Agrobacterium-mediated Transformation of Spinach using Outer Membrane Protein C (*OmpC*) gene from *Salmonella*

By

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In

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CERTIFICATION

This is to certify that this thesis entitled, **"*Agrobacterium*-mediated Transformation of Spinach using Outer Membrane Protein C (*OmpC*) gene from *Salmonella*"** is submitted by **Fatima Khalid.** It is accepted in its present form by the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirements for the degree of Master of Philosophy in Biochemistry/Molecular Biology.

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Declaration of Originality

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

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

Fatima Khalid

This thesis is dedicated whole-heartedly to

My Loving Parents

&

Husband

For their endless love, affection, support and encouragement

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LIST OF ABBREVIATIONS

A	Alpha
APCs	Antigen presenting cells
APS	Ammonium persulphate
B	Beta
BAP	Benzyl amino purine
BCG	Bacille Calmette-Guerin
Bp	Base pair
BSA	Bovine serum albumin
°C	Degree centigrade
CIN	Chromosomal Instability
cm	Centimeter
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribo-nucleic acid
d.H_2O	Distilled water
DMSO	Dimethyl sulfoxide
EDTA	Ethylene Diamine tetra acetic acid
EKT	Extracellular signal-regulated kinase
F	Forward
g	Gram
ϒ	Gamma
GALT	Gut associated lymphoid tissue
HEPA	High Efficiency Particulate Absorption
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
IAA	Indole acetic acid
IBDs	Inflammatory Bowel Diseases
IDs	Infectious diseases
IgA	Immunoglobin A

IgE	Immunoglobin E
IgG	Immunoglobin G
kb	Kilo base
KCl	Potassium chloride
L	Litre
LA	Luria-Bertani Agar
LB	Luria-Bertani broth
LFH	Laminar Flow Hood
LS	Linsmaier and Skoog
M	Molar
Mg^{+2}	Magnesium ion
mg	Milligram
mM	Millimolar
mg/ml	Milligram/milliliter
mg/mol	Milligram/mole
MS	Murashige and Skoog
MSI	Microsatellite Instability
N	Normal
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NLS	Nuclear localization signal
NN	Nitsch and Nitsch
OD	Optical density
OMPs	Outer membrane proteins
OmpC	Outer membrane protein C
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGRs	Plant growth regulators

pH	Power of hydrogen ions
Psi	Pounds per square inch
qRT-PCR	Quantitative real time PCR
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SHM	Schenk and Hildebrandt medium
SQend	Starting quantity of endogenous gene
SQtrans	Starting quantity of transgene
TBE	Tris Boric Acid EDTA
TBS-T	Tris buffered saline with tween
T-DNA	Transfer deoxy ribonucleic acid
TE	Tris EDTA
Tm	Melting temperature
TMB	3,3',5,5'-Tetramethylbenzidine
Ti plasmid	Tumor inducing plasmid
TSP	Total soluble protein
µl	Micro liter
µm	Micro meter
UV	Ultra-violet
v/v	Volume per volume
Vir	Virulence
WPM	Woody plant medium
WT	Wild type
w/v	Weight per volume

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ABSTRACT

Salmonellosis is a bacterial infection caused by *Salmonella*. Severe *Salmonella* infections lead to the development of colon cancer. The currently available vaccines against this infection are Ty21a and Vi CPS, which work only against *Salmonella typhi* and not the others serovars of *Salmonella*. Outer membrane protein C (OmpC) is an immunogenic protein of *Salmonella* and is a potential candidate for the development of sub-unit vaccine. Plants can be used as bio-factories for the 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 OmpC 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. 20 mg/L concentration of hygromycin antibiotic was found optimum for selection of transformed leaf explants. Successful stable transformation of spinach was carried out with OmpC antigen. Transformed explants were grown on selection media containing suitable concentration of hygromycin. Explants with 3 days co-cultivation time showed 50% 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 and copy number calculated was 1. Protein expression was confirmed through dot Blot, western blotting and ELISA. Taken together, successful expression of *Salmonella* OmpC antigen in plants could facilitate the development of edible and cost-effective subunit vaccine against salmonellosis.

Keywords: *Spinacia oleracea*, *OmpC* gene, Salmonellosis, *Agrobacterium*-mediated stable transformation, PCR, qRT-PCR, Dot blot, Western blotting

1. Introduction

1.1 Infectious Diseases

Diseases caused by pathogenic microorganisms such bacteria, fungi, viruses and worms are called Infectious Diseases (IDs). IDs continue to be a significant contributor of morbidity and mortality globally (Kundu *et al*., 2018). A healthy host immune system effectively suppresses the signs of disease in many circumstances (Selgelid and Enemark, 2008). Immune system recognizes pathogen associated molecular patterns (PAMPs) and anti-microbial and pro-inflammatory responses are released due to activation of signal transduction pathway (Lee *et al*., 2018). However, illnesses may appear when an infectious agent overwhelms the host's immune system or when it is weakened (Kurup and Thomas, 2020).

Worldwide, vaccination has saved countless lives by preventing the spread of numerous infectious illnesses. It is considered as a key tool in public health (Plotkin and Plotkin, 2012). Extensive research has been going on to develop vaccines against novel targets. Clinical trials are also in progress to develop cost-effective vaccines (Wagner and Weinberger, 2020).

1.2 Salmonellosis

Salmonellosis is a bacterial infection caused by *Salmonella* bacteria. The bacterium resides in the intestines of humans, animals and birds. Infection occurs by consuming contaminated food or water (Budd, 1918). Individuals with weakened immune system have high susceptibility to death (Spier, 1993). Salmonellosis occurs worldwide and approximate 95% cases are caused by contaminated food (Acheson *et al*., 2001). Estimated 1.5 million people suffer from this infection in US annually. The antimicrobial-resistant strains of *Salmonella* occur to be a serious threat to public (Frey *et al*., 2023). The bacteria enter the digestive tract and replicates inside the intestinal tissues causing the infection. This disease affects both children and adults (Goldberg & Rubin, 1988).

1.2.1 *Salmonella*

Salmonella belongs to the *Enterobacteriaceae* family. It was named after the scientist Daniel E. Salmon. It is a non-spore forming, aerobic, motile, rod-shaped (also known as bacilli) gram-negative bacteria (Lüderitz *et al*., 1966). The average size of rod ranges

from 0.7-1.5 by 2.0-5.0 µm. The bacterium is enveloped containing lipopolysaccharides functioning as an endotoxins along with outer membrane protein or porin C (Winfield and Groisman, 2003). Endotoxins are involved in causing disease, mostly toxicity is caused by presence of Lipid A moiety (Spier,1993). It is capable of replicating in a wide variety of cells, including phagocytic and epithelial cells. *Salmonella* lives inside host as facultative intracellular parasite (Finlay and Fallow, 1989). Although salmonella prefers dry environments, it may survive for several months in damp conditions as well (Wibisono *et al*., 2020).

The genus *Salmonella* contains two species: *S. enterica* (divided into 2000 serovars) and S. *bongori*. Typhoid fever is known to be caused by *S. typhi* which is *S. enterica* serovar. However, gastroenteritis has been linked to *S. typhimurium*. Certain serovars like *S. typhi*, only infect humans; however, *S. typhimurium* infects both humans and various animals (McClelland *et al*., 2001).

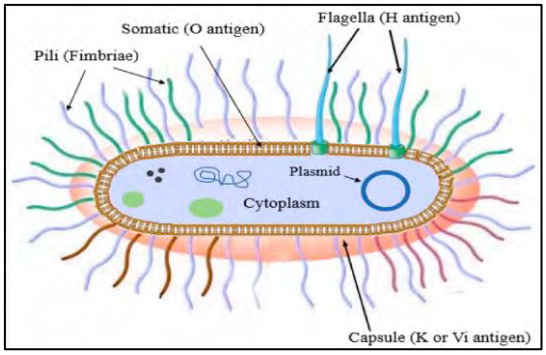

Figure 1.1: Schematic illustration of *Salmonella* (Adapted from Teklemariam *et al*., 2023).

1.2.2 Signs and symptoms of salmonellosis

The common signs and symptoms of salmonellosis are diarrhoea, abdominal cramps, fever, nausea, vomiting, chills, headache and blood in the stool. After infection, symptoms take at least a week to appear. These are only mild symptoms that occasionally occur, but they have the potential to get worse and lead to infections of the urinary tract, bones, and nervous system (Lundberg *et al*., 1999).

1.2.3 Mode of transmission of *Salmonella*

Salmonella is commonly transmitted through contaminated foods such as raw or undercooked eggs, poultry, meat and unpasteurized dairy products. Infection can also transmit through pets' contact (Sanyal *et al*., 1997). Historical analysis reveals that the

bacterium can thrive in a variety of food processing environments. When infected water, soil or surfaces come into contact with fruits and vegetables, they may also get contaminated. Drinking contaminated water is also another way through which bacteria can transmit (Levantesi *et al*., 2012). Humans come into contact with *Salmonella* as it can be found in a variety of foods, including eggs, poultry, raw dairy products, sprouts, lemons, mangoes, chocolate, and dry almonds (Waldner *et al*., 2012).

1.2.4 Vaccines against Salmonellosis

Given that salmonellosis spread worldwide, the high number of death cases reported lead to the various strategies for developing a suitable vaccine against the infection. The vaccination can induce cell-mediated and humoral responses but it is not always associated with the resistance against re-infection (Mastroeni *et al*., 1993). Due to its facultative intracellular pathogenic nature, the bacterium is able to survive extracellularly and also intracellularly in monocytes and macrophages. This makes it difficult for vaccine developers as cellular immunity mediated by both $CD4^+$ and $CD8^+$ T cells is necessary to eradicate bacteria intracellularly while humoral immunity is essential for dealing with bacteria extracellularly (Mastroeni *et al*., 2001).

1.2.4.1 Currently available vaccines

Systemic vaccination strategies have been available recently for these infections. There are two widely accessible vaccines that have been approved for use against *Salmonella* (Fraser *et al*., 2007). There are two vaccines currently available in market that provides resistance against *S. typhi* and there are none available against other three invasive serovars of *Salmonella enterica*, Paratyphi A, Typhimurium and Enteritidis (MacLennan *et al*., 2014).

These are live attenuated vaccines. *Ty21a* (Vivotif) is given orally and the other Vi capsular polysaccharide (Vi CPS) vaccine (Typherix or Typhim Vi) is given in the form of injections. The *S. typhi Ty2* strain undergoes nonspecific chemical mutagenesis, which results in the formation of a mutant galE and the production of the *Ty21a* vaccine. The galactose derivatives accumulate in the host cell due to the deficiency of UDP-galactose-4-epimerase in the mutant and as a result breakdown of bacterial cells take place. The Vi CPS vaccine derived *from S. typhi* through its purified Vi polysaccharide stimulates the production of T-cell independent antibody and this results in the maturation of class switch and affinity (Engels *et al*., 1998).

In recent years, there has been a notable advancement in utilizing *Salmonella* vaccines as a means of delivering DNA vaccines. The use of bivalent *Salmonella* strains for DNA vaccine delivery may enable the induction of immunity not only against the *Salmonella* carrier itself but also against the heterologous antigen(s) expressed by the *Salmonella* and the antigen(s) encoded by the DNA vaccine. As of now, this technology appears highly promising and presents a novel method for the delivery of mucosal vaccines (Garmory *et al*., 2002).

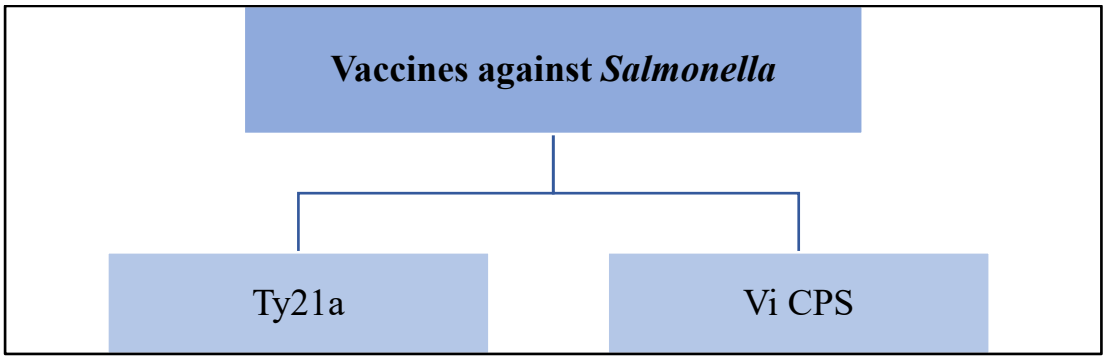

Figure 1.2: Vaccines against *Salmonella*

1.3 Colon Cancer

The uncontrolled growth of cell in colon or rectum is termed as colon cancer. It is also known as colorectal, bowel or rectal cancer. Nearly, 1 million people worldwide lose their lives to this cancer. It is the third most common cancer and one of the main causes of cancer-related mortality (Siegel *et al*., 2023). It is caused by the colon's aberrant proliferation of glandular epithelial cells that results in the development of basic polyp and then adenomatous polyps. The likelihood of polyps or adenomas developing into cancer grows with age. There are many types of colon cancer such as sporadic, hereditary and colitis-associated colon cancer (Greenwald, 1992).

There are many causes of colon cancer such as sporadic mutations, cigarette smoke, alcohol consumption, diet high in red and processed meat, chronic inflammation in Inflammatory Bowel Disease, microsatellite instability, family history, genetics and lifestyle etc (Fearon and Vogelstein, 1990). A small percentage of cancer cases are associated with bacterial infections; viruses account for the majority of cancer cases (Scherer *et al*., 2014). Bacterial infections cause specific types of cancer through inflammation, DNA damage by toxins, metabolites, altering host signal transduction during their infection cycle (Chumduri *et al*., 2016).

1.3.1 Signs and symptoms of colon cancer

The signs and symptoms of colon cancer are not noticeable in the early stages. However, as the cancer progresses, different symptoms may appear. The early cancer symptoms are mostly non-specific such as fatigue, abdominal pain, nausea, vomiting and weight loss etc. The secondary or late-stage colon cancer symptoms are more specific and are easily diagnosed. These include chronic fatigue and weakness, Inflammatory Bowl Diseases (IBDs), palpable mass in abdomen, perforation leading to bleeding and high-grade fever (Holtedahl *et al*., 2021).

1.3.2 Development of colon cancer

Colon cancer is genetically diverse and there are numerous unique mechanisms which play a role in its development. The hyperproliferation of epithelial cells due to genetic and epigenetic modifications is one of the major factors involved (Testa *et al*., 2020). These irregularly dividing cells form a benign adenoma, which may develop into cancer and spread to other areas of the body through a number of different mechanisms such as microsatellite instability (MSI), chromosomal instability (CIN) and serrated neoplasia (Malki *et al*., 2020; Katsumata *et al*., 2021).

The development of colon cancer is also facilitated by the severe salmonella infections. *Salmonella* in its host cell secrete effector proteins which excites certain pathways like AKT and ERK. These pathways play a crucial role in cancer development in pretransformed cells. Another effector AvrA is involved in exciting beta-catenin signalling that leads to colon cancer development in host cell (Lu *et al*., 2016; Duijster *et al*., 2021).

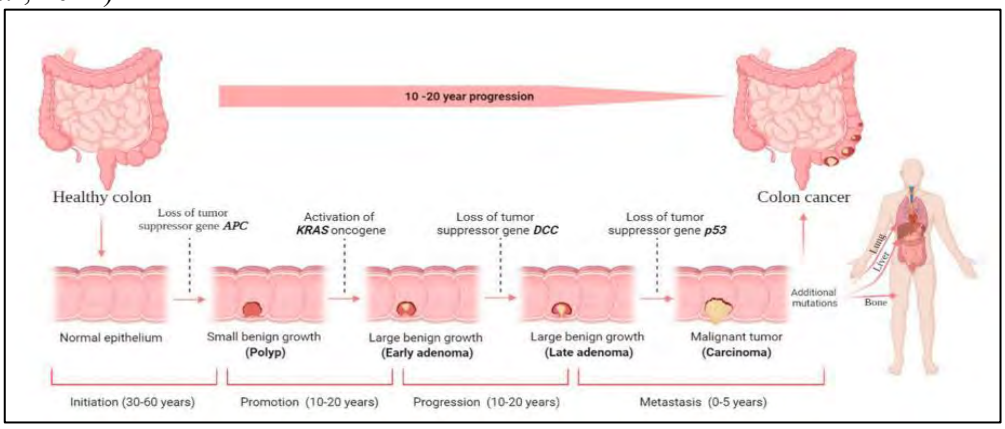

Figure 1.3: Developmental stages of colon cancer (Adapted from Hossain *et al*., 2022).

