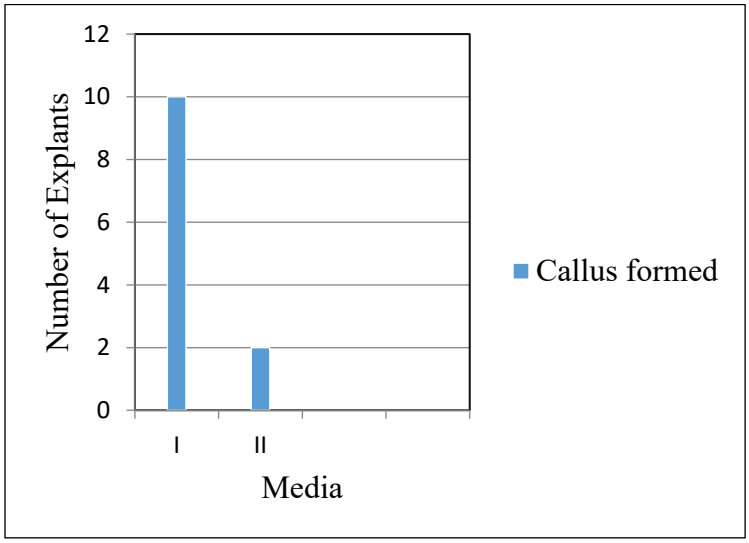


Figure 3.3: Graphical representation of percentage efficiencies of *Spinacia oleracea* explants on callogenesis media. The data shown here is from three batches (n=3).

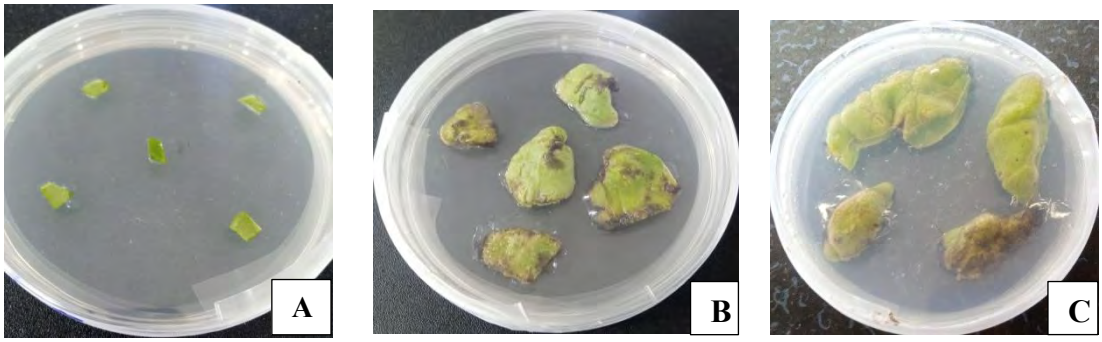
3.3. Effect of hygromycin concentration on survival of explants

To determine the effect of hygromycin on wild-type explants, explants were prepared and shifted to different hygromycin concentrations. The hygromycin concentration that bleaches out wild type explants was used for selection of transformed explants.

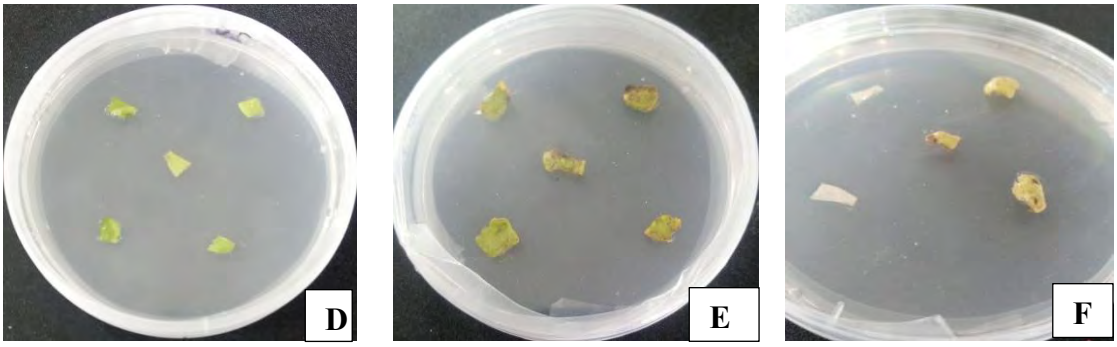
3.3.1. Effect of hygromycin concentration on *Spinacia oleracea* explants

Suitable hygromycin concentration was determined for selection of transgenic leaf explants. *Spinacia oleracea* explants were inoculated on media containing BAP and IAA with different hygromycin concentrations i.e. 0 mg/L, 15 mg/L, 20 mg/L, 25 mg/L and 30 mg/L and results were recorded. It was noticed that as the hygromycin concentration

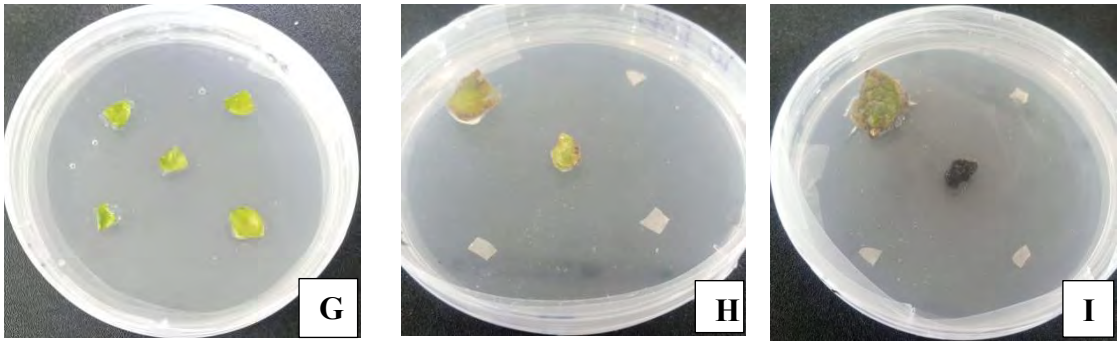
increases, regeneration ability decreases and more explants tend to bleach out. Figure 3.4 shows effect of hygromycin concentration on leaf explants of *Spinacia oleracea* and results are mentioned in table 3.2. Graphical representation is given in figure 3.5.



