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Engineering Plants as Platforms for Production of Vaccines

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Abstract

Microbial pathogens have always posed serious threats and challenges to human existence. Pathogenic microbes causing epidemic and pandemic outbreaks have the potential of effacing life on earth. Vaccines are used as prophylactic as well as treatment measures against diseases and are effective in eradicating deadly pathogens. Conventional vaccines though effective, have high production costs, involve tedious purification processes and have biosafety issues, requiring time-consuming biosafety tests for commercial production. Plant-based vaccines offer several advantages over the conventional systems such as ease of production, storage, higher yields, stability and safety. The review discusses significance, advantages, comparisons, prospects and challenges or constraints in the production of plant-based vaccines and antibodies.

Keywords

Plant Vaccines, Biopharmaceuticals, Molecular Farming, Bioreactors, Regulations

1. Introduction

"Let food be thy medicine"—Hippocrates

Man has evolved in his continuous journey of "struggle for survival" on the earth. Microbial diseases not only had adverse effects on human health and life due to the fatality rate, but also had dwindled the economies of nations world-wide. Pandemics caused by known as well as newly evolved pathogens, which are more frequent during recent times, have sent alarm signals to mankind, to prepare against more deadly pandemics in future and promote development of vaccine platforms for handling the worst outbreaks. The ongoing global catastrophe of massive scale, the COVID-19 is wreaking havoc, killing

people *en masse* without any discrimination of region or race. The world is on the war to vanquish the virus, where all ways and means to control the virus are being tested and implemented. A virus is not considered as a living organism, but is an assembly of biomolecules, an obligate parasite requiring a living host for its multiplication. Also, there is a growing concern globally of the increasing antibiotic resistance among many pathogenic bacteria. Some bacteria and fungi are as well obligate and life-threatening.

Drugs which can cure, and vaccines for prevention and treatment of fatal microbial diseases, are the prime mode of tackling a disease. When an epidemic or pandemic outbreak occurs through spread of deadly pathogen at a fast pace, as with the COVID-19, a solution or cure or treatment of the disease is desired on an emergency basis, than understanding the problem *per se* of the disease and the pathogen, through detailed scientific investigation or research. The conventional system of treatment relies on curative drugs and vaccines, which forms the first line of defense or treatment against the contagion. A vaccine may be attenuated form of the live virus or microbial pathogen, or a subunit of the virus, which can elicit immunity response with production of antibodies, that act against the viral antigens. The body's response to pathogen attack through production of antibodies constitutes active immunity. In passive immunity, the antibodies which can react or bind with the pathogen-derived antigens are administered to induce immunity. Vaccination greatly reduces disease, disability, death and inequity worldwide [1]. Diseases like smallpox have been eradicated and diseases such polio, tetanus, measles etc. are restricted by vaccination [2]. The rapid spread of severe contagious infections such as HIV, SARS, Ebola, and Zika in recent years has emphasised on the significance of global preparedness for pandemics, which necessitates extremely rapid development and comprehensive distribution of vaccines against potentially deadly, novel pathogens [3]. The challenges to develop and produce vaccines and therapeutics are immense, due to the ever-increasing or rapidly evolving pathogens, resulting in greater demand than supply. Rapid development and large-scale production of vaccines is the need-of-the-hour in case of an unexpected global calamity of a pandemic of massive scale. Plants offer a safe alternative for low-cost as well as large-scale production system for the vaccines, especially in developing countries. The review describes the strategies, advantages, challenges and prospects in the production of plant-based vaccines.

2. Vaccines: Types and Production Systems

2.1. Types of Vaccines

A vaccine provides immunity against a disease. Vaccines used for immunisation against diseases can be categorised into live attenuated vaccines, inactivated vaccines, subunit, recombinant or conjugated vaccines, toxoid-based vaccines, viral vector-based vaccines and nucleic acids-based vaccines.

Live attenuated vaccine uses weakened or attenuated form of a pathogen, that

causes a disease. They provide strong long-term immune response as they are similar to natural infection. Attenuated vaccine may have small amount of the live virus which may be risky for people with weak immune system. Also, they require cold storage facilities. Live attenuated vaccines are used against smallpox, chickenpox, Measles, Mumps and Rubella (MMR vaccine) etc. [4].

Inactivated vaccines use killed or inactive pathogen and require booster doses of vaccine for immunity [5]. Flu, pertussis, polio, rabies vaccines etc. are inactivated vaccines.

Subunit, recombinant, polysaccharide and conjugate vaccines use part of the pathogen such as protein, capsid (viral coat protein), sugar moiety etc. Subunit vaccines comprise of purified antigen(s) derived from the pathogen, while conjugate vaccines, consist of a polysaccharide component of the pathogen that is poorly immunogenic, so that it is chemically linked to a protein. Recombinant subunit vaccines are safer since they do not have a pathogen and can also be scaled up. Since subunit vaccines consist of small fractions of the pathogen, immunogenicity is greatly decreased with respect to those derived from whole cells, generating the need for co-administering adjuvants to attain immune-protection [6]. They can be used in patients with weak immune systems and require booster doses to maintain immunity. These vaccines are used against Hepatitis B, Whooping cough, Human Papilloma Virus (HPV) etc.