1.4 Outer Membrane Protein C (*OmpC*)

Outer membrane proteins (OMPs) are immunogenic proteins present on the outer membranes of gram-negative bacteria. They serve many functions such as maintaining integrity, providing semi-permeability, bacterial colony formation, protection from toxins, biles and pathogens (Huzmi *et al*., 2019). Due to presence on the outer surface, these proteins are easily recognizable by the host immune system and thus considered as highly immunogenic (Xu *et al*., 2019).

Outer membrane protein C is a porin found in the outer membrane of *Salmonella enterica* serovar *typhi*. This serovar causes typhoid fever in humans. During pathogenesis, *OmpC* is stimulated and hence it is considered as a best suitable candidate for the development of vaccine against this infection. *Salmonella* porins lead to the stimulation of both humoral as well as cell mediated immunity (Toobak *et al*., 2013). *OmpC* is a 43kDa recombinant protein. It exists in the form of a homotrimer. *OmpC* possess unique epitopes on its surface (Agarwal *et al*., 2010).

A study conducted in 2009 showed that in murine models, the denatured forms of recombinant OmpC of *S. typhi* acts as immunogenic proteins. Despite recombinant OMPs' high immunogenicity, there is no proof of their protective immunogenicity (Verma *et al*., 2009). In several studies, researchers have created multivalent subunit vaccines by combining various immunogenic antigens with *OmpC* to boost the protective impact of subunit vaccines on bacteria (Kumar *et al*., 2005).

1.5 Plant Biopharming

Plant biopharming commonly referred as molecular pharming involves the production of biological pharmaceuticals and therapeutics by using living systems as host. The living hosts help in producing economical biological products that are difficult to synthesize *in vitro* (LeBlanc *et al*., 2020). Recent developments in molecular biology and plant biotechnology includes the usage of transgenic plants (plants that have undergone genetic modification) as bioreactors for the production of recombinant therapeutic proteins (Bergougnoux, 2014).

The first biopharming procedure included the production of chimeric human growth hormone *via* tobacco and sunflower (Barta *et al*., 1986). Over the last 20 years, plants have been used to generate large amounts of pharmaceuticals, drugs, vaccines, monoclonal antibodies, and immunomodulatory proteins through the use of transient

expression systems and molecular farming (Yemets *et al*., 2014). Different types of plants have been transformed such as tobacco, maize, lettuce, barley, tomato, rice, broccoli, pitcher plants, carrot, and soybean (Kim *et al*., 2012; Miguel *et al*., 2019).

There are many benefits of using plants in biopharming as they are affordable and cheap. The infrastructure cost is minimized and there are simple requirements for biomass expansion ((Dhama *et al*., 2020).

1.6 Edible Vaccines

Plant-based edible vaccines are recombinant protein vaccines that are given orally to elicit protective immunity against specific infections. These vaccines use specific antigens produced by specific plant species (Lössl and Waheed, 2011; Naik, 2022). It has now become a common practice to develop edible vaccines against human and animal diseases. They have shown potential in the treatment of cancer, autoimmune disorders, infectious diseases, and birth control (Lal *et al*., 2007).

Using genetic engineering techniques, the genes encoding the disease-specific antigens are expressed into plant hosts to create plant-based vaccines. Plants can be directly injected with genes or they can undergo *Agrobacterium*-mediated transformation (Kurup, 2020). Genetically modified plants such as 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). The method of producing an edible plant-based vaccine that can produce an immune response is demonstrated in Figure 1.3.

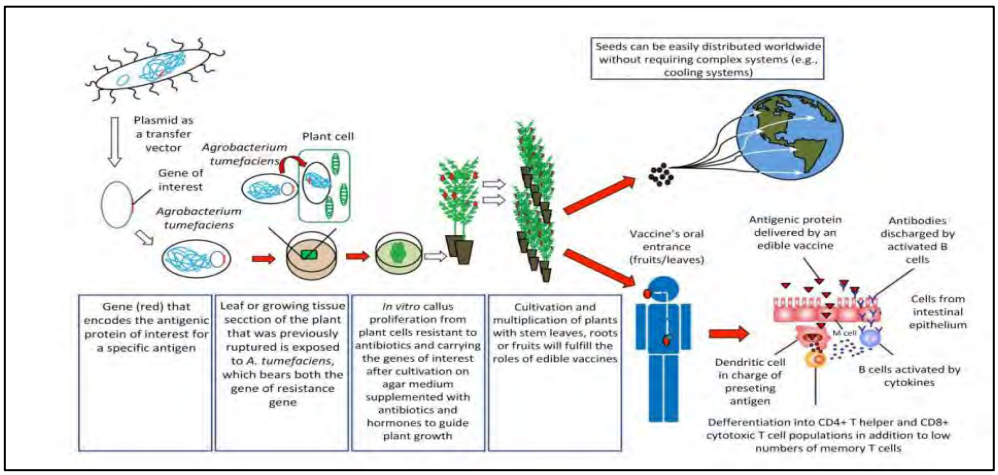

Figure 1.4: Method of producing plant-based edible vaccines and its immune action (Concha *et al*., 2017).

1.6.1 Immunological Mode of Action of Edible Vaccines

Oral administration of edible vaccines mimics the natural course of infection. The plant-based vaccines can stimulate both mucosal as well as systemic immunity as compared to the conventional vaccines (Koo *et al*., 1999). The bio-encasement and direct ingestion of plant components protect the antigen from intestinal and gastric secretions, as well as from enzymatic degradation (Gunasekaran and Gothandam, 2020). When they reach the digestive tract, the cell wall breakdowns and antigens are released. The M cells of Peyer patches and GALT (gut-associated lymphoid tissue) then take up antigens and move them to macrophages and Antigen Presenting Cells (APCs), which exhibit them to helper T cells (Th cells). Th cells play a role in the activation of B cells which release IgA, IgE, IgG antibodies and memory responses (Buonaguro and Butler-Ransohoff, 2010; Streatfield, 2006). Through the epithelial cells, these antibodies penetrate the lumen's secretions and bind to the antigens present there. Consequently, neutralization of antigens occurs in a manner similar to traditional vaccine processes (Sharma and Sood, 2010).

1.6.2 Advantages of plant-based edible vaccines

There are several advantages of using edible plants as a food source and developing vaccines for both humans and animals. The cost-effectiveness of plant-based systems is their main benefit. As opposed to expensive equipment and the infrastructure of labor-intensive fermenter-based systems, plants can be grown and scaled up as needed (Waheed *et al*., 2016). The maintenance cost of cooling chain, transportation and storage has been reduced due to the stability of antigens in plant-based system (Kolotilin *et al*., 2014). Compared to injections, oral administration is more practical and suitable for young patients. The use of syringes in conventional vaccination raises the risk of environmental contamination and second-hand ailments (Sohrab, 2020). Moreover, the rigid cell wall provides protection against the gut's acidic environment (Sack *et al*., 2015). The chances of contamination due to pyrogens and endotoxins is very low thus there is no requirement for downstream processing and stringent purification (Waheed *et al*., 2015). The transport mechanism can potentially be eliminated if naturally occurring food plants are altered to produce a particular vaccine. Transgenic seeds may be easily preserved for a long time period at room temperature and are also widely available as a source of vaccines (Mičúchová *et al*., 2022).

1.6.3 Disadvantages of plant-based edible vaccines

There are many challenges that are associated with edible vaccines. There is a need for optimal dose concentration as antigen can be expressed differently in different part s of plants (Appaiahgari *et al*., 2017). The ripeness, size of fruit and plant can lead to differentiable protein proportions (Tripurani *et al*., 2003). Plant shelf life is very crucial. In order to eliminate infection, proper storage of plant is required (Richter and Kipp, 1999). Plant-based expression also raises the risk of contaminating naturally occurring non-transgenic plants (Malabadi *et al*., 2015). The expression of antigens in chloroplast can solve this issue as there is minimal chance of transgene dispersal through pollens (Waheed *et al*., 2016). Regarding the acceptance of plant-based vaccinations, human perception and environmental concerns should also be taken into account**.**

1.7 Biotechnology and Plants

Biotechnology is defined as the use of living organisms or their derivatives to enhance human health and the environment. It involves variety of methods to utilize biological organisms along with their systems and processes for the production of valuable products (Gibbs *et al*., 1983; Verma *et al*., 2011). The term "Biotechnology" comprises of two scientific words biology and technology and this term was coined by Hungarian Engineer Karl Ereky in 1919 (Ereky, 1919). The main concept of biotechnology includes the isolation of gene of interest from an animal's cell and its insertion into a vector which then introduces the gene of interest into an expression system which can be animal, plant or microbe. This method is also known as genetic engineering or recombinant DNA technology (Squires and Slotin, 1984).

Plant biotechnology is the most dynamic and exciting domain of genetic engineering. It is also termed as Green Biotechnology (Wiel *et al*., 2016). The plants act as bio-factories for the production of vaccines, growth hormones, antibodies, subunits, antigens and enzymes through genetic manipulation (Sohrab *et al*., 2017). The production of goods and crops through agriculture is a form of old plant biotechnology (Boulter, 1995). Three research groups were able to explain *Agrobacterium*-mediated transformation in 1983, which changed the course of modern plant biotechnology (Vasil, 2008). To produce a plant-based vaccine, a gene of interest encoding an antigen protein for a given disease is incorporated into the plant genome through different

techniques (Laere *et al*., 2016). The transgenic plants are used for production of bacterial, viral, parasite and immune-contraceptive vaccines (Guan *et al*., 2013).

1.8 Plant Tissue Culture

Plant tissue culture is defined as the production of cells, tissues, organs and entire plant within sterilised and regulated environment in a growth chamber (Ahloowalia *et al*., 2004). Haberlandt, a German researcher is regarded as the father of tissue culture. He explained the theory of single-cell culture and totipotency (Krikorian & Berquam, 1969). According to Haberlandt, plants with separated cells naturally have the ability to regrow into whole plants (Haberlandt, 1902).

In this process, plant tissues are removed and are grown on specific culture media. Part of plant that has been cut and transferred to nutrient media is referred to as explant. Different parts of plants can be used such as leaf disks, roots, shoot tips, cotyledons, hypocotyls, axillary buds or zygotic embryos (Kumar and Loh, 2012). Other names for plant tissue culture include axenic, in vitro, cell and sterile culture (Thorpe, 2007). The culture media provides all the basic nutrients, hormones, water and energy for the tissue to grow into a plant (García-Gonzáles *et al*., 2010).

Large scale production and multiplication of plants is done by using plant tissue culture. Additionally, it has a wide range of industrial uses in the areas of plant propagation, secondary metabolite synthesis, disease prevention and the development of improved crops (Gamborg *et al*., 1976; Hussain *et al*., 2012).

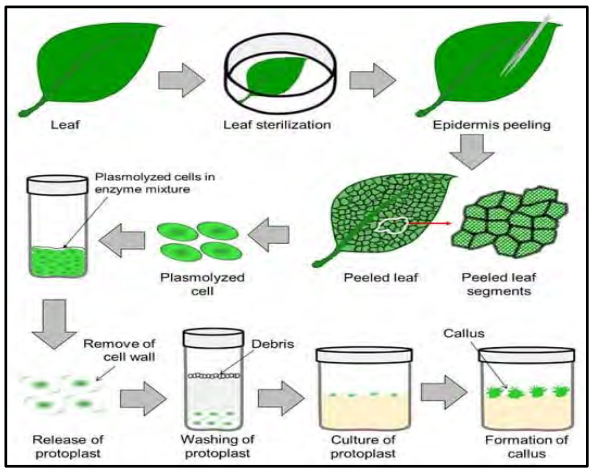

Figure 1.5: Plant Tissue Culture (Adapted from Mehbub *et al*., 2022).

1.8.1 Explants

An explant is a small part of plant that is grown in culture media and has full regeneration potential to grow into a whole plant. It can be axillary buds, cotyledons, hypocotyls, roots, shoot tips, zygotic embryos or leaf discs (Idowu *et al*., 2009). Explants are handled with care and are provided optimized environmental conditions along with required hormones and nutrients required for their growth (Gasic and Korban, 2006).

In plant transformation, the size of explant, type, age, hormones and nutrients concentration play a very important role (Chakraborty *et al*., 2020). In case of cotyledon explants, the plant should be 3–6 days old for the best results in terms of shoot regeneration (Goh *et al*., 1990). The ideal material for regeneration when leaves are the source of explants is the first fully grown leaf (Sharma *et al*., 1990). These explants can grow into a full new plant in cultured media within growth chambers irrespective of external environment conditions.

1.8.2 Tissue Culture Media

Certain types of culture media along with optimized environment, temperature and light conditions are required for the regeneration of tissue-cultured plants. The culture media contains all the essential micro- and macronutrients for the metabolism, growth and development of plant (Beyl, 2005). The basal media includes all the necessary nutrients, water and energy required for the growth of plant (Phillips and Garda, 2019).

There are many different mineral formulations available for the cultivation of plant tissues. Gamborg's B5 medium and B5 modifications (Gamborg *et al*., 1968), Murashige and Skoog media (MS) and modified MS media (MMS) (Miller *et al*., 1955), and LS (Linsmaier and Skoog, 1965), Woody Plant Medium (WPM), Driver and Kuniyuki Woody plant medium (DKW) and Schenk and Hildebrandt medium (SH medium) are examples of the major media. Carbohydrates such as sucrose are added to plant tissue culture media. MS media is now the most widely used medium for plant tissue culture (Vasil, 2008).

1.8.3 Growth Regulators of Plants

Plants produce organic compounds called plant growth regulators which influence their physiological functions, metabolic and developmental responses. They are also termed as plant growth substances, bioregulators or phytohormones (Gaspar *et al*., 1996). They

are synthetic as well as naturally occurring substances and are formed at various morphological and cytological locations (Evans *et al*., 1981).

Phytohormones affect different aspects of plant's life and parts such as flowering, aging, root growth, organ deformation and death, stem lengthening, fruit colour enhancement, stress tolerance and moisture relations in plants, maturity, disease resistance, fruit formation, maturation and many other quality characteristics (GRAHAM and Ballesteros, 1980; Harms and Oplinger, 1988).

The five main classes of naturally occurring phytohormones are Auxins, Cytokinin, Gibberellins, Abscisic acid, and Ethylene (Phillips & Garda, 2019). On the basis of their function, they are broadly divided into two categories i.e., growth promoters and inhibitors (Roberts and Hooley, 1988). Plant growth promoters that stimulate cell division, cell expansion, flowering, fruiting and seed development include auxins, gibberellins and cytokinin. Plant growth inhibitors contain abscisic acid, which encourages plant abscission and dormancy while hindering plant development (Bons and Kaur, 2020).

1.9 Plant Transformation

Plant transformation is defined as the insertion of foreign DNA into plant genome in order to generate a transgenic plant (Birch, 1997). Using this method, a desired gene with a beneficial function is isolated, ligated using an appropriate vector to produce a recombinant DNA molecule, and then the gene is inserted into the plant to develop a transgenic plant. This helps in obtaining the desired characteristics (Gheysen *et al*., 1998).

The process of transforming plants began in the late 1800s. The first incident of plant transformation was reported by three research groups in 1983. They utilized *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid for this purpose (White *et al*., 1985). The first attempt at plant transformation was reported for tobacco in 1984 (DeBlock *et al*., 1984; Horsch *et al*., 1984; Paszkowski *et al*., 1984). This method has since been extended to 35 families of plant species (Keshavareddy *et al*., 2018).

Gene expression in plant cells can be genetically altered to occur either permanently or temporarily. The transmitted gene is called 'transgene' and the organisms which are produced by successful gene transfer are called "transgenics". Through plant genetic transformation, advantageous genes for horticulture or agriculture can be directly

inserted into suitable plants, creating new, genetically modified crops (Babaoglu *et al*., 2000).

1.9.1 Methods of Plant Transformation

For successful transfer of foreign genetic material into the particular host organism, various strategies are being used**.** Two main techniques of gene transfer include:

- Direct Gene Transfer
- Indirect Gene Transfer

In direct method, DNA or RNA is inserted into the plant genome through physical and chemical procedures. Gene can be directly transferred through many procedures such as particle bombardment (biolistics), electroporation, protoplast transformation, microinjection, silicon carbide fibre, polyethylene glycol method (PEG method) and whisker-mediated transformation (Keshavareddy *et al*., 2018; Ozyigit, 2020).

In indirect method, the foreign genetic material is introduced through methods such as *Agrobacterium* and viral vector-mediated transformations. *Agrobacterium tumefaciens* is used as a vector for *Agrobacterium*-mediated stable as well as transient nuclear transformation (Hwang *et al*., 2017; Ozyigit, 2020). It has wide range of applications in pharmaceuticals including the human therapeutic proteins, vaccines and antibodies. It can also control metabolic pathway and thus helps in increasing the plant yield (Hansen and Wright, 1999; Newell, 2000).