0 mg/L hygromycin



15 mg/L hygromycin



20 mg/L hygromycin

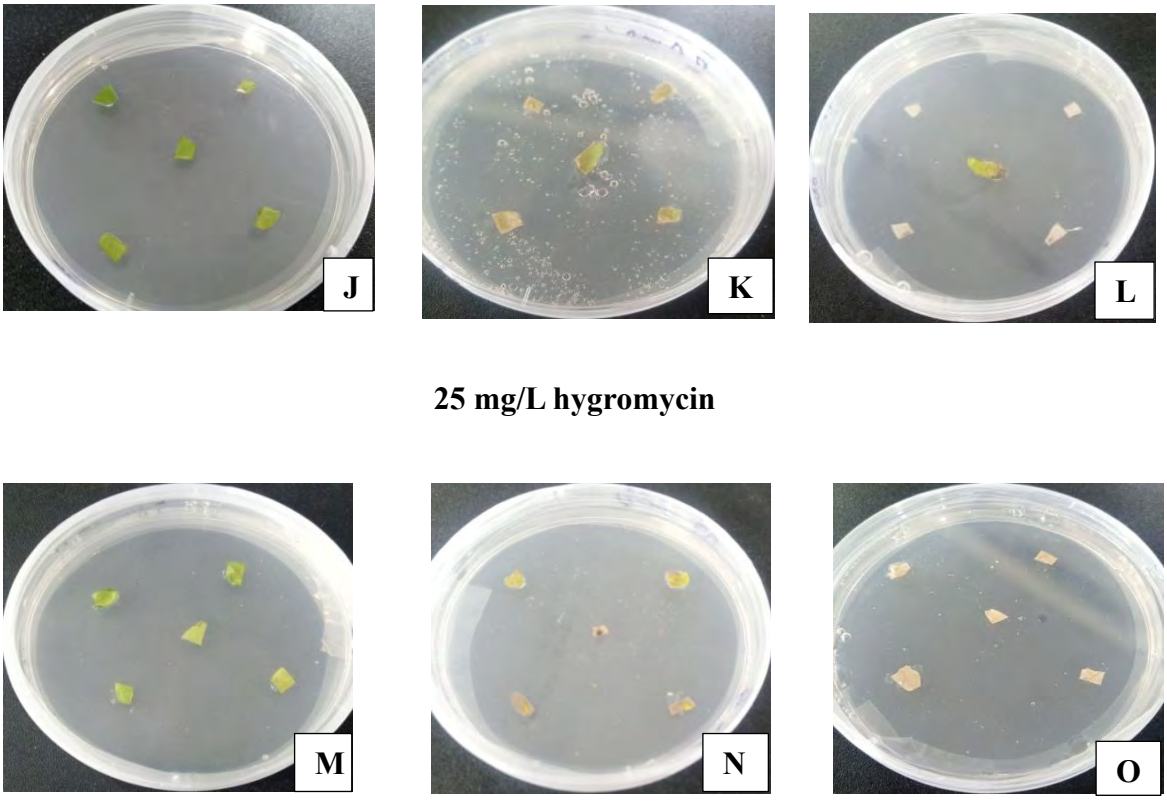


Figure 3.4: Effect of hygromycin concentration on leaf explants of *Spinacia oleracea*. Different concentrations i.e. 15 mg/L, 20 mg/L, 25 mg/L and 30 mg/L of hygromycin were added to media containing BAP and IAA and its effect on explants were recorded on different days. (A) Day 1 on 0 mg/L. (B) Day 15 on 0 mg/L. (C) Day 30 on 0 mg/L. (D) Day 1 on 15 mg/L. (E) Day 15 on 15 mg/L. (F) Day 30 on 15 mg/L. (G) Day 1 on 20 mg/L. (H) Day 15 on 20 mg/L. (I) Day 30 on 20 mg/L. (J) Day 1 on 25 mg/L. (K) Day 15 on 25 mg/L. (L) Day 30 on 25 mg/L. (M) Day 1 on 30 mg/L. (N) Day 15 on 30 mg/L. (O) Day 30 on 30 mg/L.

Table 3.2: Effect of hygromycin on leaf explants.

S. No.	Concentration of hygromycin (mg/L)	Total no. of leaf explant	Callus formed	Explants bleached	Percentage of callus formed	Percentage of explants bleached
1	0	15	15	0	100	0
2	15	15	9	6	600	40
3	20	15	6	9	40	60
4	25	15	3	12	20	80
5	30	15	0	15	0	100