Toxoid vaccines use toxins from the pathogen that causes a disease. Diphtheria and tetanus vaccines are toxoid vaccines [7].

Viral vector-based vaccines such as Adenovirus (Ad) or measles virus vectors are highly versatile platforms for vaccine development. Viral vector-based vaccines can be used for different viruses, delivered without additional adjuvants and can be administered as intramuscular, intranasal, intradermal and oral vaccination. High yield production processes and means of upscaling have been established for these vaccines so that they can be used immediately in case of a pandemic outbreak. But viral vectors are genetically modified organisms (GMOs) considered as potential risks to human health and environment and unsafe due to persistent replication of attenuated vaccines. Viral vectors can integrate into the host genome, or undergo recombination during production, leading to emergence of uncharacterised or novel pathogens. These safety concerns might also delay clinical studies in case of a pandemic. Viral vector-based vaccines are highly complex and comparatively cost-intensive [3] [8].

Nucleic acid-based vaccines employ antigen-encoding plasmid DNA or RNA or messenger RNA or viral replicons. Due to the ease of antigen manipulation they are also versatile. Vaccine can be developed against various pathogens such as virus, bacteria or parasite and administered as intramuscular or intradermal injections. A eukaryotic expression cassette carrying the antigen is inserted into a bacterial plasmid for propagation in *E. coli*. Minimal DNA constructs devoid of a bacterial backbone, such as the semi-synthetic minicircle DNA and the fully synthetic Doggybone™, have been developed to avoid safety issues related with selectable marker [9]. DNA vector vaccine provides relatively low immuno-

genicity, since DNA vaccines must cross both plasma and nuclear membranes for protein expression, unlike the RNA vaccines which upon crossing plasma membrane are translated. Encapsulation of DNA vaccines in lipid nanoparticles, adsorption to polymers and use of molecular adjuvants like cytokines can enhance the uptake of DNA vaccines and enhance the immune response. DNA vaccines have long-term persistence, however, potential risk of genomic integration of exogenous DNA into the host genome or chromosomes may result in mutagenesis and oncogenesis or new diseases. Molecular adjuvants like cytokines may also have undesirable, side-effects such as inflammation or autoimmunity. DNA vector-based antigen expression is the first effective vaccine against Ebola virus, Zika virus etc. and used against human pathogens such as HIV, influenza virus, malaria, hepatitis B virus, respiratory syncytial and herpes simplex virus [3] [10].

RNA vaccines use either non-replicating mRNA and/or self-amplifying mRNA as vaccine. Non-replicating mRNA contains antigen sequence flanked by 5' and 3' untranslated regions (UTRs). The mRNA with a protein-encoding open reading frame (ORF) flanked by a 5' cap structure, poly(A) tail at the 3' end, as well as 5' and a 3' untranslated regions are obtained by *in vitro* transcription of a cDNA template, typically plasmid DNA (pDNA) produced in *E. coli*, which is linearized using restriction enzyme and transcribed using recombinant phage DNA-dependent RNA polymerase. Self-amplifying mRNA vaccines are based on the alphavirus genome, where the genes for structural proteins are deleted and replaced with the antigen of the pathogen. Large size of these vaccines, lower yields and increased occurrence of abortive constructs are challenges to vaccine production. Extracellular ribonucleases can catalytically hydrolyze unprotected "naked" mRNA, which is also highly unstable under physiological conditions. Hydrophilicity and strong net negative charge of RNA prevents its uptake by cells after application *in vivo*. This can be overcome by complexing of mRNA with highly efficient carriers to form protamine-complexed mRNA or with complexing agents such as lipid- and polymer-based nanoparticles. mRNA can be administered as intradermal, intra venous or intra-muscular injections. RNA vaccines are used against influenza, Zika and Ebola virus infections [10] [11].

2.2. Vaccine Production Systems

In conventional method of vaccine development, a pathogen is inactivated or attenuated, concentrated and purified to develop a vaccine. The vaccine production systems can be broadly categorised into three viz., the egg-based vaccines, cell-based vaccines, and vaccines produced using investigational-manufacturing systems [12]. Each vaccine technology has its own advantages and disadvantages related to its ability to induce certain immune responses, manufacturing capacity and safety for human use. Embryonated eggs are used for vaccine production in egg-based vaccines, which is a commonly used system for production of Influenza vaccine. The virus particles are injected into eggs, incubated for virus replication and the viral antigens or vaccines are isolated and purified from eggs. But