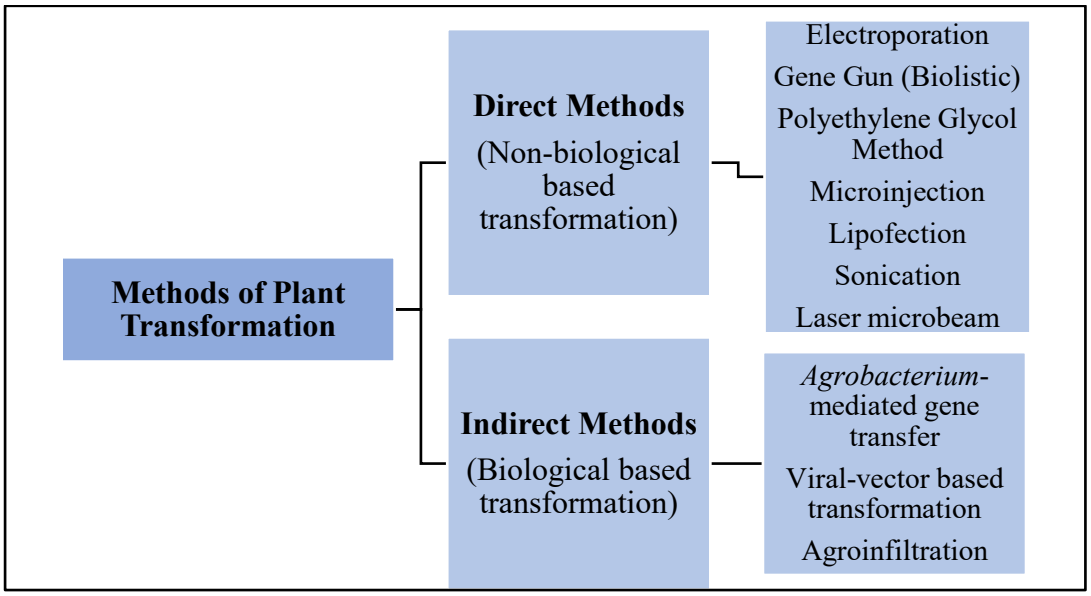

Figure 1.6: Methods of plant transformation (modified from Saifi *et al*., 2020)

1.9.2 *Agrobacterium*-mediated Transformation

Agrobacterium tumefaciens is used as a vector for the insertion of foreign gene of interest into host plant genome. It transfers the specific DNA (T-DNA) of tumor inducing (Ti) plasmid into the nucleus of infected cells (Chilton *et al*., 1977). It has extensive applications in the research. The insertion of T-DNA occurs precisely, and transformation efficiency is fairly high (Iwakawa *et al*., 2021). There are two types of potential pathways for transformation:

- Stable Transformation
- Transient Transformation

The difference between these two types of transformation is that in stable nuclear transformation, the genetic material is transferred inside the nucleus and it becomes integrated into the host cell genome and is successfully transmitted to next generations. In transient transformation, the transferred DNA resides in the host genome for a short period of time and is not passed to the next generations (Krenek *et al*., 2015; Hwang *et al*., 2017).

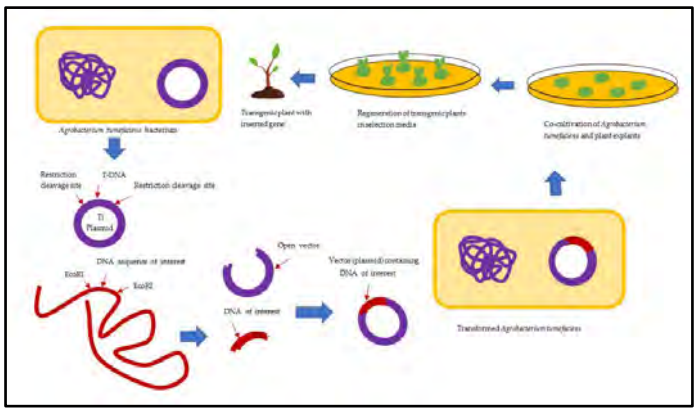

Figure 1.7: *Agrobacterium*-mediated plant transformation (Adapted from Ghimire *et al*., 2023).

1.9.2.1 Stable Nuclear Transformation

This transformation process involves the insertion of transgene (foreign DNA) into the host cell' nucleus. The transgene is passed down to the following generations in a stable way (Gelvin, 2010). *Agrobacterium* is commonly used for this purpose because this method is very simple and cost-effective (Hansen and Wright, 1999). This technique can insert up to 150kb segment of DNA into plant genetic makeup. Target protein can be produced in large quantities by using the transgenic seeds (Davies, 2010).

1.9.2.1.1 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a rod-shaped gram-negative soil bacterium. It belongs to the class *Alpha proteobacterium* of the family *Rhizobiaceae.* This phytopathogenic bacteria causes crown gall disease in many plant varieties (Zupan *et al*., 2000; Anand *et al*., 2008). The unique and highly developed process of *Agrobacterium* pathogenesis includes the transfer of genes between the bacteria and plant kingdom (Escobar and Dandekar, 2003). The oncogenic property of this bacterium is due to the presence tumor-inducing (Ti) plasmid. Ti possesses transferred DNA (T-DNA) which is then stably integrated into the plant nuclear genome and causes crown gall or tumor (Hooykaas and Beijersbergen, 1994; Niazian *et al*., 2017).

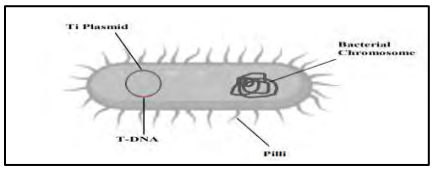

Figure 1.8: Structure of Agrobacterium tumefaciens (Ali *et al*., 2010).

1.9.2.1.2 Ti Plasmid in *Agrobacterium tumefaciens*

Ti plasmid is an extrachromosomal DNA found in the pathogenic strains of *Agrobacterium* such as *A. tumefaciens, A. rhizogenes, A. rubi* and *A. vitis*. It is approximately 200 kb in length. Researchers have taken advantage of its ability to genetically alter the host plants in order to introduce desired genes into plants (Nester, 2015).

The two important components of Ti plasmid involved in transformation are transferred DNA (T-DNA) and virulence (*vir*) region (Ziemienowicz, 2014). Vir region contains genes that encode proteins involved in the transfer of T-DNA into the host plant genome. There are many different loci located on the vir region such as virA, virB, virC, virD, virE, virF, virG. VirA and virG play a primary role in regulating gene expression (Hwang *et al*., 2017). Figure 1.8 shows the diagrammatic representation of Ti plasmid.

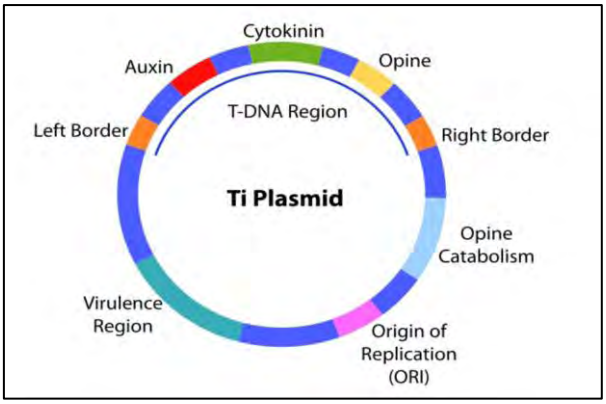

Figure 1.9: Diagrammatic representation of Ti plasmid (Alkuddsi *et al*., 2014).

1.9.2.1.3 T-DNA

T-DNA is flanked by two direct repeats of 25 bp sequence called the T-DNA right and left borders. It is integrated into the plant cell through *Agrobacterium*-mediated transformation. Two different kinds of genes present in T-DNA are the oncogenes and opine producing genes (Barampuram and Zhang, 2011). T-DNA encodes enzymes for the synthesis of opines that act as major nitrogen source (Savka *et al*., 2002). These enzymes also synthesize auxin, cytokinin and indole-acetic acid which due to enormous quantities, cause rapid growth in plants that lead to cancer development (Gaudin *et al*., 1994). Tumor formation is the proof that T-DNA integrates and expresses in the plant cells. Thus, by placing gene of interest within the T-DNA, desired characteristics in plants can be obtained (Potrykus *et al*., 1998; Alimohammadi and Bagherieh-Najjar, 2009).

1.9.2.1.4 Signal recognition in *Agrobacterium tumefaciens*

The signal transduction starts when *Agrobacterium* recognizes the chemical signals which are released as a result of interaction between plant and bacterium. These signals include phenols, sugars, low pH and phosphate signals (Brencic and Winans 2005). The most important activators of virulence and pathogenesis include acetosyringone and hydroxyacetosyringone (Stachel *et al*., 1985; Stachel & Zambryski, 1986).

1.9.2.1.5 T-DNA processing

In the T-DNA processing, Vir region of Ti plasmid plays an important role. When the host plant is wounded by the bacterium, Vir genes-encoded proteins are expressed. VirA and VirG are two regulator proteins that regulates the gene expression. The infected wounded plant releases phenols which are recognized by VirA and induces the

autophosphorylation of VirA which then phosphorylates VirG. As a result, the process of transcription of vir genes begin (Zupan and Zambryski, 1995). VirD1/D2 recognises T-DNA and cleaves it endonucleolytically. Double stranded T-DNA is cleaved by VirD1 and double stranded DNA is cleaved by VirD2. A single stranded copy of DNA called T-strand is formed that is released from Ti plasmid. While virD2 is still covalently bound at the T-strand 5' end, virD1 is released (Tzfira *et al*., 2003; Pratiwi and Surya, 2020).

1.9.2.1.6 T-DNA Migration

A. tumefaciens transfers the T-DNA into plants cell as T-strand/*vir*D2 complex. VirE2 protein also migrates during infection into host plant (Dombek and Ream, 1997). The nuclear localization signal (NLS) sequence found at the C-terminus of the VirD2 and VirE2 proteins directs the proteins and T-strand to the nucleus of the host cell (Pitzschke & Hirt, 2010). Endoplasmic reticulum helps in directing Ti plasmid into nucleus (Guo *et al*., 2019).

1.9.2.1.7 T-DNA Integration

After migrating into the host cell, the proteasomal machinery degrades the T-complex and liberates T-DNA and helps it in its integration in host genome (Tzfira *et al*., 2004). T-DNA is randomly integrated inside the nucleus but the expression of transgene is permanent, this process is known as stable transformation (Nishizawa-Yokoi *et al*., 2021).

VirD2 and virE2 are involved in T-DNA integration. The incorporation inside nucleus is carried out by Ω domain of virD2 and it shields T-DNA's right border from degradation of any kind (Lacroix & Citovsky, 2019). VirE2 along with transcription factor VIP1 and VIP2 is involved in this process and also protects left border of T-DNA (Anand *et al*., 2007; Saika *et al*., 2014).

1.9.2.1.8 T-DNA Expression

Following its integration into the plant genome, the inserted T-DNA has two possibilities. Either T-DNA is integrated into the plant genome or it is not expressed at all (Ozyigit, 2012; Primrose & Twyman, 2013). Figure 1.9 shows the T-DNA transport and integration into the host nuclear genome.

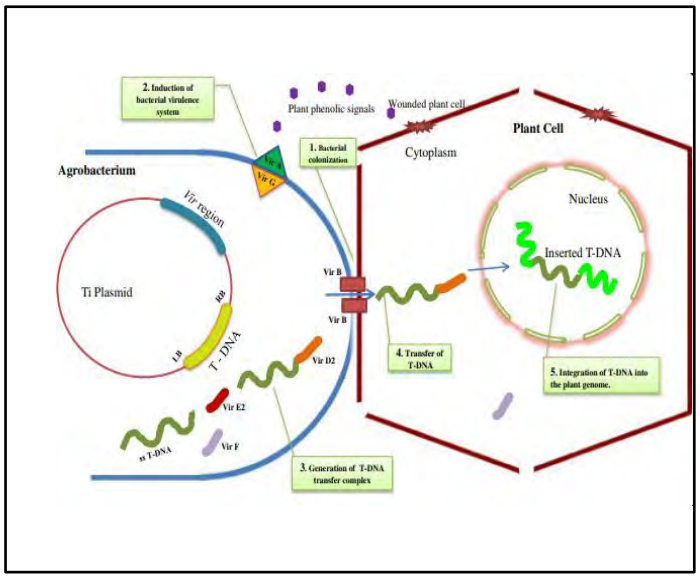

Figure 1.10: T-DNA nuclear transport and integration (Mehrotra and Goyal, 2012).

1.9.2.1.9 Mechanism of Stable Transformation

The stable *Agrobacterium*-mediated transformation consists of many steps which are demonstrated in Figure 1.10. These steps include: (1) seed sterilization and growth of plants; (2) plasmid isolation and preparation of *Agrobacterium*; (3) preparation of explants and infection with *Agrobacterium* followed by co-cultivation of explants; (4) selection of transgenic explants through optimizes antibiotic concentration; (5) callus formation and regeneration of transgenic explants; (6) acclimatization of transformed plant and molecular confirmation with PCR, qRT-PCR etc., (Asande *et al*., 2020; Pratiwi & Surya, 2020).

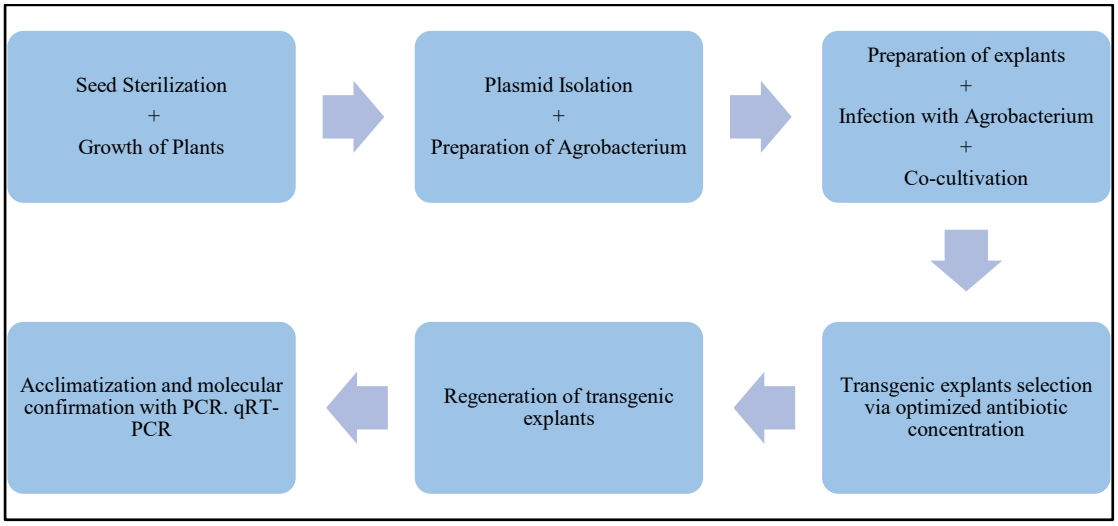

Figure 1.11: Schematic representation of *Agrobacterium*-mediated stable transformation.

1.9.2.1.10 Pros and Cons of Stable Transformation

The primary benefits of stable *Agrobacterium*-mediated transformation are its high transformation efficiency in plant cells and its ability to consistently express the desired gene's protein product (Laere *et al*., 2016). One *Agrobacterium*-transformed leaf disc can be used to create a variety of genetically modified plants and desired characteristics can be obtained (Meyers *et al*., 2010). No labour-intensive techniques and expensive instruments are required in this method (Lee and Gelvin, 2008).

The disadvantage of stable nuclear transformation is that the overall plant regeneration is quite challenging and is time consuming as compared to other methods (Waheed *et al*., 2016). Gene silencing and transgene expression may also occur (Horn *et al*., 2004; Fahad *et al*., 2015).

1.9.2.2 Transient Transformation

In *Agrobacterium*-mediated transient transformation, the permanent integration of transgene does not take place inside the host nuclear genome and central dogma expression pattern is not followed (Chen *et al*., 2011; Fahad *et al*., 2015). The transient expression usually peaks 2-4 days post-infection in plant cells and last for 10 days by gradually decreasing with each transformed cell (Kapila *et al*., 1997).

Successful transient transformation has been reported in many plant species such as tobacco, lettuce, rose, maize, sunflower, aspen and switchgrass (Lizamore and Winefield, 2015).

The transient nuclear transformation can be done through various methods such as biolistic method, polyethylene glycol (PEG) mediated transformation, electroporation, and agroinfiltration using *Agrobacterium* (Tsuda *et al*., 2012; Tan *et al*., 2013).

1.9.2.2.1 Agroinfiltration

Agroinfiltration is a very efficient method of delivering the gene of interest inside the host cell. This technique is simple, very quick and straightforward (Simmons *et al*., 2009; Zhao *et al.,* 2017). The desired targeted gene is infiltrated into leaf extracellular space either through vacuum or physical infiltration (Donini and Marusic, 2019).

Studies have shown various methods for agroinfiltration process. Syringe (agroinjection) and vacuum infiltration are the most extensively used methods (Kaur *et al*., 2021). *Agrobacterium* is injected into plant leaves using needleless syringe. As the

Agrobacterium enters the leaf's intercellular space, the leaf's light green colour begins to darken, indicating a successful and efficient infiltration (Santi *et al*., 2008).