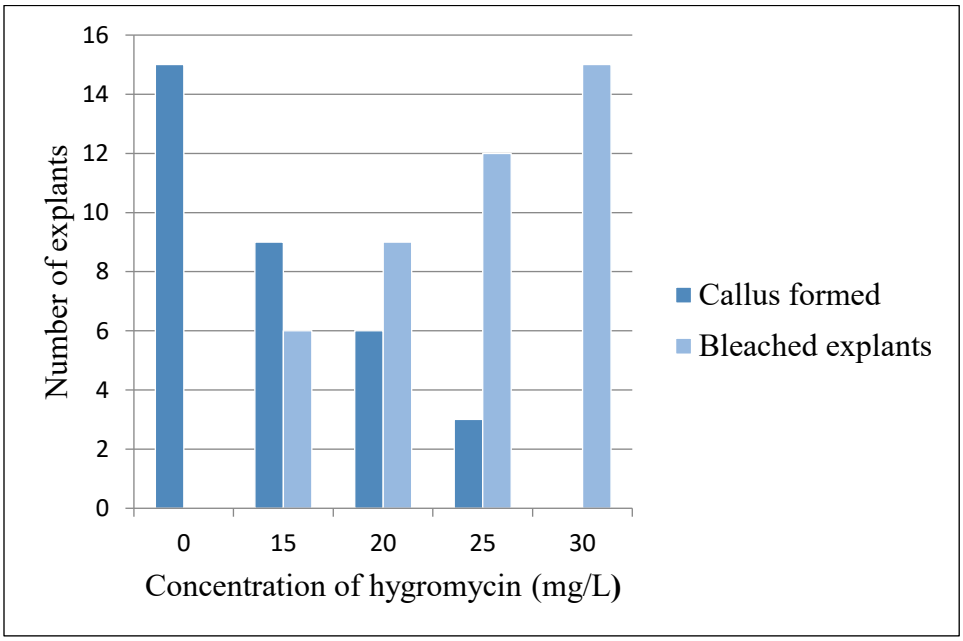


Figure 3.5: Graphical representation of effect of hygromycin concentration on callus formation of explants. The graph represents data from three batches (n=3).

3.4. *Agrobacterium*-mediated transformation

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

Leaves of *Spinacia oleracea* were used for *Agrobacterium*-mediated stable transformation. Leaf explants were prepared by cutting them into smaller pieces. *Agrobacterium tumefaciens* containing pGWB5 carrying *OmpK* gene was used to infect the explants. After infection, explants were kept on co-cultivation plates and then shifted to selection plates containing hygromycin.

3.4.1.1. Optimization of infection time

In the experiment, leaf explants were divided into two groups. Both groups were infected with *Agrobacterium*. Some of the explants were treated with bacterial culture for 8 minutes and others were treated for 10 minutes. The explants were observed for 7 days (figure 3.6). It was noticed that as the infection time increases, it gets more difficult to get rid of excess bacteria and regeneration efficiency decreases. Thus, 8 minutes infection time was considered an optimum time for transformation of leaf explants. Data regarding the infection time is provided in table 3.3 and graphically represented in figure 3.7.

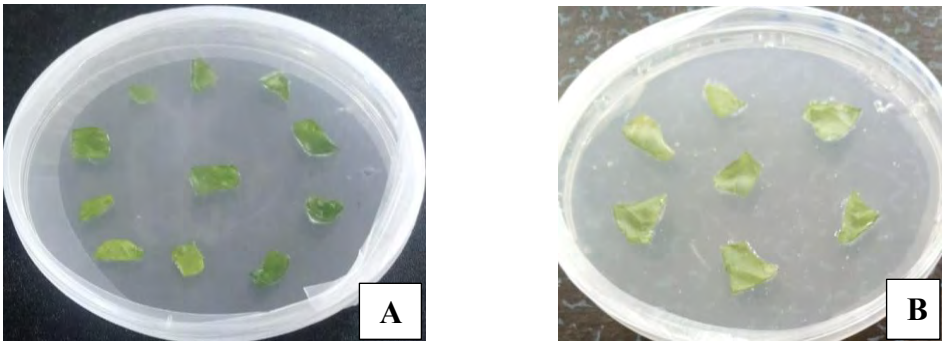


Figure 3.6: Leaf explants after infection on co-cultivation media. Explants were treated with *Agrobacterium tumefaciens* culture for different time periods. (A) Explants treated for 8 minutes. (B) Explants treated for 10 minutes.

Table 3.3: Effect of infection time on callus formation of explants.

Infection time	No. of leaf explants	Callus formed	Callus formation efficiency
8 minutes	11	3	27.27%
10 minutes	7	0	0%

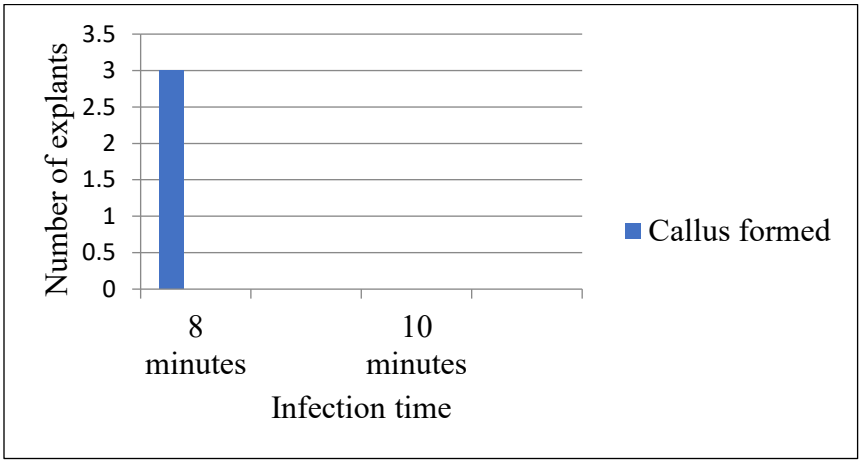


Figure 3.7: Graphical representation of effect of infection time on callus formation efficiency of explants

3.4.1.2. Optimization of Co-cultivation time

After infection, explants were kept on co-cultivation media in dark for 2 and 3 days. Explants were observed after specific co-cultivation time. It was noticed that as the co-cultivation time increases, regeneration of leaf explants also increases. Explants that were placed on co-cultivation media for 3 days showed a higher callus formation efficiency compared to explants that were kept for 2 days as shown in figure 3.8. Results for optimization of co-cultivation time are given in table 3.4 and graphically represented in figure 3.9.