this method cannot be used for all strains of the virus, large number of eggs are required and involves time-consuming regulatory processes. Cell-based production systems such as mammalian cell culture systems could be used for production of viral antigens of subunit vaccine because they can produce high titres (1 - 5 g/L) of complex proteins with mammalian glycan structures, but require costly infrastructure for production and monitoring for safety, since mammalian cultures are prone to contamination with mammalian pathogens and oncogenic agents and are poor in scalability [13]. Recombinant subunit vaccines produced in genetically modified cells have better safety, less antigenic competition, specificity and the ability to differentiate between infected and vaccinated animals. The gene encoding a protective antigen is expressed in a heterologous system and the resulting protein is purified and administered as a vaccine [14]. Investigational manufacturing systems such as bacteria, yeast or insect cells and plants are used for production of recombinant vaccines. *Escherichia coli* was the bacterial system which was used earlier for production of recombinant subunit vaccines [15]. But protein folding and post-translational modifications do not occur correctly in bacterial system. Hence eukaryotic cell systems like yeast which were simple, use inexpensive culture media for growth and carry out folding and N glycosylation of proteins, were used. *Saccharomyces cerevisiae* was used for production of hepatitis B virus surface antigen particles or vaccine [16]. However, the glycan structures in yeast system differ from that of mammals. Insect cell cultures though less expensive also have low scalability.

The conventional vaccine production approaches such as egg-based and cell-based production systems were followed for eradication of smallpox and for controlling polio, tetanus, measles etc. But the conventional method of whole pathogen cultivation for vaccine production may not be feasible during a disease outbreak because of low producibility, requirement of *in vitro* conditions, high biosafety level and specialised labs for cultivation. Also, there is a risk of reversion of the attenuated inactive form of the pathogen to a highly pathogenic form, no protective responses as in Ebola or undesirable side effects as in case of formalin-inactivated respiratory syncytial virus. Currently, the average development time for conventional vaccines from preclinical phase is more than 10 years [3]. In case of an outbreak, time is a major constraint, requiring development of vaccine at a fast pace, in large quantities and with nominal side-effects. Other challenges during an outbreak are unpredictablility in pathogenicity, mutation rate and adaptability of the novel pathogen. Already licensed vaccines would take 3 - 5 months between identification of a pandemic influenza and vaccine distribution, which would cause wide global spread of the pandemic virus.

2.3. Plant Vaccines

Plants can be engineered and used as production platforms for low-input, large-scale production of vaccines or pharmaceuticals with immense scalability [17]. In plant vaccines, the biomass or purified fractions are intended to serve

as elicitors of protective immunity throughout the administration by distinct routes.

The genes encoding the antigen protein of the pathogen causing a specific disease are integrated into the plant genome through artificial methods, where the plant produces the antigen protein which confers immunity, when purified from plant and administered as vaccine, or directly consumed as an edible vaccine. The plants act as bioreactors for these pharmaceuticals or therapeutically important proteins, that can be used for humans as well as animals. Plant-based vaccines are, biologically active and produced inexpensively as well as in substantial amounts to elicit an immune response [18]. Plants thus offer a less-expensive production system and an effective and efficient delivery system.

Plant vaccines are effective, feasible alternatives for resource-poor or low-income countries which do not have powerful healthcare infrastructure to produce their own vaccines nor have benefited from the current vaccination programs due to the expensive vaccine development technologies [2].

2.3.1. Strategies of Production of Plant Vaccines

Production of plant vaccines involves two components 1) Research and development and 2) Commercial production. Generally, vaccine development has 6-phases according to the Center for Disease Control and Prevention (CDC), USA. These are exploratory, preclinical, clinical development, regulatory review and approval and finally manufacturing and quality control [19].

In exploratory phase, research and development on synthetic or natural antigens or weakened strains of the pathogenic virus are carried out to treat or prevent a disease. In the pre-clinical phase, tissue culture or cell culture systems and animal testing are undertaken, to verify the effectiveness of the candidate vaccine to provide immunity. In the third phase of clinical development, a proposal or application describing the research findings and for conducting clinical trials, is submitted by the vaccine manufacturing firm to the sanctioning authority. Once proposal to conduct clinical trials are approved, human testing or trials are conducted in 3 stages. In Phase I, the candidate vaccine is administered to a small group of people (<100) to know the safety. Phase II involves larger group of subjects in hundreds to know about safety immunogenicity, immunization schedule, dosage etc. Still larger subject group of thousands are covered in Phase III trials where side-effects, safety and effectiveness of the candidate vaccine is assessed. This is followed by regulatory review and approval where application for licence for manufacturing by the firm is scrutinised for approval. Next step is manufacturing the vaccines and then quality control to monitor the performance, safety and effectiveness of the vaccine. Development of plant vaccine also should necessarily involve all these steps. In addition, regulatory processes are involved to address the issue of gene escape, biosafety, environmental and health hazards, before for release of transgenic plant with the vaccine, which is time-consuming. Development of plant vaccines involves research and development, assessment of quality and safety of product, obtaining approval from

regulatory bodies, production and delivery of the vaccines or therapeutic proteins and assessing the effectiveness of the product in disease control, through proper monitoring (Figure 1).