In vacuum infiltration, the target recombinant DNA-carrying Agrobacterium strain is first submerged in an infiltration medium containing plant leaves. After that, the submerged plants are placed in a vacuum chamber with negative air pressure (Bechtold and Bouchez, 1995).

1.9.2.2.2 Pros and Cons of Transient Transformation

There are many advantages of transient transformation such as it is very flexible, fast, less-labour intensive and more cost-effective (Heenatigala *et al*., 2018). The transgene expression is very high and required protein can be obtained within some days after infection (Nosaki and Miura, 2021). Moreover, no tissue culture is required for this purpose (Fischer *et al*., 1999).

The disadvantage of transient expression is that it is not suitable for organs like roots, woody tissues and waxy leaves. During host cell replication, the transgene expression is lost and (Li *et al*., 2021). The large-scale production of recombinant protein is not possible with this technique (Fischer *et al*., 2012).

1.10 *Spinacia oleracea*

Spinach, scientifically known as *Spinacia oleracea*, is a vegetable with dark green leaves. It is a common vegetable and its leaves are consumed either fresh, cooked or they can be preserved through freezing, canning and dehydration (Roberts *et al*., 2016). This potent medicinal plant, recognized as a powerhouse of nutrients, is abundant in essential nutritional elements (Morelock *et al*., 2008). The term spinach has originated from the Persian word “ispanai” which means “green hands”. Over the time, it evolved into the Latin term "spanachia," eventually giving rise to the English words “spinage” and “spinach” (Naseer *et al*., 2019). Its leaves are enriched with nutrients and has wide range of applications in nutritional and culinary contexts. It is a valuable part of a balanced and health-conscious diet because of its phytochemical makeup and potential health advantages (Rashid *et al*., 2020).

1.10.1 Taxonomic Classification

Spinach is a leafy edible plant which used to be the member of family Chenopodiaceae, but in 2003 it it was added in family Amaranthaceae. There are over 2400 species and

160 genera in this family. Spinach belongs to the subfamily Chenopodioideae (Metha *et al*., 2014).

Table 1.1: Taxonomic classification of *Spinacia oleracea*

Kingdom	Plantae
Phylum	Tracheophytes
Class	Magnoliopsida
Order	Caryophyllales
Family	Amaranthaceae
Genus	*Spinacia*
Species	*oleracea*

1.10.2 Morphology

The leafy green stem of spinach is smooth, piped, succulent and can reach maximum height up to 30 centimetres. As an annual flowering plant, it is identified by simple, alternate leaves that exhibit varying morphologies, ranging from smooth to crinkled, depending on the specific variety (Eftekhari *et al*., 2010). The leaves are 2-30 cm in length, 1-15 cm in width. The leaves develop from the centre of the plant. Large leaves are located near the base while smaller ones are present at peduncle. Spinach is a dioicous plant having both male and female flowers. The flowers are yellowish green in colour and after maturing, they carry little fruit bunch with seeds within them (Ebadi-Segheloo *et al*., 2014). Seeds are mostly sown near the end of winter or early spring. The optimum growth of spinach requires cool weather, fertile soil and mild temperature (Kilickan *et al*., 2010).

1.10.3 Types of Spinach

There are three main varieties of spinach based on their morphology: savoy, semi-savoy and flat-leafed and these are further divided into cultivars and sub-cultivars (Nešković & Ćulafić. 1988).

- **Savoy**

Compared to the other two varieties, it is more productive and handles cold efficiently. It consists of wrinkled leaves. Regiment and Bloomsdale are the two cultivars included in this variety

- **Semi-savoy**

It is a very productive variety of spinach with slightly crinkled leaves. They typically have superior disease and bolt resistance. It can grow in spring, summer and fall. Tyee, Catalina, Indian Summer and Teton are the cultivars included in this variety.

- **Flat-leafed**

It has smooth, flat leaves and is considered as a best choice for processed spinach. Space spinach and Red Carnival are the cultivars included in this variety.

Figure 1.12: Types of Spinacia oleracea. (A) Savoy. (B) Semi-savoy. (C) Flat-leafed spinach.

1.10.4 Centre of Origin and World distribution

The exact origin of spinach is not known but it is said to have been discovered for the first time some 2,000 years ago in Iran (Morelock and Correll, 2008). The absence of references to spinach in Greek and Roman cultures suggests that its expansion likely occurred later in history. The earliest documented evidence of spinach dates back to the 4th century AD in Mesopotamia (El Faiz, 1995). The spread of spinach from its origin to different geographical locations is still unknown to a great degree. Spinach distribution in the west may have resulted from its expansion into the Muslim regions (Sneep, 1983). Recent evidence suggests that the Moors brought spinach to Europe via the Iberian Peninsula. Moorish Spain had been growing spinach since the eleventh century. (Al- ‘Awwâm, 2000; Heine, 2018). The Pyrenees Mountain Range has provided the most important paleoethnobotanical evidence (Hallavant & Ruas, 2014). There is very less evidence indicating the spread of spinach. Earliest evidence suggests that spinach has been brought to China via Nepal in the 7th century (Laufer, 1919). Yet, the initiation of spinach in Nepal remains unclear (Ribera *et al*., 2021).

The historical data cited above indicates that spinach followed two distinct routes. It was firstly distributed from Southern and Eastern Asia and then to Africa, Mediterranean and Northern Europe and later on brought to America (Laufer, 1915).

Spinach is closely related to *Spinacia tetrandra* and *Spinacia turkestanica.* It has been proposed that these could be the ancestors of wild spinach, either one or both. phylogenetics studies have shown that *S. oleracea* is more genetically closer to *S. turkestanica* instead of *S. tetrandra.* Thus, *S. turkestanica* is most likely to be the ancestor of spinach. Currently, the population of wild spinach have increased since the Centre for Genetic Resources Netherlands had organised two expeditions to gather more data (Ribera *et al*., 2021).

Spinach may be grown in a variety of regions. Low salinity soil is considered to be the best for its growth as high salinity soil or irrigation water reduces its harvest. It is considered as the "Queen of vegetables" by Arab and is mostly consumed in form of salads and soups. Noodles, steamed buns, and mixed spinach meatballs are produced on an industrial scale (Zhou *et al*., 2023). Major manufacturers of this economically significant crop include California, Texas, Arkansas, Oklahoma, Maryland, Virginia, New Jersey and Colorado (Correll *et al*., 1994).

1.11 Selection of *Spinacia Oleracea*

Spinacia oleracea is a green vegetable enriched with iron, vitamin A and other important nutrients such as beta-carotene, lutein, xanthin, and flavonoids. Among green vegetables, spinach stands out for its high nutritional content, antioxidant properties, and vitamin content and is regarded as the world's healthiest vegetable by WHO (Swiader *et al*., 1992).

Genetically modified spinach is prioritized for the production of plant-based edible vaccines. Vaccine production for HIV using HIV-1 Tat protein as a potential candidate and anthrax using spinach is being tested (Saxena and Rawat, 2014; Naik, 2022).

1.12 Aims and Objectives

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

- To carry out *Agrobacterium*-mediated stable nuclear transformation of the leaf explants of *Spinacia oleracea*.
- To verify the integration of *OmpC* transgene in *Spinacia oleracea* through several molecular biology techniques such as PCR and qRT-PCR.
- To estimate and quantify the OmpC protein by dot blot, western blotting, and ELISA.

2. Materials and Methods

The current study was performed in Plant Biotechnology Laboratory, Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. This research study aimed to optimize the best shooting regeneration medium for wild-type *Spinacia oleracea* leaves, and to develop an efficient protocol for *Agrobacterium*-mediated stable transformation of *Spinacia oleracea* with plasmid containing *OmpC* gene from *Salmonella enterica*. The integration and expression of *OmpC* gene was confirmed using polymerase chain reaction (PCR) and various other techniques of molecular biology in transformed plants. All utilized materials and methods followed in the present study are illustrated in this chapter.

2.1 Materials

2.1.1 Laboratory Instruments

All laboratory instruments were used after getting the required instrument's instruction manual for information and guidelines. The information on various instruments which were used for this research work is given in table 2.1.

Table 2.1: Laboratory Instruments

Appliances	**Company**
Autoclave	Yamato, USA
Weighing balance	Ohaus Corp, USA
Blotting apparatus	Major Science, USA
Centrifuge	Eppendorf, Germany
Conventional PCR	Applied biosystems, USA
Electroporator	Biorad, USA
Freezer (-20°C)	Dawlance, Pakistan
Freezer (-70°C)	VWR, USA
Gel casting instrument	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	Thermo Scientific multiskan GO
Vertical Gel electrophoresis apparatus	Cleaver Scientific, USA
Vortex	Scientific Industries, USA
Water bath	Precision, 180 Series, USA

2.1.2 Laboratory Glass and Plastic Wares

The research was conducted using various kinds of glass and plastic items. Plastic wares such as pipette tips, eppendorf, PCR tubes, petri plates etc. were discarded after one time use. Glassware such as 9 cm glass plates, magenta boxes, flasks, beakers, measuring cylinders etc were all reused. They were washed by using commercial liquid detergent (Max) and were dipped in the bleach solution overnight. The apparatus was dried in a hot oven at 150 ℃. It was then properly wrapped 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
Gloves	Qualtex, Malaysia
Magenta-boxes (Jars)	Pakistan
Microtiter plates	Costar, USA

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

2.1.3 Ready to use Laboratory Kits and Reagents

A section of high-quality, cost-effective, convenient supply of ready-to-use laboratory solutions and kits were used for this experimental work which were purchased from different companies. The information of respective reagents and kits is given in Table 2.3.

Table 2.3: Ready to use Laboratory Reagents and Kits

Reagents	**Company**
Anti-His antibody	Abcam, USA
Chemiluminescence system	ECL® PLUS; GE Healthcare
dNTP Set	Thermofisher, USA
Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG	Abcam, USA
Ni-NTA Agarose	Thermofisher, USA
Protein ladder	Thermofisher, USA
RNase A	Bioworld
SYBR green	Thermofisher, USA
Taq DNA Polymerase (5U/µl)	Thermofisher, USA

1 Kb DNA ladder	Thermofisher, USA
3,3′,5,5′-tertamethylbenzidine (TMB)	Thermofisher, USA
4-10% NuPAGE® Bis-Tris Gel	Thermofisher, USA

2.1.4 Chemicals and Reagents

Chemicals and Reagents of high-grade purity were used in this experimental work. They were purchased from different chemical manufacturers and suppliers. All solutions were made by using distilled water as solvent. The detail of chemicals and reagents is summarized in Table 2.4.

Table 2.4: Chemicals and Reagents

Chemicals	**Company**
Agar (Bacteriological grade)	Bioworld, Dublin, Ireland
Ammonium persulphate (APS)	AnalaR^{TM}, England
Ammonium sulphate ($(NH_4)_2SO_4$)	GPR, BDH laboratory supplies, UK
Agar Plant TC	Phytotech lab, US
Bovine serum Albumin (BSA)	MP Biomedical, USA
Boric Acid	AnalaR^{TM} England
Bromophenol Blue	Sigma-Aldrich, USA
β-mercaptoethanol	Biochem, USA
Calcium Chloride	AnalaR^{TM}, England
Cefotaxime	Sanofi-Aventis Pakistan
Chloroform ($CHCl_3$) Analytical grade	Sigma-Aldrich, USA
Coomassie	AnalaR^{TM} England
CTAB	Oxford lab chem, India
Dimethyl sulfoxide (DMSO)	Biochem, USA
Ethanol 99.7-100% pure v/v	Merck, Germany
Ethidium Bromide	Sigma-Aldrich, USA

Ethylenediamine tetra-acetic acid (EDTA)	AnalaR™ England
Glacial Acetic acid	Merck, Germany
Glucose	GlaxoSmithKline, UK
Glycerol (87%)	AnalaR™ 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™ England
LB Broth (Miller)	Microgen India
Magnesium chloride 6-hydrate ($MgCl_2.6H_2O$)	AnalaR™ England
Methanol	Sigma-Aldrich, USA
Phenol	Merck, Germany
Potassium Chloride (KCl)	AnalaR™ England
Propanol-1 (Propa-1-ol)	AnalaR™ England
Propanol-2 (Propa-2-ol)	AnalaR™ England
Sodium dodecyl Sulphate (SDS)	Phytotechnology Laboratories, USA
Sodium acetate (CH_3COONa)	AnalaR™ England
Sodium Chloride (NaCl)	AppliChem, USA
Sodium Hydroxide Pellets	Merck, Germany
Sodium metabisulphite	Sigma-Aldrich, USA
Sucrose	AnalaR™ England
Sulphuric acid	Sigma-Aldrich, USA
Tris Base	Invitrogen, USA
Tris-(hyroxymethyl) aminomethane	Phytotechnology Laboratories, USA

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

2.1.5 Primers

A primer is a short nucleic acid sequence that serves as a starting point for DNA synthesis. DNA polymerase enzymes require a primer to be bound to the template DNA strand in order to add nucleotides to the 3′-end of the nucleic acid strand to begin the DNA synthesis. In this research two sets of different primers were used.

- *OmpC* nuclear forward primer
- *OmpC* nuclear reverse primer
- *OmpC* internal forward primer
- *OmpC* internal reverse primer

The description of these primers is given in table 2.5

Table 2.5: Sequence of Primers

Primers	**Sequence**
OmpC-nuclear forward primer	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGAAAGTTAAAGTACTGTCCCTCCTGGTACCAGCTCTGCTG-3′
OmpC-nuclear reverse primer	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTGATGGTGATGGTGATG-3′
OmpC-internal forward primer	5′-TTGGTAAAGTTGATGGCCTG-3′
OmpC-internal reverse primer	5′-GCCGACATCAACATATTTTACGAT-3′

2.2 Methods

2.2.1 Workplace Safety Measures

To achieve the ultimate goal of disinfection and to prevent contamination and cross-contamination sterile conditions were maintained throughout the experimental work, particularly during seed sterilisation, seed germination, plant tissue culture and

streaking of *Agrobacterium* culture on Luria Agar plates**.** Following actions were taken for this purpose:

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

2.2.2 Sterilization of Laminar Flow Hood

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 MS, Luria-Bertani broth (LB), Luria Agar (LA) were used for seed germination, plant tissue culture, bacterial inoculation and bacterial streaking under optimized conditions.

2.2.3.1 Plant Culture Media

2.2.3.1.1 Murashige and Skoog Media (MS Media)

Different strengths of MS media (Full MS and half MS) were used for seed germination and plant regeneration (table 2.6). Full MS media was utilized for the germination of spinach seeds as well as for the regeneration of spinach plant tissues. For its preparation, MS and sucrose were weighed and dissolved in the required amount of distilled water in a media bottle. The pH was adjusted in the range of 5.75 to 5.85 via a pH meter with the help of 0.1 N HCl or 1 M NaOH solutions. Lastly, agar was weighed and added to the bottle. The medium was sterilized under controlled conditions, 121 ºC temperature, and 15 psi for 20 minutes, in an autoclave. The media was poured in jars (50 ml each) within sterile environment of LFH. Jars and plates were wrapped with parafilm and stored in the growth chamber at 25±2°C. The composition of different MS media is given in Table 2.6.

Table 2.6: Chemical composition of plant culture media

Components	**Concentration (g/l)**
Full MS Media	
MS Basal Salt	4.4
Sucrose	30
Agar	0.8 %
Liquid ½ MS Media	
MS medium w/ vitamins	2.2
Sucrose	30

2.2.3.2 Bacterial culture media

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

2.2.3.2.1 Luria Broth Media

Luria Broth media was utilized for bacterial growth (inoculation) in flasks. For its preparation, in a flask, LB was weighed (table 2.7) and dissolved in the required amount of distilled water. The flask was tightly covered with aluminium foil with the help of

paper tape. At standard conditions, the prepared media was sterilized through autoclave and stored at room temperature.

2.2.3.2.2 Luria Agar Media

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

Table 2.7: Chemical composition of bacterial culture media.

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

2.2.4 Preparation of Stock Solutions

The composition of various hormones and antibiotics used in this research study is mentioned in table 2.8.

Table 2.8: Composition of stock solutions of hormones and antibiotics

Stock Solutions	**Concentration**
Hormones	

BAP	1 mg/ml
IAA	1 mg/ml
Antibiotics	
Acetosyringone	100mM
Cefotaxime	250 mg/ml
Hygromycin	50 mg/ml
Kanamycin	50 mg/ml
Rifampicin	20mg/ml

2.2.4.1 Preparation of Stock Solutions of Plant Hormones

2.2.4.1.1 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 eppendorf and wrapped with parafilm. They were stored at -20 °C.

2.2.4.1.2 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.