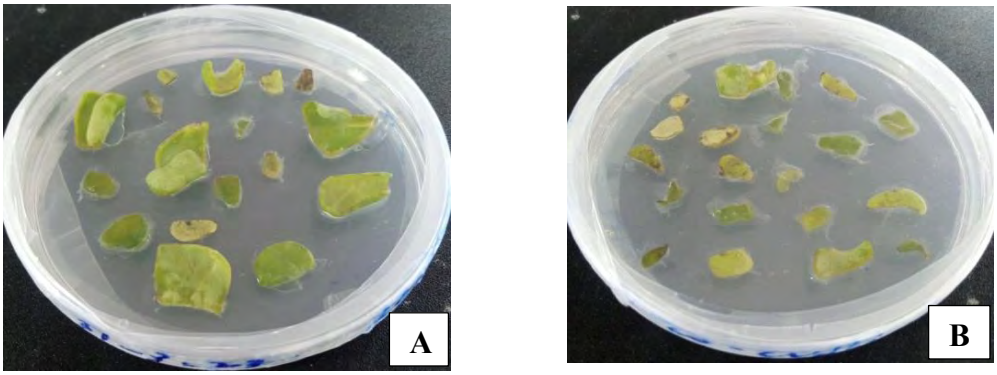


Figure 3.8: Explants after co-cultivation time. Different co-cultivation time was provided to leaf explants. (A) Explants after 3 days co-cultivation time. (B) Explants after 2 days co-cultivation time.

Table 3.4: Effect of co-cultivation time on callus formation efficiency of explants.

Co-cultivation time	Total no. of leaf explants	Callus formed	Callus formation efficiency
2 days	20	3	15%
3 days	17	9	52.94%

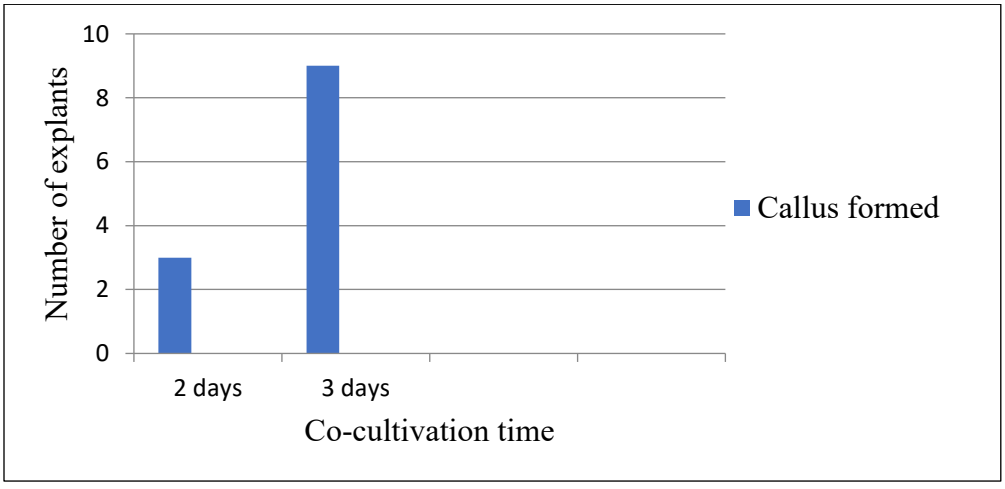


Figure 3.9: Graphical representation of effect of different co-cultivation time (2 and 3 days) on callus formation efficiency of explants.

From the results, it was concluded that infection time of 8 minutes and co-cultivation time of 3 days enhances the transformation efficiency of leaf explants. Thus, these optimum conditions were used to transform leaf explants of *Spinacia oleracea*.

3.4.1.3. Transformation

Leaf explants were transformed with *OmpK* gene (present in pGWB5 vector) via *Agrobacterium*-mediated stable transformation. Conventional PCR was used to confirm the successful transformation and integration of *OmpK* transgene. Different molecular biology techniques were used for detection of gene expression and protein levels.

3.4.1.3.1. PCR confirmation of plasmid containing *OmpK* gene

Sambrook and Rusell (2001) protocol was used for plasmid isolation. Agarose gel electrophoresis was used for confirmation of isolated plasmid. Presence of *OmpK* gene in plasmid was confirmed by conventional PCR by using specific primers for amplification of transgene. The PCR product was loaded onto agarose gel for confirmation of our gene of interest. *OmpK* internal forward and reverse primers were used for confirmation of *OmpK* gene in the plasmid. Gel results showed the presence of transgene in the isolated DNA samples (Figure 3.10). Transgene integration was confirmed through *OmpK* internal primers.

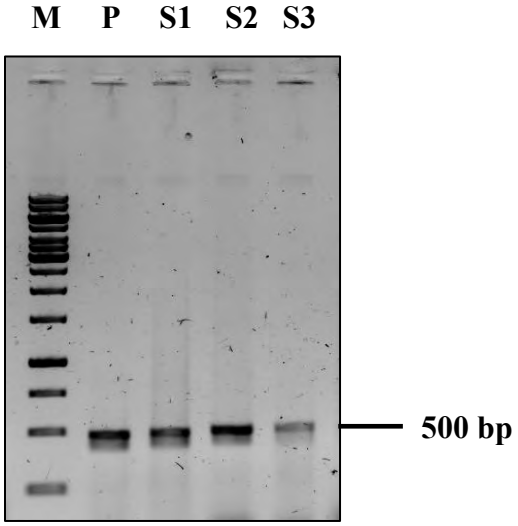
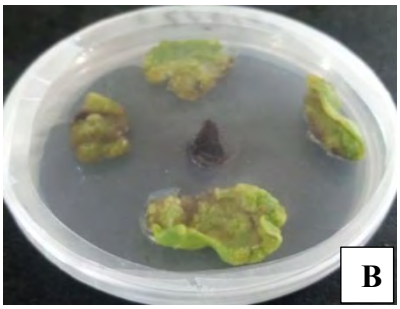