Production of plant vaccines comprises of various steps such as selection and design of antigen from the pathogen, selection of a suitable vector for the antigen and the most suitable host plant for vaccine production, transformation of plants using the vector carrying the antigen, regeneration of the transgenic plants after confirmation of expression of antigen in the host plant and evaluation and characterisation of the purified antigen or immunogen or whole plants (in case of edible vaccines) for the immunogenicity or immune-protectiveness. These are discussed below.

1) Selection and design of antigen/ vaccine

The first step in vaccine production involves selection of the protective antigen from the pathogen and designing immunogen using bioinformatics, genomics and proteomics tools. Immuno-protective epitopes can be identified by assays such as phage display technology and requires fully annotated genome sequence of the pathogen, a heterologous protein expression system and a model that mimics human immunological mechanisms. A design based on highly immunogenic carriers for the elicitation of effective immune responses to unrelated antigens is important. After design of immunogen, the transgene encoding the antigen must be designed and synthesised using recombinant DNA technology. Flanking restriction sites have to be included to facilitate the molecular cloning construct expression vectors, codon bias should be matched with that of the expression host, and undesired introns or unstable RNA motifs should be removed to optimise gene expression in the specific host [20].

2) Selection and design of vector

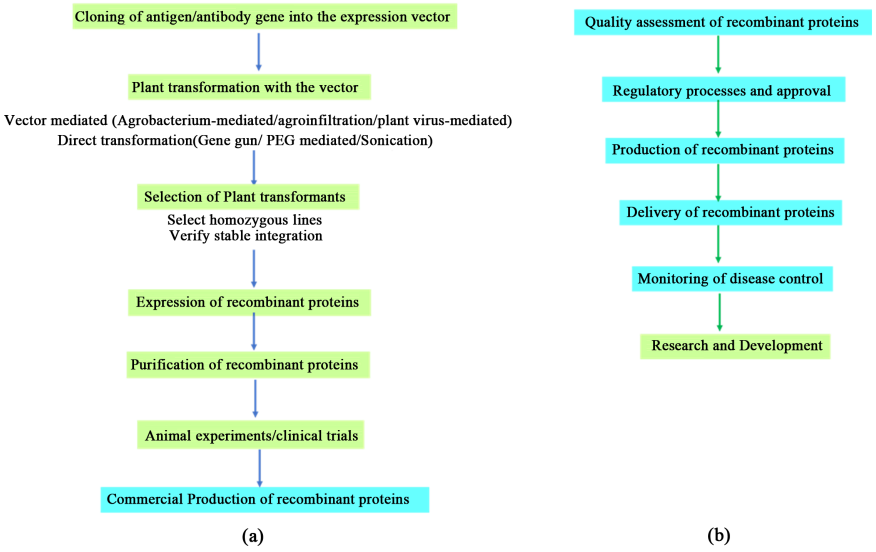

Figure 1. Steps in development and production of plant-based vaccines or recombinant therapeutic proteins (a) Research and development and (b) Commercial production of plant-based therapeutic proteins.

A specific expression vector for plant-based expression of antigen has to be selected or designed. Expression cassettes can be driven by constitutive promoters or, alternatively, by inducible or specific promoters such as seed-specific promoters, for selective expression of the antigen protein in a particular tissue or organ, in order to maximize accumulation or for higher yield, long-term storage at ambient temperature and to avoid harmful deleterious effects on the plant host [21]. Also, transcription machinery such as T7 RNA polymerase expressed from the nuclear plant genome to enhance the expression of a transgene, 5' UTR for translational efficiency, 3' UTR for transcript polyadenylation and mRNA stability, expression cassette-flanking regions, which mediate homologous recombination events for site-specific integration of the expression cassette etc. can be engineered [22]. Many plant expression vectors are commercially available.

3) Choice of plants

The plants to be chosen depend on the amenability to transformation and regeneration *in vitro*. Availability of or development of a stable transformation as well as regeneration protocol is pre-requisite for choosing a species or plant for production of plant vaccines. The expression strategy, life cycle, biomass yield, containment, and scale-up cost are the factors to be considered while choosing a plant system [23]. They should also have fast growth and high biomass production. *Nicotiana benthamiana* and *N. tabacum* are widely used for molecular farming for these reasons. Non-food crops or model systems such as tobacco, duck weed, *Arabidopsis* etc do not accumulate high amount of protein and increase extraction costs of vaccines due to high proportion of phenolic compounds. Earlier, tobacco and potato were the systems of choice for production of many plant-based recombinant proteins, due to the ease of genetic modifications. Food crops have higher proportion of stored protein, are safe for human consumption, can be directly ingested as oral vaccines, but raises concerns on potential of contamination of food and feed crops. However, for edible vaccines, fruit crops are preferred since they can be consumed directly without cooking, else heat during cooking may destroy the protein antigen. Many plant species including maize, carrot, tomato, soybean, lettuce, potato, and alfalfa are used now, since they offer better yields with no toxic compounds, making possible oral immunization using raw plant materials. Papaya and banana are good candidates for rapidly producing cheap vaccines in developing nations since they have high quantity of vitamin "A" and have sterile condition as in banana. Also, the genes are not transferred from one plant to another [2] [24].