2.2.4.2 Preparation of Stock Solutions of Antibiotics

2.2.4.2.1 Acetosyringone

In order to prepare 100 mM stock solution of acetosyringone, 196.2 mg acetosyringone powder was weighed. It was dissolved in 10 ml DMSO in 15 ml falcon tube within the sterile conditions of LFH. It was then shaken for several minutes. Upon complete

dissolution, this stock solution was filter sterilized using a syringe filter that was 0.2µm in diameter. 1.2 ml aliquots were created in eppendorf tubes and were stored at -20 ºC.

2.2.4.2.2 Cefotaxime

In order to prepare 250 mg/ml stock solution of cefotaxime, 1 g 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.

2.2.4.2.3 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. Upon complete dissolution, 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.

2.2.4.2.4 Kanamycin

In order to prepare 50 mg/ml stock solution of kanamycin, 500 mg kanamycin powder was weighed and added in 15 ml falcon tube within LFH. 10 ml autoclaved distilled water was added. The falcon tube was shaken for several minutes. 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.

2.2.4.2.5 Rifampicin

For the preparation of 20 mg/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.

2.2.5 Plant Material

Spinach seeds were used in this research work which were purchased from Awan Seed Store, Islamabad, Pakistan. These seeds were stored in dry place at room temperature in 15 ml falcon tube sealed with parafilm.

2.2.5.1 Sterilization of Spinach seeds

Spinach seeds were surface sterilized within sterile conditions of LFH. Sterilization was done to minimize the possibility of bacterial and fungal contamination and to enhance the seed germination efficiency. Seeds were sterilized in 15 ml falcon tube with 0.2% mercuric chloride for 3 minutes followed by washing thrice with autoclaved distilled water for 5 minutes respectively. Sterilized seeds were then dried on autoclaved filter paper.

2.2.5.2 Germination of Spinach seeds

Spinach seeds were germinated on full MS media (Table 2.6) which was prepared and autoclaved under standard conditions. All the apparatus for germination except the seeds were kept in UV for 20 minutes in LFH. The media was poured into jars. After seeds inoculation (5-6 seeds per jar) the jars were sealed with parafilm and were kept in growth room at 25 ºC.

2.2.5.3 Preparation of explants

A small fragment of node and leaf of plant called explant was used for the regeneration of plant using tissue culturing. In this research work, 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 were used for cutting the leaves and slicing them into 1.5 to 2 cm pieces.

2.2.5.5 Regeneration of leaf explants

Full MS media (Table 2.6) containing BAP and IAA was used for the regeneration of spinach explants. Composition of media for regeneration of explants is given in table 2.9.

Table 2.9: Composition of media for regeneration of spinach explants

MS Strength	Hormone	Working Concentration
Full MS	BAP	1 mg/L

	IAA	0.5 mg/L

2.2.6 Optimization of Hygromycin

The hygromycin concentration for spinach leaf explants was optimized to determine the optimum amount of hygromycin required for the selection of transformed spinach leaf explants. 20 mg/L was found to be the optimum concentration. It was added to regeneration media containing MS media along with hormones and poured into petri plates within LFH. Leaf explants were cut with the help of scalpel and blade and were placed in petri plates containing the regeneration media. The plates were sealed with parafilm and were kept in growth chamber at 25 ℃. Sub-culturing was done every 2 weeks. Optimization was performed in replicates, with each replicate containing 4-5 leaf explants.

2.2.7 Media preparation for Transformation

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

2.2.7.1 Preparation of Co-cultivation media

For co-cultivation media preparation (Annexure 2.1), 200 µM acetosyringone and hormones (optimized concentrations of BAP and IAA) were added to the slightly warm autoclaved Full MS media in the reagent bottle within LFH. The media was poured into petri plates and upon solidification the 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 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 2.1) was prepared by adding hygromycin (20 mg/L), cefotaxime (250 mg/L) and hormones to full MS media. Media was poured in petri plates and were wrapped with parafilm upon solidification. It was then stored in growth room at 25 ± 2 °C until further use.

2.2.8 First Transformation: genetically modified *Agrobacterium tumefaciens*

In this bacterial transformation, *Agrobacterium tumefaciens* were transformed with a binary vector pGWB5 which contained our integrated gene of interest i.e. *OmpC.*

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. A single colony of bacteria was picked by sterile loop and inoculated in flask containing LB media along with 50mg/L kanamycin and 50mg/L rifampicin. Flask was then placed in shaking incubator until growth appears. Value of OD 600 was measured using spectrophotometer (Thermo Scientific Multiskan GO).

2.2.8.2 Preparation of Electro-competent cells

For the preparation of electro-competent cells of *Agrobacterium* GV3101 strain, the bacterium inoculum was transferred into 50ml falcon tube and centrifuged at 2400 rpm for 20 minutes at 4 °C. The pellet was dissolved in 40 ml chilled distilled water. Again, centrifugation was done at 2400 rpm for 20 minutes at 4 ºC 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. The pellet was finally dissolved in 500 µl ice-cold 10% glycerol (10 ml glycerol: 90 ml distilled water) following centrifugation. After gentle mixing, 60 µl aliquots were formed and stored at -70 °C.

2.2.8.3 Electroporation

Electro-competent cells were transformed with a plasmid (7µl) by electroporation using the electroporator (Bio-Rad, USA), in accordance with the protocol mentioned in the manual. After thawing electrocompetent cells on ice, 7µl of plasmid was added to 50µl of these cells and the sample was shifted to the cuvette. The electroporator was set according to the manufacturer instructions. Electric shock of 1.8 kilovolts (KV) was given by pressing the PULSE button on the electroporator. 400 µl LB media was added to the cuvette. The sample was transferred into new eppendorf tube and incubated at 37 °C for three hours. 30 µl of the 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 2.10: Antibiotics for the selection of transformed *Agrobacterium tumefaciens*

Antibiotics	Stock Concentration	Working Concentration
Kanamycin	50 mg/ml	50 mg/ml
Rifampicin	50 mg/ml	20 mg/ml

2.2.8.4 Binary vector pGWB5 containing *OmpC*

Agrobacterium tumefaciens strain GV3101 was transformed with pGWB5 binary vector which was containing our gene of interest i.e., *OmpC.* The transformed *Agrobacterium tumefaciens* was used for transformation of *Spinacia oleracea.*

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 (Table 2.7). This LB media contained 50 mg/l kanamycin and 50 mg/l rifamycin for selection. 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 liquid ½ MS containing the pellet and 200 µM acetosyringone. The infection time was for 8 minutes. After infection, the media was discarded and explants were dried on the filter paper. Upon drying they were shifted to the co-cultivation plates. The plates were then sealed with parafilm and were kept in dark in growth chamber for different time periods i.e., 2 to 3 days. Control (wild-type explants) were also shifted to co-cultivation media. The procedure was done in replicates. Following formula was used for calculating regeneration efficiency of explants:

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

2.2.9.1.2 Selection

After incubating the leaf explants on co-cultivation media for 3 days, they were washed thrice (5 minutes each) with liquid half MS containing 400 mg/L cefotaxime and fourth time with autoclaved distilled H_2O for 5 minutes. The explants were then shifted to selection plates containing 20 mg/L hygromycin and 250 mg/L cefotaxime after being dried on autoclaved filter paper. Each plate containing 5-6 infected leaf explants was wrapped with parafilm and placed in growth room at 25 ℃. After a week the infected explants were washed as per above mentioned washing protocol. The whole work was done in sterile conditions of LFH.

2.2.10 Molecular Biology Techniques to verify Transformation

In this current study, integration of *OmpC* transgene and transformation of spinach plants were confirmed by conventional PCR analysis and copy number was determined through real-time PCR (qRT-PCR). Bradford Assay was done for the estimation of protein after extraction from plants. In transgenic plants, expression of *OmpC* protein was confirmed by performing dot blot assay and western blotting. Indirect Enzyme-Linked Immunosorbent Assay (ELISA) was performed to quantify the *OmpC* protein in total soluble protein, extracted from transgenic plants.

2.2.10.1 Plasmid isolation from transformed *Agrobacterium tumefaciens*

According to the Rusell and Sambrook (2001) protocol, plasmid isolation was carried out. Inoculated LB (OD_{600} 0.6-0.8) was transferred to 50 ml falcon tube and centrifuged for 10 minutes at 4°C, 14000 rpm. Pellet was collected and dissolved in 400 µl of solution I (Annexure 2.2). After vortexing, it was shifted to two eppendorfs and 400 µl solution II (Annexure 2.2) was added. After incubating on ice for 5 minutes, 200 µl solution III (Annexure 2.2). It was incubated on ice for 5 min at -20 ℃. Centrifugation at 4 °C, 14000 rpm for 5 minutes was done. 600 µl of supernatant was collected in eppendorf tube and equal volume of phenol-chloroform (Annexure 2.2) was added. Two layers i.e., aqueous and organic were formed. 600 µl of upper aqueous layer was collected and an equal volume of chilled isopropanol was added followed by incubation 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 pellet was washed with 1 ml 70% ethanol.

Again, the centrifugation was done for 2 minutes at 14000 rpm. Ethanol 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 Extraction of Plant genomic DNA

The genomic DNA was extracted from transgenic and wild-type plants using Cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). Temperature of water bath was pre-adjusted at 65 °C and CTAB solution (Annexure 2.3) was heated for 1 hour. Leaf explants were ground into fine powder in liquid nitrogen by using sterile pre-chilled pestle and mortar. The powdered sample was then transferred into 1.5 ml eppendorf tube and 700-900 µl CTAB solution was added. The homogenate was vortexed and placed in water bath for 60 minutes at 65 °C. After cooling, 600 µl of phenol-chloroform-isoamyl (Annexure 2.3) was added to each sample. Centrifugation was performed at 4 °C, 14000 rpm for 15 minutes. Supernatant was collected to a new eppendorf tube and equal volume of chilled isopropanol was added. The sample was incubated at -20 °C for 3 hours or overnight. Next day, the sample was centrifuged at 4 °C, 14000 rpm for 5 minutes. Supernatant was discarded and the pellet was washed with 1 ml 70% ethanol and centrifuged at 14000 rpm for 2 min at 4 °C. Supernatant was discarded and pellet was dried. T.E buffer (Annexure 2.3) 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

The concentration of DNA extracted from spinach was determined by Colibri micro volume spectrophotometer by using a highly sensitive, nanodrop micro-volume quantification (Desjardins and Conklin, 2010) method. Before using, the spectrophotometer ’s top and bottom panels were wiped using 2-3 µl de-ionized H_2O. When the lever arm was fully closed, deionized water came into contact with the upper panel. After that, liver arm was raised and both optical panels were cleaned with dry, clean lab wipes free of fur. After turning the instrument on, the nucleic acid option was selected. Blank reading was obtained by adding 1 µl of TE buffer (Annexure 2.4) onto the lower optical panel and both panels were cleaned afterwards. For the quantification of DNA samples, right constant was selected. On lower optical panel, 1µl of DNA sample was poured and “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 was performed for the confirmation of integration and transformation of *OmpC* gene into the nuclear genome of *Spinacea oleracea.* 25 µl volume of master mix was prepared for 1X PCR. Positive (*OmpC* containing plasmid) and Negative (wild type) controls were used. The expected size of amplicon was 1180 bp by using *OmpC* nuclear primers and 890 bp by using *OmpC* internal forward and reverse primers. The primers are mentioned in table 2.5.

Table 2.11: For 1X PCR, composition of master mix

Reagents	Concentration (µl)
PCR water	17.5
Taq. Buffer	2.5
MgCl2	1.5
dNTPs	0.5
Forward primer	0.5
Reverse primer	0.5)
Taq. Polymerase	0.5
Template	01

Table 2.12: PCR standard conditions.

Steps	Temp (℃)	Time (min: sec)
Initial Denaturation	95	05:00
Final Denaturation	95	00:45
Annealing	68.5/55	01:20/01:00
Initial Extension	72	02:00
Final Extension	72	10:00
Hold	04	∞

The annealing temperature for *OmpC* nuclear primer pair was 68.5 ºC and in case of *OmpC* internal primer pair it was 55 ºC.

2.2.10.5 Agarose Gel Electrophoresis and Imaging

PCR results were confirmed through agarose gel electrophoresis. PCR products of both transgenic and wild-type samples were loaded into wells. 7µl PCR product was mixed with 3µl loading dye (Annexure 2.4) before loading. 1kb ladder (Cat: SM0314, Thermo Scientific, USA) was used to check size of DNA sample. After carefully loading samples into distinctive wells, lid of gel tank was closed and electrophoresis was carried out for 60 minutes, 110 V and 500 mA. DNA bands were seen using gel documentation system under UV light.

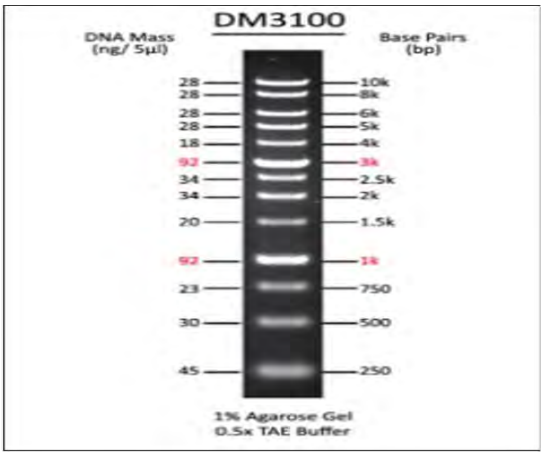

Figure 2.1: 1 kb DNA ladder for agarose gel electrophoresis (Taken from www. Gentechbio.com)

2.2.10.6 Quantitative Real Time PCR (qRT-PCR)

Following a successful transformation, the copy number of integrated *OmpC* gene within plant cell was confirmed using the qRT-PCR method described by Wen *et al.* (2012). qRT-PCR was performed using MyGo Pro Real time PCR (Stokesly Middlesbrough, UK). Both transformed and wild-type DNA samples were used. 1:10 and 1:100 dilutions were prepared for each sample. A fluorescent dye, SYBR Green (Cat No: K0221 Thermo Scientific, USA) was used for qRT-PCR. Composition of master mix is given in table 2.13. *OmpC* internal primers were used in this technique, their sequence is given below

OmpC internal forward primer 5′-TTGGTAAAGTTGATGGCCTG-3′

OmpC internal reverse primer 5′-GCCGACATCAACATATTTTACGAT-3′

Each reaction was subjected to qRT-PCR assay in triplicate. Standard curves were drawn for endogenous *β-actin* gene and *OmpC* transgene. A linear relationship was

established between the input amount of DNA and the level of fluorescence. The composition of master mix and standard conditions for qRT-PCR are given below in table 2.13 and table 2.14 respectively.

Table 2.13: Composition of master mix for qRT-PCR

Reagents	Concentration (µl)
PCR water	4
Forward Primer	0.25
Reverse Primer	0.25
SYBR Green	5
Template	0.5

Table 2.14: Standard Conditions for qRT-PCR

Programs		Ramp (˚C/s)	Temp (℃)	Hold (s)
Hold		04	95	600
Three-steps amplification	Denaturation	05	95	10
	Annealing	04	59	45
	Extension	05	72	15
Pre-Melt Hold		05	95	15
High-Resolution Melting	Initial stage	04	57	60
	Final stage	0.25	95	15

2.2.10.7 Protein Extraction

Total soluble protein was extracted from the leaves of transgenic and wild-type spinach plants. 100 mg of plant material was ground in liquid nitrogen by using mortar and pestle. 500 µl protein extraction buffer (Annexure 2.5) was added to the powdered sample. The samples were homogenized and transferred to 1.5 ml eppendorf tube. It was vortexed and centrifugation was done at 20,000 g, 4 ℃ for 10 minutes. Pellet was discarded and supernatant was taken in fresh eppendorf and again centrifuged at 20,000 g, 4 ℃ for 10 mins in order to remove cellular debris. The supernatant was collected in fresh eppendorf tube and was stored at -20 ℃.

2.2.10.8 Bradford Assay

Bradford Assay was performed to determine the total soluble protein (TSP) concentration after protein extraction. A standard curve was drawn by using Bovine Serum Albumin (BSA) as a standard. BSA stock solution of 2 mg/ml was prepared and five serial dilutions were made (Annexure 2.6). Protein extraction buffer (Annexure 2.5) was used as blank. 20 µl of blank, BSA dilutions and samples were loaded into separate wells of 96 well plate. Then, 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. Using the absorbance values of BSA dilutions, a standard curve was plot and was used to calculate the concentration of each protein sample using the TREND formula on Excel sheet.