Figure 3.10: PCR confirmation of *OmpK* gene in plasmid. (M) 1 kb gene ruler. (P) Positive control. (S1, S2, S3) Plasmids

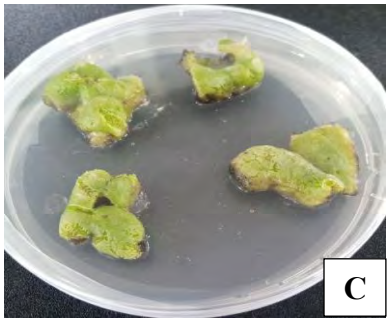
3.4.1.3.2. Transformation procedure

Following optimization of different factors, transformation was carried out under optimized conditions. Leaf explants were infected with *Agrobacterium* containing *OmpK* gene for 8 minutes. Treated samples were placed in dark for 3 days (co-cultivation period). After 3 days, explants were washed with liquid ½ MS containing cefotaxime to remove excess bacteria. They were then shifted to selection plates containing 20 mg/L hygromycin (Figure 3.11).

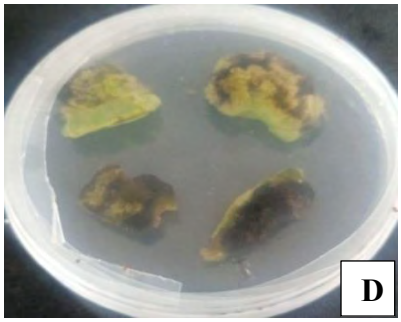
Transformed batch 1



Transformed batch 2



Transformed batch 1



Transformed batch 2

Figure 3.11: Transformed explants on selection plates. (A & B) Transformed explants on selection plates after 2 weeks. (C & D) Transformed explants on selection plates after 4 weeks.

3.4.1.3.3. PCR confirmation of transgene using different *OmpK* primers

3.4.1.3.3.1. PCR confirmation of transgene using *OmpK* internal primers

CTAB method described by Murray and Thompson (1980) was used for extraction of plant genomic DNA. Before PCR confirmation, extracted DNA was checked through agarose gel electrophoresis (figure 3.12). *OmpK* internal forward and reverse primers specific for our gene of interest (*OmpK*) were used. Figure 3.13 shows the gel image with amplified band of 500 bp, which confirms the presence of *OmpK* transgene. Consequently, successful transformation of *Spinacia oleracea* was confirmed.

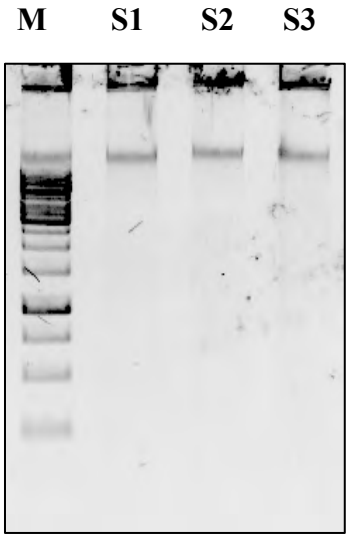


Figure 3.12: Confirmation of DNA isolated from plant samples. (M) 1 kb gene ruler. (S1) Sample 1. (S2) Sample 2. (S3) Sample 3.

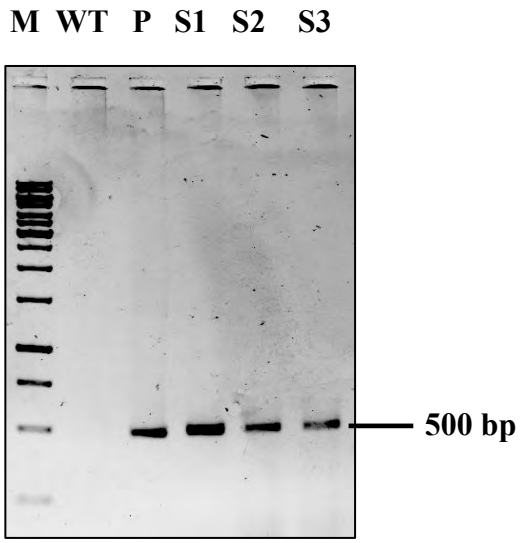


Figure 3.13: PCR Confirmation of transgene using *OmpK* internal primers. (M) 1 kb gene ruler. (WT) Wild-type sample as negative control. (P) Positive control. (S1) Sample 1, (S2) Sample 2, (S3) Sample 3.

3.4.1.3.2.2. PCR confirmation of transgene using *OmpK* nuclear primers

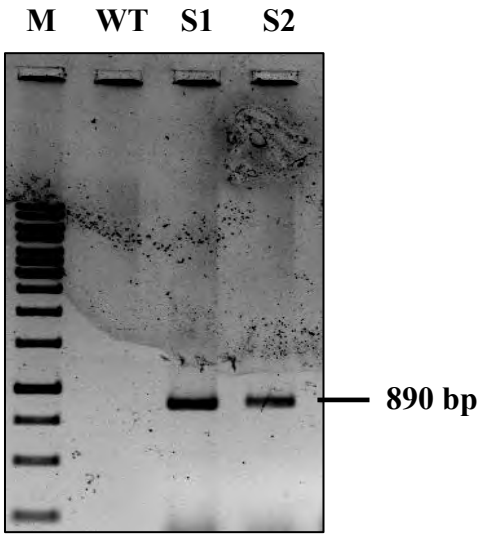


Figure 3.14: PCR Confirmation of transgene using *OmpK* nuclear primers. (M) 1 kb gene ruler. (WT) Wild-type sample as negative control. (S1) Sample 1. (S2) Sample 2.