4) Transformation of plants

Plants can be transformed with antigen containing recombinant vector through direct gene delivery (biolistic method and PEG (Polyethylene Glycol)-mediated protoplast transformation or by vector-mediated (*Agrobacterium*-mediated or plant virus-mediated) nuclear or by plastid transformation. Stable nuclear or plastid integration of the foreign gene occurs through these methods. Chloroplast transformation is a rapid and low-cost production technique due to high copy number of plastids in a plant cell, has potential to express

multiple genes in plastids, ensures site-specific integration of transgene in the plastid genome, eliminates gene silencing effect and ensures natural transgene containment. Maternally inherited plastids prevent gene flow through pollen or pollination. However, functional heterologous proteins requiring complex post-translational modifications cannot be produced by chloroplast transformation since glycosylation process does not occur in plastids. Vaccines derived from chloroplast were developed against bacterial diseases such as cholera, lyme disease, anthrax, tetanus, and plague, and against viral diseases such as rotavirus and canine parvovirus (CPV). Subcellular targeting signal sequences such as vacuolar or cell wall targeting signals improve antigen accumulation. *Agrobacterium*-mediated method is widely used for plant transformation to create edible vaccines or plant-based vaccines. Another modification of *Agrobacterium*-based transformation is agroinfiltration. Using agroinfiltration methods such as syringe and vacuum infiltration, *A. tumefaciens* suspension is infiltrated into the intracellular spaces of desired parts of the plants for transient expression of desired protein or transgene. The expression level of antigen protein in the plant cells is increased [2] [12].

Stable plant transformation for production of vaccines is a time-consuming process, absorbing much time to generate the plants and has containment issues such as transgene escape etc. Transient expression of antigens and use of plant cell culture bioreactors as well as greenhouse-grown plants are alternative methods to express vaccine or therapeutic proteins in plants.

5) Regeneration of plants

Proliferation of the transformed cells through a selective condition in the presence of a selective agent, to regenerate plants from successfully transformed cells is important. *In vitro* conditions including plant growth regulators and culture conditions such as light, temperature etc. that direct the regeneration processes or morphogenetic response of the tissue is to be optimized for the selected plant. Somatic embryogenesis and organogenesis are the two pathways for plant regeneration. In direct somatic embryogenesis, the embryo is formed directly from a cell or group of cells without the production of an intervening callus, while in the indirect somatic embryogenesis, callus is first produced from the explants. In organogenesis, organs are produced from callus or explant. Regeneration steps are avoided in transient expression systems, where whole plants are used and the transgene or DNA is not stably integrated in the genome nor inherited, but expressed temporarily in the host.

6) Evaluation and characterisation of plant vaccine or immunogen

Enzyme-linked immunosorbent assay (ELISA) and western blot assays are used to quantify the foreign protein or antigen in the plant. Immunogenicity of the plant vaccine is assessed in the preclinical level, when test animals are subjected to a defined immunization scheme and antibody levels and proliferation of specific immune cells are often evaluated by ELISA and splenocyte proliferation assays. Immuno-protective potential of the vaccine is evaluated by scoring of deaths in vaccinated and unvaccinated test animal groups or by measuring

humoral or cellular immune responses. A small number of vaccines or candidates have been tested for immunogenicity in humans. Clinical trials utilizing transgenic plants for vaccines mostly consist of either the leaves or fruits from the plants [6].

Generation of transformed plant takes much time. Molecular farming technologies other than transgenics include plant cell suspension cultures, plant virus-based transient expression systems and use of plant viruses as vaccines.

2.3.2. Plant Cell Culture Bioreactors for Vaccines

Plant cell suspension culture using protoplast or cell culture, or hairy root culture, combines the advantages of higher eukaryotic cells (efficient protein folding and post-translational modification), with the use of simple and inexpensive growth media, to make them suitable for the production of recombinant proteins. Plant cell suspension cultures, rather than whole plants are cultivated under aseptic conditions using classical fermentation technology, are easy to scale-up for manufacturing, and the regulatory requirements are similar to the existing regulations, established for well-characterized production systems based on microbial and mammalian cells. Plant cells cultures have a quick development cycle, have contained production and suitable for transient expression for rapid production of high protein yields of vaccines and prophylactic antibodies required during medical emergencies or outbreaks such as the Ebola virus disease in West Africa, but are the least scalable [25]. Also, large number of bacteria introduced into the leaves increases the endotoxin load [26]. Cell suspension cultures generated *de novo* by transformation of wild-type cells are always polyclonal because transformation is not 100% efficient and the transgenic cell lines can also undergo somaclonal variation, to generate cell populations with heterogeneous expression levels, necessitating screening and selection of the productive cell lines, optimizing the expression construct and culture conditions. Plant glycans also can affect the stability and functionality of recombinant proteins. Plant-based systems still face one major bottleneck that needs to be overcome—their lower yields compared to mammalian cell cultures [13]. The first licensed recombinant pharmaceutical protein derived from plants, Taliglucerase alfa (Elelyso®) was produced in plant cell suspension cultures. Vaccine against Newcastle disease virus (NDV) produced in tobacco suspension cultures by Dow Agrosciences, LLC, Indianapolis, USA was the first tobacco cell-based vaccine approved by the FDA against Newcastle disease virus in poultry. *Physcomitrella patens* (an alga) suspension cultures were used to produce α-galactosidase for Fabry disease and βa for Gaucher disease by Greenovation Biotech BMBH company in Germany [27]. Cells from tobacco, rice, medicago, carrot, tomato, sweet potato, soybean, Siberian and Korean ginseng are already used for production of Hepatitis B vaccine, Hepatitis antibody, Anti-HIV antibody, Norwalk virus capsid protein, immunomodulators, growth hormone, lactoferrin, interferons etc. Screening of high producing genotypes, selection of adequate medium, and optimization of the culture environment for plant cell culture may increase pro-