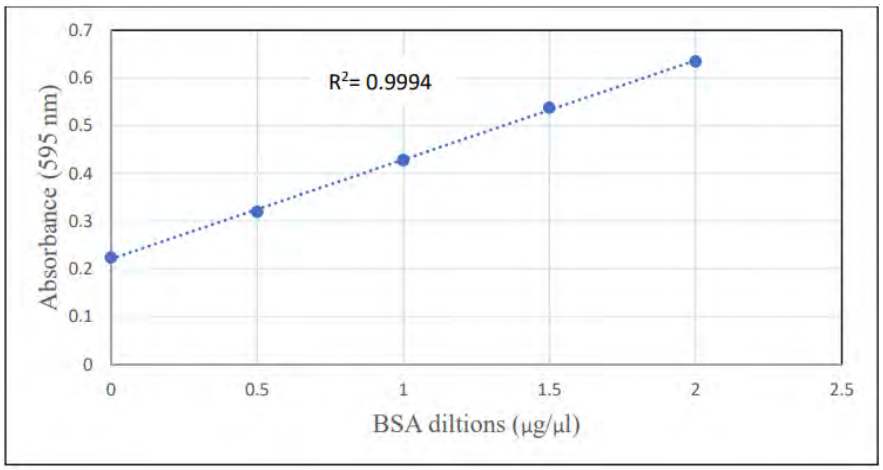

Figure 2.2: BSA Standard curve for protein quantification.

2.2.10.9 Dot Blot Assay

Dot Blot Assay is very simple and quick and performed on the same principle as many other immunological techniques; a particular antigen is recognized and bound by a particular antibody. Expression of OmpC protein in transgenic plants was confirmed through this assay. Different steps such as blotting, blocking, primary antibody treatment, secondary antibody treatment, 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 this purpose. Forceps were used to remove the protective covering of NCM and 2 µl of wild and transgenic protein samples

were blotted onto membrane. The blotted sites were marked with pencil. The membrane was incubated for one hour at 25 °C.

2.2.10.9.2 Blocking

For non-specific blocking of the sites, 20 ml blocking buffer (Annexure 2.7) was loaded onto membrane and incubated for 30-60 minutes at 25 ℃ with gentle shaking. After incubation, the blocking buffer was discarded. To avoid excess BSA binding, the membrane was washed with TBST buffer (Annexure 2.8) three times for 10 mins.

2.2.10.9.3 Primary Antibody Treatment

His tag antibody (Abcam, USA) was used as primary antibody. Working dilution 1:1000 of primary antibody (Annexure 2.9) was made from stock in TBST. (Annexure 2.8). The membrane was incubated for 30 mins at room temperature. It was then washed thrice with TBST after incubation.

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 2.10) was made from stock in TBS-T buffer (Annexure 2.8). The membrane was incubated with a secondary antibody for 30 mins with gentle shaking at room temperature followed by washing thrice with TBST.

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

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed to separate the purified proteins on the basis of their size. Nitrocellulose membrane (NCM) was used for transfer of separated proteins. Following the successful transfer, NCM was incubated with primary and secondary antibody under optimized conditions for the detection of targeted protein. Protein samples from transformed

spinach tissue were used for western blotting. Wild-type spinach was used as negative control.

2.2.10.10.1 SDS-PAGE

The apparatus was cleaned and assembled properly. Two types of gels were prepared i.e. 10% resolving gel and 5% stacking gel. 10% resolving gel (Annexure 2.11) was first prepared and poured between the two glass plates. 1 ml isopropanol was poured onto resolving gel in order to linearize it. Gel was allowed to set completely. Isopropanol was removed and 5% stacking gel (Annexure 2.12) was prepared and poured above the resolving gel. The comb was inserted in order to make wells after gel was completely set.

2.2.10.10.2 Preparation and Loading of protein samples

The protein samples were prepared by using 4X sample buffer (Annexure 2.13). 4 µl sample buffer and 20 µl protein were mixed in eppendorf tubes. For protein denaturation, the samples were heated at 90 ℃ for 10 mins before loading. Purified protein from two transgenic lines was loaded in two lanes with negative control. In one well, 4 µl prestained PAGE ruler (Cat No. 26616, ThermoScientific, USA) was loaded.

2.2.10.10.3 Electrophoresis

After the gel was set, the assembly was placed in gel tank containing the running buffer (Annexure 2.14). Comb was removed and wells were washed with running buffer. The samples were loaded and the lid of gel tank was closed and positive and negative electrodes of gel tank were connected to power supply. The gel was run initially for 20 minutes at 80 volts and then at 110 volts for 90 minutes.

2.2.10.10.4 Protein Transfer

Semi-dry method was used for protein transfer. A semi-dry blotting apparatus was used for this purpose. For transfer of one gel, 8 whatman's filter paper were cut equal to the size of the separating gel. The filter papers and NCM were soaked in 1X transfer buffer (Annexure 2.15). 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). A roller was used to remove the air bubbles. 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 the non-specific primary antibody binding. The membrane was kept in blocking buffer (Annexure 2.7) for 60 mins with continuous shaking at room temperature. After one hour, blocking buffer was removed and membrane was washed thrice with TBST buffer (Annexure 2.8) to remove excess BSA binding.

2.2.10.10.6 Primary Antibody Treatment

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 incubated with primary antibody overnight at 4°C. After treatment, membrane was washed thrice with TBST buffer (Annexure 2.10).

2.2.10.10.7 Secondary Antibody Treatment

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Abcam, USA) was used as the secondary antibody. The membrane was incubated with secondary antibody (Annexure 2.10) for 1.5-2 hours with continuous shaking at room temperature. After treatment, membrane was washed three times with TBS-T (Annexure 2.8).

2.2.10.10.8 Visualization

Chemiluminescent substrate (Cat No: WBKLS0500, Merck Milipore, Germany) was used for visualization. Equal volumes of luminol and hydrogen peroxide 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 Enzyme-Linked Immunosorbent Assay (ELISA)

To determine the protein concentration, Indirect ELISA was performed. A 96 well plate was used. Samples were prepared by using ELISA Extraction buffer (Annexure 2.16). Samples were loaded into separate wells and plate was incubated for one hour at 37°C. After one hour, it was washed thrice with wash buffer (Annexure 2.8). Each well received 200 µl of Blocking solution. After one hour incubation at 37 °C, the plate was washed thrice with washing buffer (Annexure 2.8). 50 µl primary antibody (Annexure 2.9) was added to each well and one hour incubation was done at 37 °C. The wells were washed thrice with washing buffer and 50 µl secondary antibody was added in each well. To remove unbound antibody, the plate was washed thrice with washing buffer

after one hour incubation. Enzyme specific substrate, TMB (Cat No. A3840, Applichem, Germany) was added to each well and plate was incubated at room temperature. When the blue colour appeared after 20 to 30 minutes, the reaction was stopped by adding 0.1 N H_2SO_4 (stop solution), and absorbance was measured at 595 nm.

Annexures

Annexure 2.1: Composition of media required for transformation

Media	Components	Concentration (g/L)
Co-cultivation Media	Full MS	4.4g
	Sucrose	30g
	Agar	0.8%
	Distilled water	1L
	BAP	1000µl
	IAA	500µl
	Acetosyringone	200µM
Selection Media	Full MS	4.4g
	Sucrose	30g
	Agar	0.8%
	Distilled water	1L
	BAP	1000µl
	IAA	500µl
	Hygromycin	400µl
	Cefotaxime	1000µl

Annexure 2.2: Solutions composition for plasmid isolation

Solutions	Components	Concentration
Solution I	Glucose	50mM
	Tris	25mM, pH 08
	EDTA	10mM, pH 08
Solution II	SDS	1 %
	NaOH	0.2 N
Solution III	Sodium Acetate	3 M (pH: 4.8)
Phenol-Chloroform	Phenol	25 ml
	Chloroform	25 ml
70% Ethanol	Ethanol	70ml
	Distilled Water	30ml
T.E. Buffer	Tris HCl (pH 8)	01M
	EDTA	0.5M

Annexure 2.3: Solutions for DNA extraction from plants

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

Annexure 2.4: Solutions for Agarose Gel Electrophoresis

Components	Concentration
TBE buffer recipe 10X (pH 8.0)	
Tris Base	110g/L
Boric Acid	55g/L
EDTA	9.3g/L
1X TBE Buffer	
10X TBE Buffer	10ml
Distilled water	90ml
Ethidium Bromide (10mg/ml)	
Ethidium	1g
Distilled water	100ml
Loading Dye	
Bromophenol Blue	0.25g
Distilled water	100ml

Annexure 2.5: Protein Extraction Buffer

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

Annexure 2.6: BSA Dilutions for Bradford Assay

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

Annexure 2.7: Blocking Buffer

Components	Concentration
BSA	5g
TBS-T	100ml

Annexure 2.8: Washing Buffer (TBS-T)

Components	Concentration
Tris HCl	3.08g/L (pH 7.6)
NaCl	8.77g/L
Tween 20	1ml/L
Distilled water	1L

Annexure 2.9: Dilution for Primary Antibody

Components	Concentration
His Tag Antibody	3µl
Sodium Azide	0.015g
BSA	1g
TBS-T Buffer	30ml

Annexure 2.10: Dilution for Secondary Antibody

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

Annexure 2.11: Resolving Gel

Components	Concentration
Distilled water	4.17ml
30% Stock Acrylamide Solution	3.35ml
4X Resolving Tris Solution	2.5ml
10% Ammonium Persulfate	100µl
TEMED	20µl

Annexure 2.12: Stacking Gel

Components	Concentration
Distilled water	6.76ml
30% Stock Acrylamide Solution	2ml
0.5M Stacking Buffer	3ml
10% SDS	0.12ml
10% Ammonium Persulfate	0.12ml
TEMED	20µl

Annexure 2.13: 4X Sample Buffer

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

Annexure 2.14: Running Buffer

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

Annexure 2.15: Transfer Buffer

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

Annexure 2.16: ELISA Extraction Buffer

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

3. Results

In this study, 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 *OmpC* gene integration into *Spinacia oleracea*. Dot blot, western blotting and ELISA were performed for detection of OmpC protein.

3.1 Seeds Germination

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

3.1.1 Sterilization of *Spinacia oleracea* seeds

Spinach seeds were surface sterilized through a treatment with 0.2% mercuric chloride ($HgCl_2$) for 3 minutes. After washing thrice with distilled water, seeds were dried on an autoclaved filter paper. The seeds were inoculated on both strengths of MS media i.e., ½ MS and full MS media, and were observed for 30 days of inoculation as shown in figure 3.1.

3.1.2 Germination efficiency of spinach seeds

Sterilized spinach seeds were grown in half MS and full MS media within the sterile conditions of LFH. The experiment was done in triplicates. A total of 40 seeds were used. 20 were inoculated on half MS media and 20 on full MS media. Each jar consisted of 5 or 6 seeds. The germination efficiency for seeds inoculated was calculated after 30 days of inoculation of seeds in both media.

From the results, it was observed that seeds sterilized with 0.2% $HgCl_2$ and germinated on full MS media had a high germination rate. The results also showed that the germination efficiency was different for both media i.e., 75% and 50% for full MS and half MS respectively as shown in Table 3.1 and represented graphically in figure 3.2. The germination rate of seeds was quite different. It was observed that seeds inoculated on full MS media germinated faster compared to the ones on half MS as shown in figure 3.1. Thus, for later experiments, spinach seeds were inoculated in full MS media.

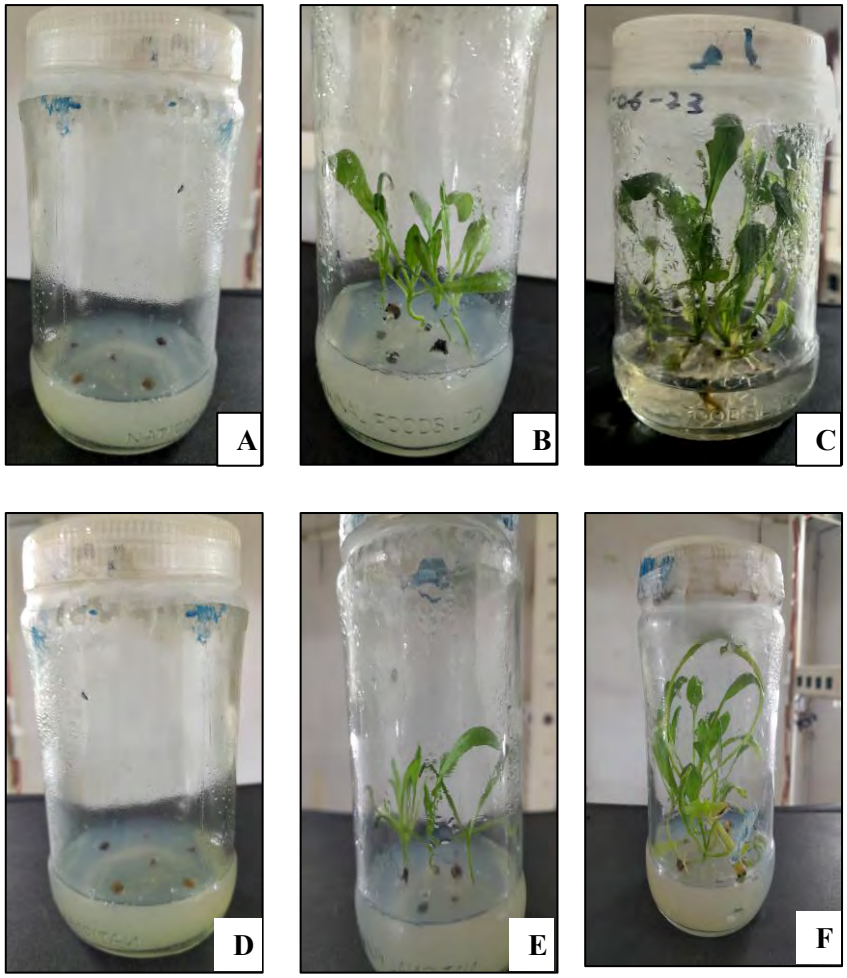

Figure 3.1: Spinach seeds germinated on different strengths of MS media (½ MS and full MS media). (A) Day 1 on full MS. (B) Day 15 on full MS. (C) Day 30 on full MS. (D) Day 1 on ½MS. (E) Day 15 on ½ MS. (F) Day 30 on ½MS.

Table 3.1: Germination efficiency of *Spinacia oleracea* on different MS strengths

Sr. No.	MS Strength	Total Seeds Inoculated	Seeds germinated	Germination efficiency
1.	½MS	20	10	50%
2.	Full MS	20	15	75%

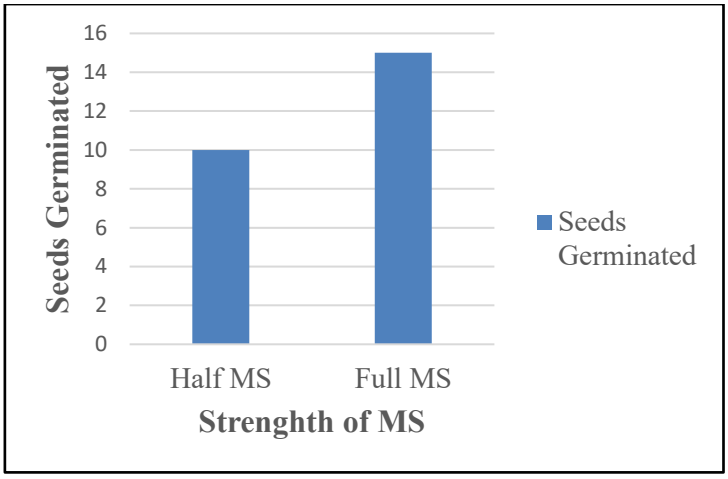

Figure 3.2: Graphical representation of seeds germination efficiency on both MS strengths (½ MS vs full MS media). The graph represents data from three batches (n=3).

3.2 Callogenesis of *Spinacia oleracea*

Spinach leaf explants were regenerated on optimized regeneration media (Full MS with vitamins along with a calculated amount of BAP and IAA). The experiment was done in triplicates. After inoculation, the explants were observed for 4 weeks. The media containing 1 mg/L BAP and 0.5 mg/L IAA was effective for development of explants (Figure 3.2). The callus was formed in almost each of the leaf explants. Callus formation efficiency of leaf explants of *Spinacia oleracea* after 30 days of inoculation is given in Table 3.2.

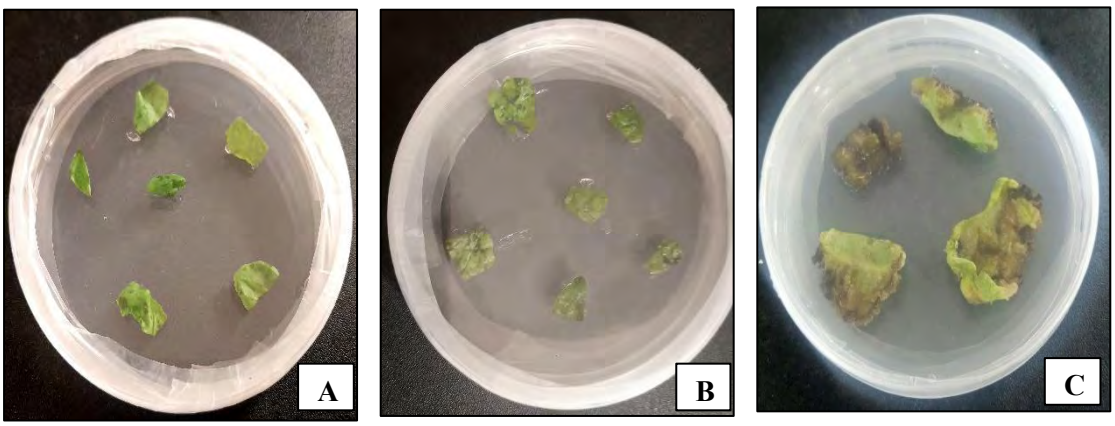

Figure 3.3: Media for callus formation of spinach leaf explants. (A) Day 1 on media. (B) Day 15 on media. (C) Day 30 on media.