3.4.1.4. Calculation of *OmpK* copy number in transgenic *Spinacia oleracea* by qRTPCR

Copy number of transformed samples was calculated in accordance with the protocol established by Schmittgen and Livak (2008). CQ values of endogenous gene and transgene were obtained (Table 3.5), which were used to plot the standard curve. This provided the correlation coefficient values for *OmpK* transgene and *β-actin* endogenous gene. These values were used to calculate the copy number for *OmpK* gene which was equal to 1.

Table 3.5: Average CQ values of transgene vs endogenous gene.

Sample	Average CQ values for transgene	Average CQ values for *β- actin*
OmpK	23.66398	27.45105895
OmpK 1:10	24.28553	28.37581828
OmpK 1:100	24.55122	31.41727384

3.4.1.5. Dot Blot

Dot blot was used to detect the presence of protein within samples. Primary antibody and secondary antibody were used for detection of OmpK protein. Both wild-type and transgenic samples were spotted on the blot. Unlike wild-type, chemiluminescence was observed for transgenic sample (Figure 3.15). This confirmed the presence of transgenic protein in our sample.

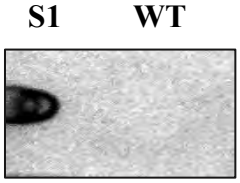


Figure 3.15: Confirmation of presence of OmpK protein in transformed samples. (WT) Wild-type. (S1) Sample 1.

3.4.1.6. Western blotting

Western blotting was used to check the expression of *OmpK* transgene. Size of monomeric form of OmpK protein is equal to 30.21 kDa. Dimeric form of OmpK protein with size equal to 61 kDa was detected in our samples as shown in figure 3.16. By using specific antibodies, band was observed for transgenic samples while no band was observed for wild-type samples (figure 3.16). Detection of 61 kDa protein confirms the successful stable transformation of *Spinacia oleracea* leaf explants.

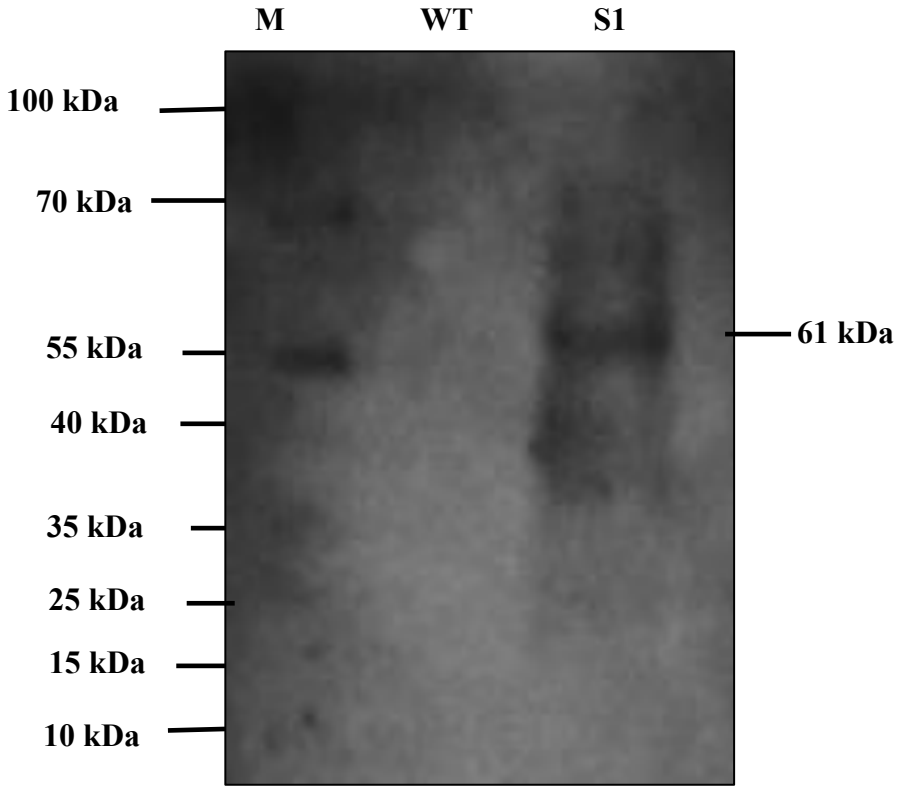


Figure 3.16: Confirmation of protein by western blotting. (M) Protein marker. (WT) Wild-type. (S1) Transformed sample 1.

3.4.1.7. ELISA

Indirect ELISA was used to confirm the presence of OmpK antigen within our transgenic samples. Total soluble protein extract from transgenic plant was used. As the TMB substrate was added, blue color appeared in all samples except for blank and wild-type samples. Addition of 0.1 N H_2SO_4 (stop solution) changed this blue color to yellow. The

data presented in figure 3.17 shows an increased absorbance of transgenic samples compared to wild-type. Among the two samples, S2 shows the highest absorbance which indicates that it contains highest amount of antigen.