duction [28].

2.3.3. Plant Virus-Based Expression Systems

The plant virus-based expression systems can be used as transient expression systems, which use whole plants and avoid tedious regeneration processes. This is desired when rapid protein yield is needed and overcomes the difficulties of stable transformation. The plant virus-based expression systems can be either epitope presentation systems or polypeptide expression systems. Short antigenic peptides are fused with the coat protein and are displayed on the surface of assembled viral particles in epitope presentation systems. In polypeptide expression systems, whole unfused recombinant proteins are expressed and accumulated in plants. But insert size and limited host range are major constraints in this system. Recombinant plant virus-based nanoparticles (PVNs) made from genetically engineered isometric or helical viruses, with antigenic epitopes from pathogens elicit effective immune responses. Self-assembling coat proteins from viruses form the protein nanostructures free of genetic material and are referred to as virus-like particles (VLPs), which are non-infectious and lack replication potential. VLPs with replication potential in plants are virus nanoparticles (VNPs). Non-enveloped helical plant viral capsids which are flexible and stable in terms of expressing foreign genes, are ideal platforms for epitope presentation system. Helical plant viruses, such as, bamboo mosaic virus (BaMV), cardamom mosaic virus (CdMV), johnsongrass mosaic virus (JGMV), papaya mosaic virus (PapMV), papaya ringspot virus (PRSV), plum pox potyvirus (PPV), potato virus X (PVX), potato virus Y (PVY), tobacco etch virus (TEV), tobacco mosaic virus (TMV), and zucchini yellow mosaic virus (ZYMV) have been genetically engineered to display immunogenic epitopes on their surfaces for vaccination against several diseases [29].

Tobacco mosaic virus (TMV)-based expression vectors are the most widely used vectors to produce foreign proteins in plants. First-generation viral vectors retain infectivity in the plant, but have raised safety concerns. Second-generation viral vectors called viral "deconstructed" vectors have minimum of viral elements required for replication of the vector, and most DNA delivery to the target plant is *via* non-viral elements. A launch vector is developed with characteristics of *Agrobacterium* binary plasmid and plant virus expression vector. Tobacco Mosaic Virus (TMV) is the viral vector widely used for the expression of foreign proteins in plants. Single-stranded positive-sense RNA genome of TMV encodes viral replicase genes for virus replication, while cell-to-cell movement protein (MP), and coat protein (CP) genes are needed for recombination, effective spread and survival of the virus in the environment. The target antigen gene that replaces CP gene which is inserted into a unique cloning site, under the transcriptional control of the coat protein sub-genomic mRNA promoter replicase, MP and viral replicase gene required for the replication of TMV gene was inserted between left and right border sequences (LB and RB) of the *Agrobacterium* binary plasmid to form a launch vector, which is transferred into plant cells

by agroinfiltration. Multiple single-stranded DNA (ssDNA) copies of sequence between LB and RB are generated and released. Thus, viral vector is launched into plant tissue [30].

These types of vectors have been used as an expression system for monoclonal antibodies due to their high and stable levels of protein expression. The viruses can infect the plant, producing a systemic infection, generating multiple copies of the genome. Leaves can be harvested after few weeks post-infection, followed by antigen purification. Antibodies against West Nile virus were produced in *Nicotiana benthamiana* developed by agroinfiltration [31].

2.3.4. Edible Vaccines

Edible vaccines include all vaccines produced in an edible format (i.e., part of a plant, its fruit, or sub products derived from that plant which, upon oral ingestion, stimulates the immune system. Edible does not necessarily mean nutritious, tasty, or organoleptically pleasing since edible vaccines need only be safe (non-toxic) for human consumption [32]. The term edible vaccine was coined in 1990 by Charles Arntzen [33]. Once an edible vaccine is consumed, the outer wall of plant cells protects the antigens from degradation by gastric secretion, delivering the antigens to the intestinal mucosal surfaces, where they are absorbed by different mechanisms to stimulate a strong and specific immune response [34]. Edible vaccines are attractive choices to reduce cost of production as they obviate the need for extraction and purification of antigens as well as sophisticated immunisation and storage facilities. Edible vaccines produced against Coronavirus (MERS-CoV) (outbreak from 2012 to 2016) in tomato and corn, against cholera (outbreak of 2010 to 2013 and 2015) in potato, tomato and algae and against rubella in tomato were already tested in animals. Probiotic commensal bacteria such as *Listeria monocytogenes*, *Streptococcus gordonii* and *Lactobacillus casei* can be genetically modified and used as edible vaccine against influenza, HIV and anthrax [35].