Table 3.2: Callus formation efficiency of spinach leaf explants

Type of explant	Media	Total no. of leaves inoculated	No. of Callus formed	Callogenesis efficiency
Leaves	Full MS vitamins along with BAP and IAA	12	9	75%

3.3 Effect of Hygromycin concentration on survival of explants

3.3.1 Effect of hygromycin concentration on *Spinacia oleracea* explants

The hygromycin concentration for the leaf explants of Spinacea oleracea has already been optimized by preparing and shifting explants to different hygromycin concentrations i.e., 0 mg/L, 15 mg/L, 20 mg/L, 25 mg/L and 30 mg/L. The hygromycin concentration that bleaches out wild type explants i.e., 20 mg/L concentration was found to be the best and it was used for selection of transformed explants. It was noticed that increased hygromycin concentration decreases the regeneration ability and more explants tend to bleach out. 0 mg/L plates were used as negative control whereas, 20 mg/L concentration of hygromycin in plates was used as positive control. Figure 3.4 shows the effect of different concentrations of hygromycin on spinach leaf explants with the results tabulated in Table 3.3. A graphical representation of these results is given in Figure 3.5.

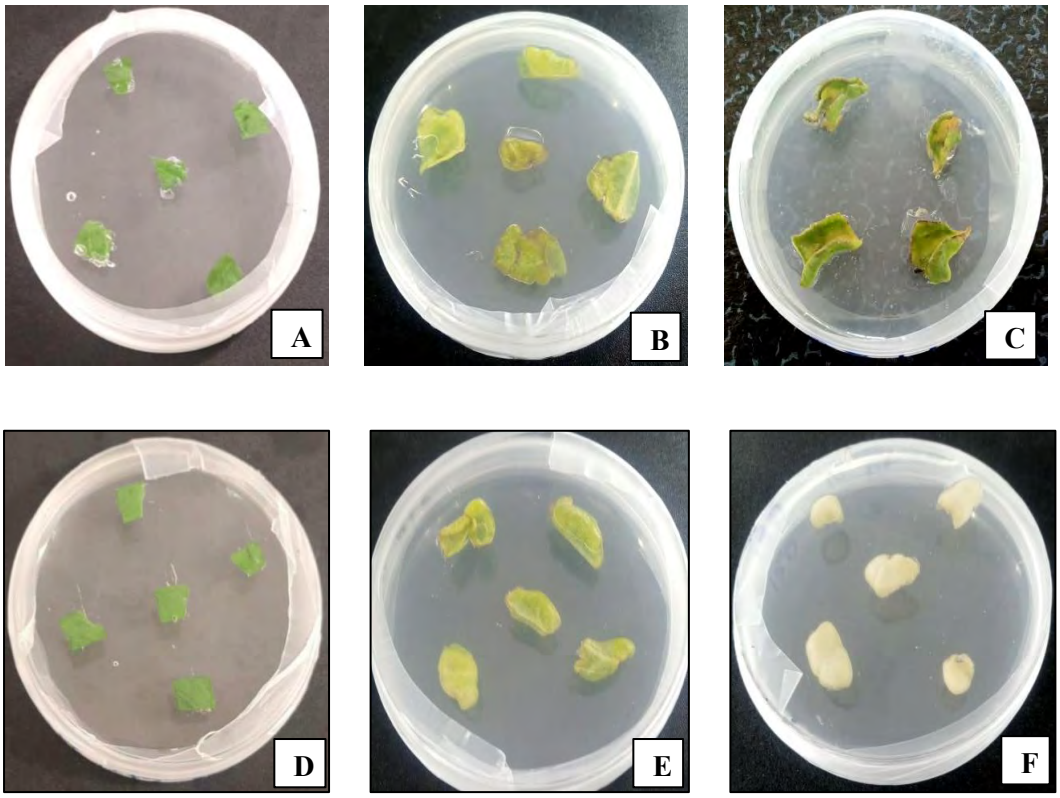

Figure 3.4: Effect of hygromycin concentration on leaf explants of *Spinacia oleracea.* (A) Day 1 on 0 mg/L. (B) Day 15 on 0 mg/L. (C) Day 30 on 0 mg/L. (D) Day 1 on 20 mg/L. (E) Day 15 on 20 mg/L. (F) Day 30 on 20 mg/L.

Table 3.3: Effect of hygromycin on leaf explants

Sr. No.	Hygromycin Concentration (mg/l)	Total no. of explants	Callus Formed	Explants bleached	Percentage of callus formed	Percentage of explants bleached
1.	0	15	11	0	73.33	0
2.	20	15	0	13	0	86.66

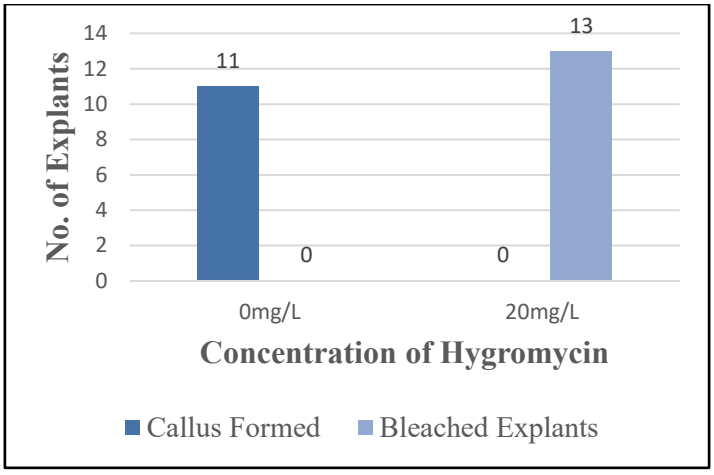

Figure 3.5: Graphical representation of effect of hygromycin concentration on callus formation of explants. The graph represents data from three batches (n=3).

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 *OmpC* gene was used to infect the explants. After infection, explants were kept on the co-cultivation plates and shifted to selection plates.

3.4.1.1 Optimization of infection time

In this current study, 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). The results showed that the increase in infection time makes it difficult to get rid of excess bacteria and leads to decreased regeneration efficiency. Thus, 8 minutes infection time was considered an optimum time for transformation of spinach leaf explants. Data regarding the infection time is provided in table 3.4 and is graphically represented in figure 3.6.

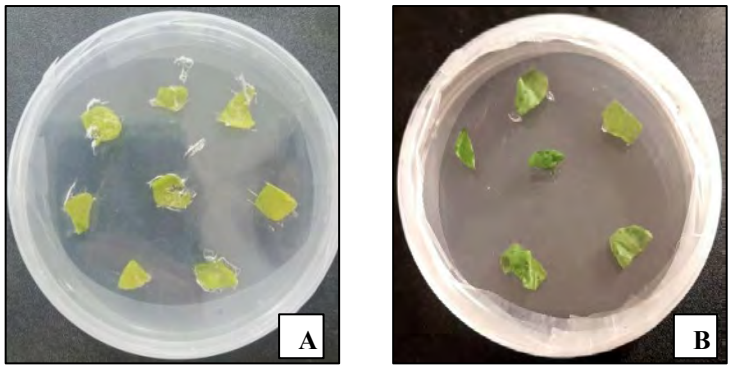

Figure 3.6: Leaf explants after infection on co-cultivation media. Explants were treated with *Agrobacterium tumefaciens* culture for different time periods. (A) Explants treated for 8 minutes. (B) Explants treated for 10 minutes.

Table 3.4: Effect of infection time on callus formation of explants

Infection time	No. of leaf explants	Callus formed	Callus formation efficiency
8 minutes	8	3	37.5
10 minutes	7	1	14.28

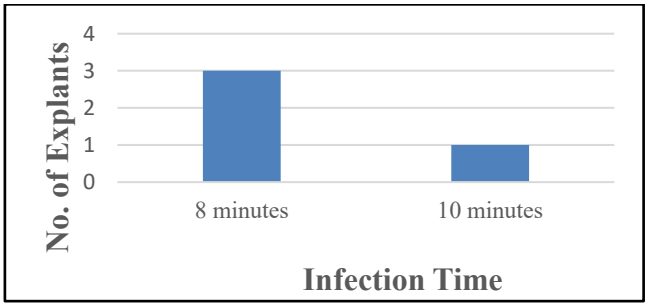

Figure 3.7: Graphical representation of effect of infection time on callus formation efficiency of explants.

3.4.1.2 Optimization of Co-cultivation time

After infection, the leaf 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 with the increase in co-cultivation time, the regeneration efficiency of leaf explants also increases. Explants that were placed on co-cultivation media for 3 days showed higher callus formation efficiency as 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 3.5 and graphically represented in figure 3.9.

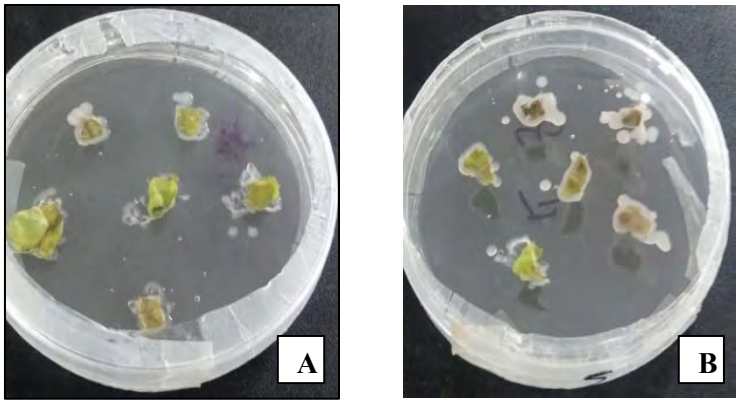

Figure 3.8: Explants after co-cultivation time. Different co-cultivation time was provided to leaf explants. (A) Explants after 3 days co-cultivation time. (B) Explants after 2 days co-cultivation time.

Table 3.5: 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	6	1	16.6
3 days	6	3	50

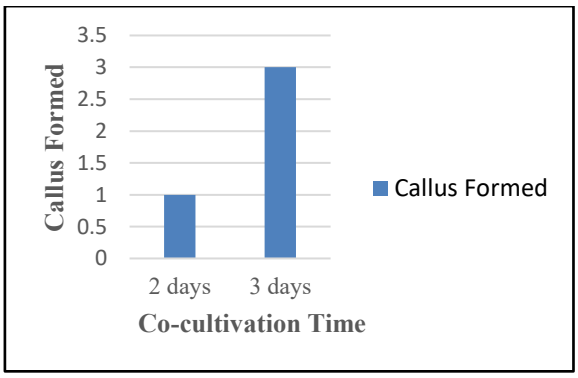

Figure 3.9: Graphical representation of effect of different co-cultivation time (2 and 3 days) on callus formation efficiency of explants.

3.4.1.3 Transformation

Leaf explants were transformed with *OmpC* gene (present in pGWB5 vector) via *Agrobacterium*-mediated stable transformation. Conventional PCR was used to confirm the successful transformation and integration of *OmpC* 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 *OmpC* gene

Plasmid isolation was done according to protocol by Sambrook and Rusell (2001). The plasmid was isolated after the transformation of PGWB5 containing the *OmpC* gene transformed in the *Agrobacterium* strain GV3101. Agarose gel electrophoresis was used for confirmation of isolated plasmid. The presence of the *OmpC* gene in the plasmid was confirmed by conventional PCR by using specific primers for amplification of the transgene. The PCR product was loaded onto an agarose gel for confirmation of gene of interest. *OmpC* internal and nuclear forward and reverse primers were used for the confirmation of the *OmpC* gene in the plasmid. Gel results showed the presence of the *OmpC* gene in the isolated plasmid (Figure 3.6).

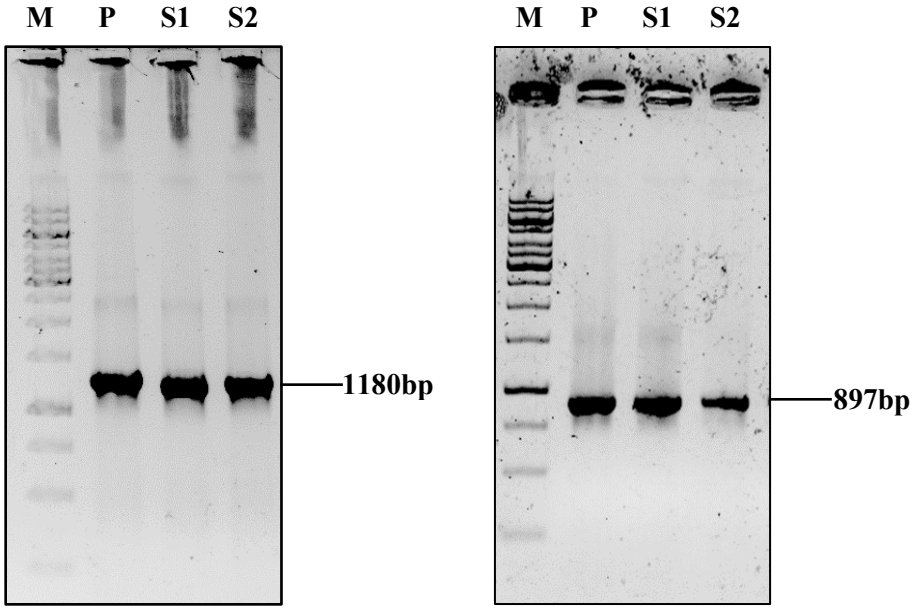

Figure 3.10: PCR confirmation of *Omp*C gene in plasmid. M: 1 kb gene ruler, P: positive control and S1, S2: plasmids.

3.4.1.3.2 Stable nuclear transformation procedure

Following optimization of different factors, transformation was carried out under optimized conditions. Leaf explants were infected with *Agrobacterium* containing *OmpC* 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 the excess bacteria. They were then shifted to selection plates containing 20 mg/L hygromycin (Figure 3.11).

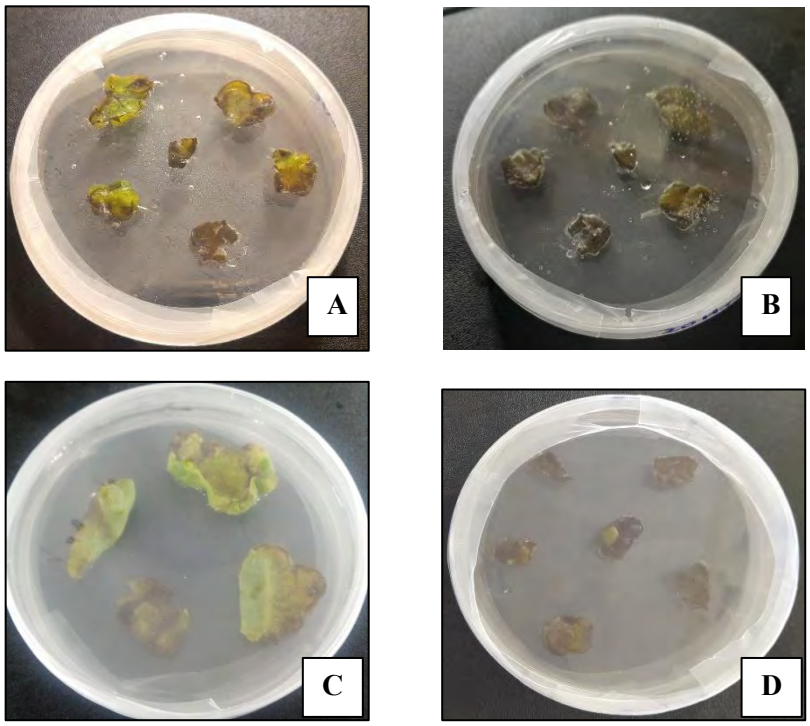

Figure 3.11: Transformed explants on selection plates. (A) Transformed batch 1 on selection plates after 2 weeks. (B) Transformed batch 1 on selection plates after 4 weeks (C) Transformed batch 2 on selection plated after 2 weeks (D) Transformed batch 2 on selection plates after 4 weeks.