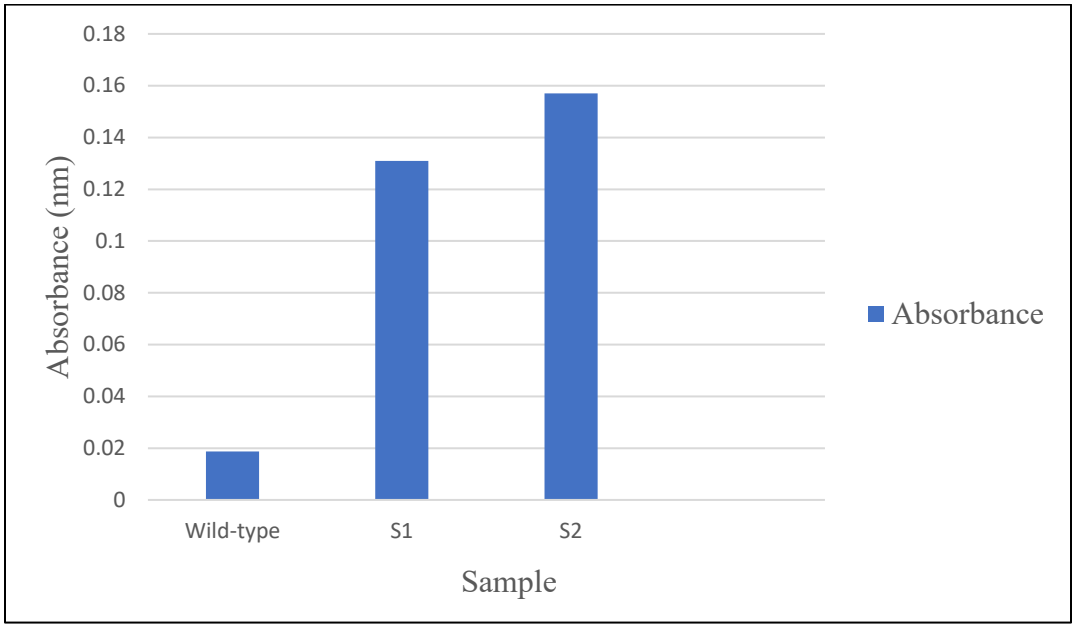


Figure 3.17: Graphical representation of ELISA of wild-type and transgenic *Spinacia oleracea* samples.

CHAPTER # 04

4. DISCUSSION

Vibriosis is caused by Gram negative bacterium *Vibrio anguillarum*. This lethal hemorrhagic septicaemic disease occurs in fish, bivalves and crustaceans. Vibriosis is regarded as major obstacle to aquaculture since it increases the susceptibility of disease outbreaks, resulting in significant economic losses and large-scale deaths worldwide (Irshath *et al*., 2023). Mortality rates range from 30% to as high as 100% (Austin *et al*., 2007).

Use of antibiotics and chemotherapeutics for treatment of vibriosis is linked with serious disadvantages such as drug resistance, antibiotic residues remain in fish muscles; compromising the health of consumers and environment safety (Manyi-Loh *et al*., 2018). Vaccination is the best strategy for development of immune defense mechanisms in fish against pathogenic microorganisms; that attack economically significant aquaculture species. Vaccination is an effective approach for preventing infectious diseases like vibriosis in fish; leading to social, economic and environmental maintenance of aquaculture production (Ma *et al*., 2019).

Outer membrane protein K (pOmpK) is an immunogenic protein found in *Vibrio anguillarum's* outer membrane. It could serve as subunit vaccine candidate for *Vibrio anguillarum*. OmpK stimulates antibody-mediated as well as cell-mediated immunity and can serve as DNA vaccine candidate (Xu *et al*., 2019).

According to World Health Organization (WHO), development of cost-effective vaccines against numerous infectious diseases is required (WHO, 1999). In developing countries, IDs are responsible for high rates of morbidity and mortality. Therefore, more attempts are to be done for fulfilling vaccine demands for these countries. Vaccines currently used against multiple IDs face the problem of safety, high production and distribution costs, lack of scalability and maintenance of cooling chain (Lössl and Waheed, 2011). In order to overcome these hurdles associated with using bacteria, yeast and insects as expression systems for vaccine production, an alternative strategy is required. Plants offer an alternative approach; they can be used as cheap bio-factories for edible vaccine development. Because of their safety, efficacy, cost-effectiveness, ease of administration, and ease of storage, plant-based

vaccines hold great promise (Lal *et al*., 2007). Thus, spinach has been used for expression of OmpK antigen in an attempt to develop cost-effective plant-based edible vaccine.

4.1. Choice of *Spinacia oleracea* for expression of foreign antigens

Plants that have prolonged shelf life, fast growth rate and can be stored for very long time without any degradation are considered the ideal candidates for edible vaccine production (Khalid *et al*., 2022). In present study, *Spinacia oleracea* has been used for producing plant-based edible vaccine. This green leafy vegetable is rich in iron, vitamins and minerals. Its genome has been studied extensively. It grows in a very short time and can be cultivated in all seasons. All these benefits make *S. oleracea* an appropriate plant to be used for the expression of vaccine antigens (Naik, 2022).

4.2. Optimization of tissue culture

Regeneration of explants *in vitro* depends on several factors. These include light, humidity, nutrients, phytohormones, and temperature and pH conditions. Regulated environment and appropriate concentration of PGRs is important for proper callus and shoot formation. Various combinations of PGRs were used to determine their effect on plant growth. Hormones including BAP, IAA, Kinetin and 2,4-D were used. Only leaf explants were used in this study. Two combinations used in present research study were BAP and IAA (1mg/L and 0.5 mg/L) and Kinetin and 2,4-D (2mg/L and 0.5mg/L). Leaf explants excised from spinach plant inoculated on media containing BAP and IAA showed maximum callus formation with efficiency of 66.67%.

4.3. Optimization of hygromycin concentration

In the present study, hygromycin concentration was optimized for the selection of transformed leaf explants of *Spinacia oleracea*. Five different concentrations i.e. 0 mg/L, 15 mg/L, 20 mg/L, 25 mg/L and 30 mg/L were used for optimization purpose. With the increase in hygromycin concentration, increase in bleaching effect was observed. Leaf explants grown on media containing BAP and IAA supplemented with 0 mg/L hygromycin showed prominent callus formation and callus formation efficiency was found to be 100%. As the hygromycin concentration was increased,

more bleaching was observed. 15 mg/L hygromycin concentration showed 40% bleaching effect. 20 mg/L and 25 mg/L hygromycin concentration showed 60% and 80% bleaching effect. Maximum bleaching was observed in leaf explants that were placed on media containing 30 mg/L. 20 mg/L hygromycin concentration was chosen for selection of transformed explants. Similar results were shown by Chin *et al*. (2009).