2.3.5. Plantibodies

Antibodies constitute the humoral adaptive immune system which specifically recognize and bind to target antigens or toxins of pathogens. An antibody has a binding region or paratope which binds to the epitope or antigenic determinant on the antigen and varies depending on the conformation of the epitope. The constant region of antibody (located on the heavy chain) determines the class and subclass of antibody. For binding of antigen and antibody, the shape of the paratope must fit the epitope, so that several non-covalent bonds can form simultaneously. Antibody-coding genes from mammals/humans can be engineered into plants to make antibodies and antibody fragments called the plant-derived antibodies or plantibodies [36]. In 1990, plants were first considered as a potential host for producing antibodies and the word "plantibody" was coined.

Plants are capable of synthesizing and assembling virtually any kind of anti-

body molecule, ranging from the smallest antigen-binding domains and fragments to full-length and even, multimeric antibodies. The antibodies are purified from plants through filtration, chromatography etc. [37]. The first plantibody produced in tobacco, CaroRx®, is a clinically advanced anti-*Streptococcus mutans* secretory immunoglobulin that binds to the bacterium, thus protecting humans from dental caries [38]. Later a humanised antibody against herpes simplex virus glycoprotein B was expressed in soybean [39]. Plantibodies have also been produced against anthrax, Ebola and various forms of cancer in humans. The drug, called ZMapp, contains a cocktail of three humanized anti-Ebola virus mAbs and was developed by Mapp Biopharmaceutical Incorporated, San Diego. 360 million doses of plantibodies against anthrax can be produced from a single acre of tobacco while 1.5 kg of antibodies is provided per acre of corn, compared to vaccines. However, the introduced plantibodies are flushed through a person's system relatively quickly, in a matter of hours or days, which necessitates the patient to take doses indefinitely.

2.3.6. Human Interferons

Higher levels of accumulation of human interferons were obtained by targeting the hIFN-γ protein to endoplasmic reticulum (ER) or apoplastic space than in cytoplasm of tobacco. The protein was biologically active and protected from infection generated by vesicular stomatitis virus (VSV) [40].

2.3.7. Other Pharmaceuticals

Plants are natural reservoirs of compounds or metabolites which have antimicrobial (antiviral, antibacterial, antifungal, anti-parasitic) properties. However, the production of such compounds may be low in their natural source. The plants that synthesize these compounds do so in low concentrations and grow slowly resulting in only minute quantities of the desired compound [41]. Engineering the biosynthetic pathways for these compounds into heterologous plants optimized for molecular farming could boost supplies and reduce costs [42]. Anti-cancer drug Taxol (paclitaxel) and artemisinin, a crucial anti-malarial compound are few examples of such pharmaceutical compounds [43].

2.4. Status of Production of Plant Vaccines

The attempt to produce vaccines in plants was made by Hiatt and co-workers in 1989. The first plant made vaccines (PMVs) were described by Curtiss & Cardineau in 1990 [44]. First demonstration of subunit vaccine or antigen expression in plants was that of *Streptococcus mutans* surface protein antigen (SpaA) in tobacco in 1990. Large amounts of antigens could be produced for parenteral and oral applications. Stimulation of a mucosal immune response could prevent *S. mutans* that causes dental caries, from colonizing the tooth and thus prevent tooth decay [45]. This research resulted in the first patent (US patent No. 5,654,184) related to the plant-based vaccine technology [46]. The concept of using engineered or transgenic plants to produce and deliver subunit vaccines

was introduced by Charles Arntzen [47]. Production of hepatitis B and heat-labile toxin B subunit in potato tubers as well as potato plants was also initiated. Direct consumption of transgenic plant parts could reduce high production costs like purification, storage and transportation costs. In 1998, the first human trial of edible vaccine was carried out with raw potato expressing a part of *Escherichia coli* toxin that causes diarrhea. It was for the first time proved by National Institute of Allergy and Infectious Diseases (NIAID) that significant immunogenicity can be induced safely by an edible vaccine. After two years, antigens of Norwalk virus (that causes diarrhoea) were expressed in potato [48]. The norwalk virus antigens were expressed in transgenic tomatoes as well. Similarly, rabies glycoprotein and hepatitis B surface antigen were expressed in spinach and lettuce respectively. Plant as a bioreactor is cheaper, easy-to-handle, requires no sophisticated or cold storage facilities, easy to scale up, cost-effective or less-expensive production, involves effective, convenient and easy route of administration. Bacterial, viral, parasitic and immune-contraceptive vaccines can be produced in plants as edible vaccine [12].