3.5 Confirmation of stable transformation of *Spinacea oleracea* with *OmpC*

3.5.1 PCR Confirmation of transgene

Plant genomic DNA was extracted by the CTAB method described by Murray and Thompson (1980). Before PCR confirmation, extracted DNA was checked through agarose gel electrophoresis (Figure 3.12). The presence of the *OmpC* gene in *Spinacea oleracea* was confirmed by conventional PCR by using specific primers for amplification of the transgene. *OmpC* nuclear forward and reverse primers as well as *OmpC* internal forward and reverse primers were used for the confirmation of *OmpC* transgene. The PCR product was loaded onto an agarose gel for confirmation of our gene of interest. Figure 3.13 shows the gel image with amplified bands of 1180 bp with *OmpC* nuclear forward and reverse primers and 897 bp with *OmpC* internal forward and reverse primers, which confirms the presence of *OmpC* transgene. Consequently, successful transformation of *Spinacia oleracea* was confirmed.

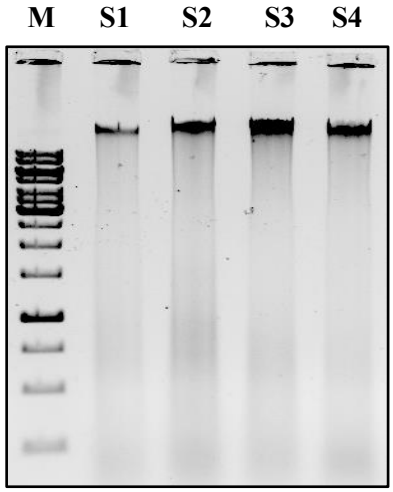

Figure 3.12: Confirmation of DNA isolated from plant samples. M: 1 kb marker, S1: sample 1, S2: sample 2, S3: sample 3 and S4: Sample 4.

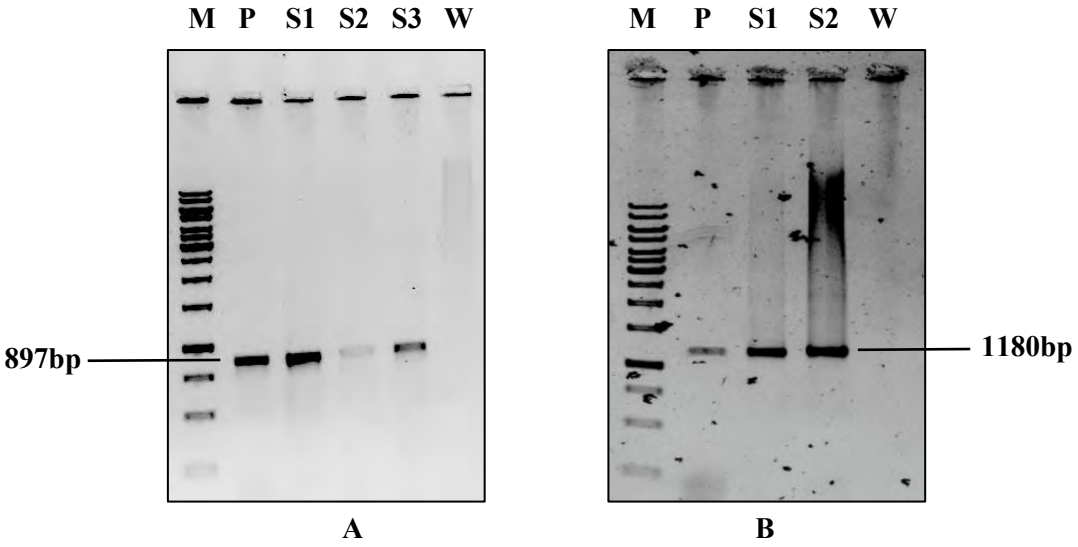

Figure 3.13: PCR confirmation of *OmpC* transgene in *Spinacea oleracea*. (A). PCR confirmation of *OmpC* transgene with *OmpC* internal forward and reverse primers (B). PCR confirmation of *OmpC* transgene with *OmpC* nuclear forward and reverse primers M: 1Kb marker, W: negative control wild-type plant, P: positive control and S1, S2, S3: transformed plant DNA samples.

3.5.2 Calculation of *OmpC* 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 3.6). These values were used to plot the standard curve.

Table 3.6: Average CQ values of transgene vs endogenous gene.

Sample	Average CQ values for transgene	Average CQ values for β- actin
OmpC	17.0897349	29.82290853
OmpC 1:10	22.62138332	31.82694862
O*mpC* 1:100	28.60989578	32.77485378
OmpC 1:1000	39.62329885	33.31442407

This provided the correlation coefficient values for *OmpC* transgene and *β-actin* endogenous gene. SQ values were used to calculate the rline which provided us with the copy number of the transgene by using the formula:

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

Copy number of *OmpC* transgene was equal to 1.

3.5.3 Dot Blot

A Dot blot was done prior to Western Blotting to check the presence of protein within the samples. Specific primary and secondary antibodies were used for the detection of *OmpC* protein. Both wild type and transformed samples were spotted on the blot. Unlike wild-type, positive results were observed for both samples (Figure 3.14). This confirmed the presence of transgenic protein in the samples.

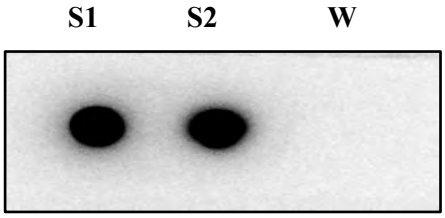

Figure 3.14: Confirmation of the presence of OmpC protein in transformed samples by dot blot. S1: sample 1, S2: sample 2, W: Wild type.

3.5.4 Western Blotting

The presence of OmpC protein was confirmed through Western Blotting. Size of monomeric form of OmpC protein is equal to 42.2 kDa and trimeric form is 126 kDa. Monomeric form of OmpC protein with size equal to 42.2 kDa was detected in our

samples as shown in Figure 3.15. Specific antibodies were used for the detection of protein in these samples. By using specific antibodies, band was observed for transgenic samples while no band was observed for wild-type samples (Figure 3.15). Detection of 42.2 kDa protein confirms the successful stable transformation of *Spinacia oleracea* leaf explants.

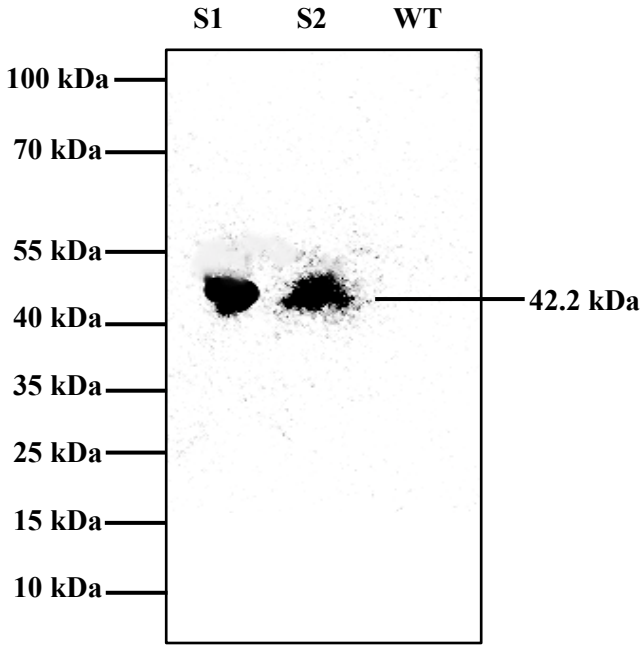

Figure 3.15: Expression of protein by western blotting. S1: transformed sample 1, S2: transformed sample 2 and WT: Wild-type.

3.5.5 ELISA

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

The data presented in Figure 3.16 shows an increased absorbance of transgenic samples having OmpC protein compared to the wild-type one. Among the two samples, S2 showed the highest absorbance which means that it contains the highest amount of antigen.

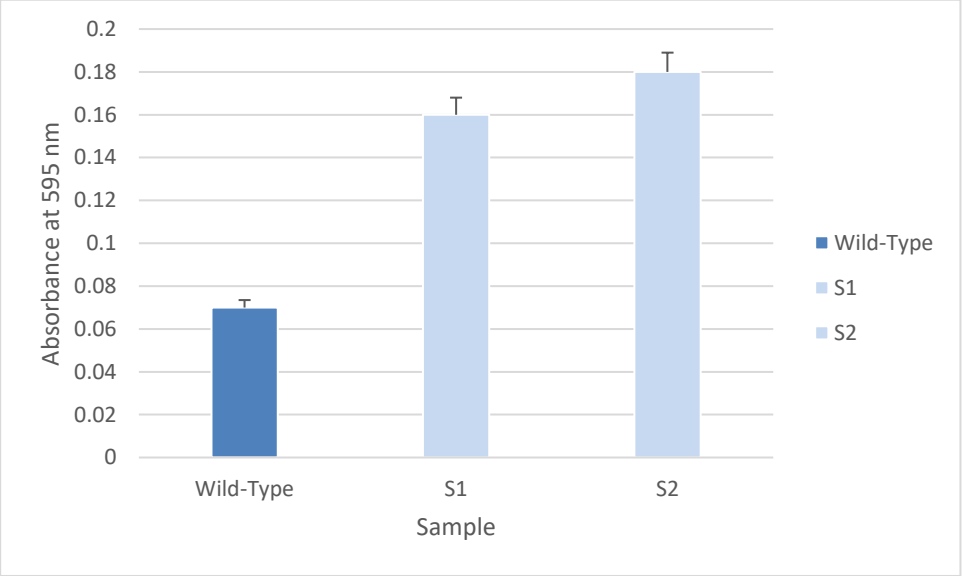

Figure 3.16: Graphical representation of ELISA of wild-type and transgenic *Spinacia oleracea* sample

4. Discussion

Salmonella bacterium is the source of the bacterial infection known as salmonellosis. Humans, animals and birds' intestines are the home to this bacterium. The bacteria invade the digestive tract and replicates inside the intestinal tissues causing the infection. Salmonellosis mainly spreads through the consumption of contaminated food and water (Waldner *et al*., 2012). The important serovar of *Salmonella* involved in human infections is *Salmonella enteritidis.* In 2023, estimated 90 million cases of salmonellosis were reported worldwide with approximately 1.5 million deaths cases due to non-typhoidal *Salmonella* infections (Galán-Relaño *et al*., 2023).

Colon cancer is the 3rd most prevalent type of cancer and is one of the leading causes of cancer-related mortality (Siegel *et al*., 2023). Severe *Salmonella* infections also contribute to the development of colon cancer. As bacteria enters the host body, it secretes a number of proteins that can trigger and activate cancer causing pathways like AKT and ERK (Duijster *et al*., 2021).

Use of antibiotics and chemotherapeutics for treatment of salmonellosis is linked with many disadvantages such antibiotic resistance and limited antibiotic availability in some areas (Wick, 2011). Vaccination is an effective approach against infectious diseases like salmonellosis. The vaccines are intended to boost the immune system's capacity to identify and neutralise particular bacterial infections.

The production of a novel, safe, and more reliable vaccine is required for eradicating infectious diseases especially in developing countries. Development of subunit vaccines is an effective and promising approach to boost the immune system. Outer Membrane Protein C (pOmpC) is an immunogenic protein found in the outer membrane of *Salmonella*. It could serve as subunit vaccine candidate for *Salmonella.* pOmpC stimulates antibody-mediated as well as cell-mediated immunity and can has a potential to serve as DNA vaccine candidate (Guo *et al*., 2013).

Currently available vaccines against numerous IDs have issues with safety, high production and distribution costs, limited scalability, and cooling chain maintenance. It becomes difficult for developing countries to cope with the vaccine production as they face other problems such as poverty, malnutrition etc (Lössl and Waheed, 2011). In order to overcome all these issues, plants offer an alternative approach; they can be used as cheap bio-factories for the development of plant based edible vaccines. Therefore,

spinach has been used for the expression of OmpC antigen in an attempt to develop cost-effective plant-based edible vaccine.

4.1 Selection of *Spinacia oleracea* for the expression of foreign antigens

Plants that have extended shelf life, fast growth rate and long-term storage without any degradation are considered to be the ideal candidates for edible vaccine production (Abd-Aziz *et al*., 2022). In the current study, *Spinacia oleracea* has been used for producing an edible plant-based vaccine. Spinach is a leafy green vegetable grown all over the world in all seasons. It is very rich in iron, vitamins and nutrients. Its genome has been studied extensively. And all these traits make spinach favourable to be used for expression of foreign proteins and for the production of vaccines (Mane *et al*., 2015).

4.2 Optimization of germination media for spinach seeds

The ability of a seed to grow *in vitro* depends on several parameters. These include light, humidity, nutrients, phytohormone concentration, ambient temperature and pH conditions. For proper sprouting of seeds and growth of plants, regulated environment and appropriate concentration of plant culture media is very important. Different concentrations of MS media were used to determine their effect on the efficiency of seed germination. Two combinations of MS media i.e., full MS basal and half MS basal were used. The seeds inoculated on full MS basal showed 75% germination efficiency and the rate of growth was faster as compared to those germinated on half MS media.

4.3 *Agrobacterium*-mediated stable transformation of *Spinacia oleracea*

Agrobacterium-mediated stable transformation efficiency is influenced by several factors. These factors include optical density of *Agrobacterium* culture, infection time and co-cultivation period. The current study describes the best possible conditions for transformation of *Spinacia oleracea.* A crucial element is the choice of O.D. Optical density was measured at wavelength 600 nm OD_{600}. It was found that OD_{600} within range of 0.6-0.8 was found to be effective for transformation and lead to increased transformation efficiency. O.D. levels above 0.8 negatively affect the explants and lessen the efficacy of the *Agrobacterium*'s infection. Similar results were shown by Niazian *et al*. (2019).

Infection time is another factor that influences the transformation efficiency. Two different time periods i.e. 8 and 10 minutes were used to infect the explants respectively

and their resulting callus formation efficiency was calculated. Explants given an 8-minute infection treatment demonstrated a greater callus development efficiency compared to explants treated for ten minutes. Thus, it was concluded that the ideal infection time was 8 minutes. Similar results were shown by Lin *et al*. (2016).

Agrobacterium's transformation efficiency is also impacted by the co-cultivation time. Two different time durations were used i.e. 2 and 3 days. Explants treated with 3 days co-cultivation time showed higher callus formation efficiency of 50% compared with explants that were given 2 days co-cultivation time had 16.6% callus formation efficiency. Thus, 3 days co-cultivation time period was considered as the best time for efficient transformation of spinach. Similar results were showed by Naderi *et al*. (2012).

One important aspect influencing the transformation efficiency prior to infection is the pre-culturing of explants on regeneration media containing hormones. It was found that pre-culturing for 1-2 days was an effective strategy for increasing the transformation efficiency. Similar results were also shown by Geetha *et al*. (1999).

In our study, successful *Agrobacterium*-mediated stable transformation of *Spinacia oleracea* with *OmpC* gene was achieved. Leaf explants were treated for 8 minutes with *Agrobacterium* containing *OmpC* gene. After infection, explants were co-cultivated on media containing BAP and IAA supplemented with 200 µM acetosyringone and were 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's* overgrowth.

Different molecular biology techniques were performed to confirm the successful integration of transgene in spinach such as conventional PCR, qRT-PCR, dot blot assay and western blotting. PCR was performed using *OmpC* specific primers. Wild-type samples were used as negative control. The desired size of amplicon was 897 bp using *OmpC* internal forward and reverse primers. *OmpC* nuclear forward and reverse primers gave amplified product of 1180 bp. No band was observed for wild-type samples.

Using the Kang *et al*. (2012) methodology, qRT-PCR was carried out to determine the copy number of transgene. It was found that positive results occurred for our transgene and no results were obtained for wild-type samples. Copy number of transgene was

calculated in comparison with copy number of endogenous gene *β-actin*. Copy number of *OmpC* transgene was found to be 1.

Dot blot and western blotting were performed for the validation of protein expression. Total soluble protein was isolated from both wild-type and transformed samples. Transgenic samples showed chemiluminescence signal when dot blot was viewed whereas the wild type samples did not show any chemiluminescence signal. For western blotting, SDS-PAGE was run and protein was transferred from gel to nitro-cellulose membrane. The blot was incubated with primary and secondary antibody and finally with substrate. Upon visualization, transgenic samples showed a monomeric 42.2 kDa band confirming the presence of OmpC protein.

OmpC antigenic protein expression was also verified by ELISA. Protein samples from stably transformed explants showed a colour change when TMB substrate was added unlike blank and wild-type samples. This confirmed the presence of OmpC antigen within transgenic samples.

4.4 Conclusion and Future Perspectives

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

- Spinach seeds germinated more efficiently on full MS media.
- Parameters like co-cultivation period, infection time and OD_{600} of *Agrobacterium* culture were 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 proved to be more efficient for seeds germination. 20 mg/L hygromycin was found to be the best concentration for selection of transgenic leaf explants. 8 minutes infection time and 3 days co-cultivation period was shown to enhance the transformation efficiency. Taken together, this study might help to create an edible and a cost-effective vaccine against Salmonellosis in future which might reduce the risk of *Salmonella* infections.

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