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

Several factors influence *Agrobacterium*-mediated stable transformation. These factors include optical density of *Agrobacterium* culture, infection time and co-cultivation time. The present study explains the best possible conditions for *Spinacia oleracea*. Optical density was measured at wavelength 600 nm OD_{600}. OD_{600} within range of 0.6-0.8 was found to be effective for transformation and caused enhanced transformation efficiency. Similar results were shown by Niazian *et al*. (2019).

Infection time influences the transformation efficiency. Two different time periods i.e. 8 and 10 minutes were used. 2-3 weeks old *in vitro* grown explants were infected for 8 and 10 minutes, respectively and their callus formation efficiency was calculated. Explants treated for 8 minutes infection time showed higher callus formation efficiency in comparison with explants that were treated for 10 minutes. Hence, it was deduced that 8 minutes was the best infection time. Similar results were shown by Lin *et al*. (2016).

Another factor that affects transformation efficiency is co-cultivation time. 2 and 3 days co-cultivation time was used. Explants with 3 days co-cultivation time showed higher callus formation efficiency of 52.94% compared with explants that were given 2 days co-cultivation time had 15% callus formation efficiency. Hence, 3 days (72 hours) co-cultivation time was more suitable for efficient transformation of *Spinacia oleracea*. Similar results were shown by Gul *et al*. (2015).

Pre-culturing the explants on hormones containing media also enhances the transformation efficiency. Pre-culturing for 1-2 days was found to be beneficial strategy in enhancing the transformation efficiency.

In our study, successful *Agrobacterium*-mediated stable transformation of *Spinacia olercaea* with OmpK antigen was achieved. Leaf explants were treated for 8 minutes with *Agrobacterium* containing *OmpK* gene. After infection, explants were co-cultivated on BAP and IAA containing media supplemented with 200µM acetosyringone, kept in dark for 72 hours (3 days). After co-cultivation time, explants were shifted to selection media. The selection media containing 20 mg/L hygromycin was also supplemented with 400 mg/L cefotaxime, which prevents *Agrobacterium* overgrowth.

Several molecular techniques that were used for confirming successful transformation and transgene integration include PCR, qRT-PCR, Dot blot, Western blotting and ELISA. PCR was performed using *OmpK* specific primers. Wild-type samples were used as negative control. The desired size of amplicon was 500 bp using *OmpK* internal forward and reverse primers. *OmpK* nuclear forward and reverse primers gave amplified product of 890 bp. No band was observed for wild-type samples.

To determine the copy number of transgene, qRT-PCR was performed in accordance with Wen *et al*. (2012) protocol. Copy number of transgene was calculated in comparison with copy number of endogenous gene *β-actin*. Copy number of *OmpK* transgene was found to be 1.

We performed Western blotting and Dot blot for validating the protein expression. Total soluble protein was isolated, SDS-PAGE was run, protein was transferred from gel to nitrocellulose membrane, blot was incubated with primary and secondary antibody and finally with substrate. Upon blot visualization, transgenic samples showed chemiluminescence signal unlike wild-type sample. Dot blot analysis gave the same results. Positive results were obtained for stably transformed explants.

ELISA was also performed to measure the antigenic protein present within samples. Protein samples from stably transformed explants showed a color change when TMB substrate was added unlike blank and wild-type samples, which confirmed the presence of OmpK antigen within transgenic sample. It was observed that S2

transgenic sample showed higher absorbance at 595 nm compared with S1, indicating that S2 contains more concentration of antigen.

4.5. Conclusion and Future Perspectives

We have optimized several factors that are important for *Agrobacterium*-mediated stable transformation of *Spinacia oleracea*.

- Full MS media containing 1 mg/L BAP and 0.5 mg/L IAA could be used for good callogenesis of spinach leaf explants.
- Hygromycin concentration for selection of transformed leaf explants was optimized.
- Parameters like co-cultivation period, infection time and OD_{600} of *Agrobacterium* culture was optimized for successful stable transformation of *Spinacia oleracea*.

The present study provides optimized parameters for *Agrobacterium*-mediated stable transformation of *Spinacia oleracea*. Full MS media containing 1 mg/L BAP and 0.5 mg/L IAA gave good callus formation efficiency. 20 mg/L hygromycin was found to be the best concentration for selection of transgenic leaf explants. 3 days co-cultivation period and 8 minutes infection time was shown to enhance the transformation efficiency. Taken together, this study can prove beneficial for development of cost-effective edible vaccine against *Vibrio anguillarum* that can prevent the risk of fish vibriosis.

CHAPTER # 05

5. REFERENCES

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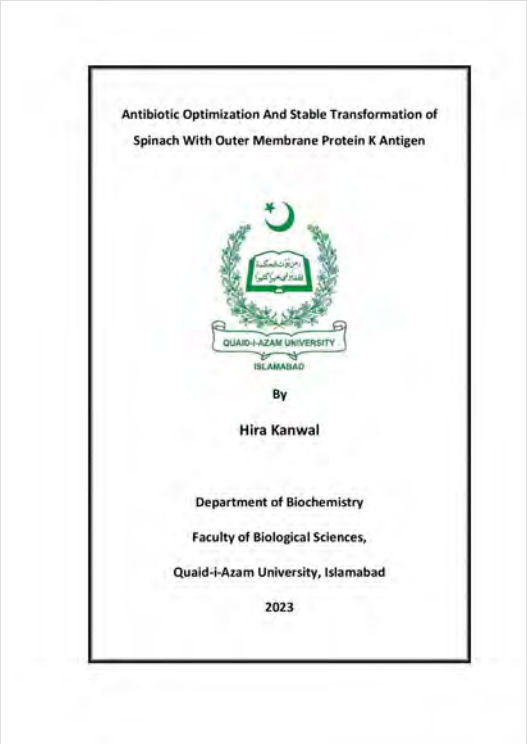

Antibiotic Optimization And Stable Transformation of Spinach With Outer Membrane Protein K Antigen

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