The first chimeric gene expressed in plants was human growth hormone expressed in sunflower and tobacco plants, transformed through *Agrobacterium*-mediated transformation. Mouse monoclonal antibody (mAb) was produced in tobacco leaf. First generation plant-based vaccines were produced against influenza virus, human papilloma virus and norovirus by modifying PVX or TMV. In second generation plant-based vaccines, deconstructed viral vectors devoid of different viral elements needed for its replication and infectivity were used. Recombinant viral vectors with heterologous coat protein, with cell surface presentation of foreign antigen in the viral coat protein and sub-genomic promoters etc were more stable, environmentally safe and provided high yield.

Potato and tobacco were used as model organisms initially in development of plant-based vaccines. Potato was used as model plant in edible vaccine production since it was easy and efficient to transform, tuber-specific promoters could be used to express transgene/antigen gene, outcrossing risk was low, clonal propagation to produce stable transgenics was possible, tubers could be eaten (cooked), were used in food industry and tubers could be stored for long periods without refrigeration. Tobacco and alfalfa have leaves which are major source of biomass; banana, tomato, apple, guava and strawberry were the fruit crops; peanut, corn, soybean and chickpea were seed-based crops; cabbage, lettuce, potato, carrot and spinach are the vegetable plants which were used for the production of vaccine antigens.

Plant vaccines have been developed for many human diseases such as hepatitis B, Human Immunodeficiency virus (HIV), rabies etc. More than 25 vaccines licensed for use in humans with many more in the development pipeline [14]. In March 2018, Medicago Inc. conducted phase III clinical trials to develop flu vaccine in tobacco and is expected to be launched in the market by 2020-2021. In

June 2018, researchers from the University of Nottingham, Malaysia, launched a project to develop plant-based vaccine against dengue fever, caused by *Aedes* mosquitoes. Researchers from Arizona State University's (ASU) Biodesign Institute, developed a norovirus vaccine from tobacco plant in August 2018. Other than tobacco, many edible crop plants are also used such as the dengue virus vaccine produced in lettuce through chloroplast transformation in 2016. Medicago Inc., iBio Inc., Icon Genetics-GmbH, Creative Biolabs etc. are involved in plant-based vaccine development. The Queensland University of Technology, Australia plans to use the genome sequence information of *Nicotiana benthamiana* to use the plant as biofactory to produce antibodies, vaccines and therapeutics, to develop protein-based diagnostic products in bulk quantities at a low cost against COVID-19 or similar viruses or pathogens.

Although plant-based human vaccines are not approved yet, vaccines against influenza, norovirus, hepatitis B virus, rabies virus etc. have been successfully produced in various transgenic plants and tested for their safety and efficacy under clinical trials [49].

Veterinary Vaccines

Vaccines against several animal diseases have been developed in plants, tested in animal models and in target animal species with the disease. Vaccines against anthrax, Bovine Herpes virus 1, enterotoxigenic *E. coli* etc. were produced in tobacco. The Foot and Mouth Disease (FMD) virus VP1 epitope were expressed in alfalfa and *Arabidopsis* leaves and potato tubers, while the FMD virus polyprotein P1-2A/protease 3C was expressed in tomato. Epitopes of mink enteritis virus, murine hepatitis virus and rabbit haemorrhagic disease virus were produced through cowpea mosaic virus display in cowpea, Tobacco Mosaic Virus epitope display in tobacco and Plum pox virus epitope display in tobacco respectively. The infectious bronchitis virus S1 glycoprotein was expressed in potato tuber while transmissible gastroenteritis coronavirus glycoprotein N terminal domain was expressed in maize grains [14]. Bovine trypsin derived from maize has been commercialized since 2002 [49]. Neutralizing antibody responses were elicited against homologous and heterologous Newcastle Disease virus by inoculating plant-produced fusion protein (F) antigen (transmembrane glycoprotein), into Specific Pathogen Free (SPF) chickens [50]. Newcastle disease vaccine derived from tobacco cells was first approved for poultry use by United States Department of Agriculture [49].

In addition to expression of antigen for vaccine production (**Table 1**), pharmaceuticals such as antibodies, enzymes, therapeutically important proteins or peptides and growth hormones are produced in plants [49] [76].

2.5. Advantages of Plant-Based Vaccines

2.5.1. Low Cost of Production

The vaccines used for immunisation against contagious disease are mostly costly and not easily accessible. On the contrary, the plant bioreactors are cost-effective

Table 1. Plant based vaccines and their status.

Sl No	Vaccine/Antigen	Pathogen/Disease	Crop	Transformation method	Reference	Remarks
			Viral			
1	Norwalk virus capsid protein	Norwalk virus causing Gasteroenteritis	Tobacco leaves, Potato tubers	*A. tumefaciens*	[51]	Immunogenic in humans and mice
2	Respiratory Syncytial viral G and F protein	Respiratory Syncytial virus	Alfalfa mosaic virus display in Tobacco	Viral vector	[52]	
3	Rotavirus VP6 protein	*E. coli*, rotavirus and *Vibrio chlolerae*	Potato tubers	*A. tumefaciens*	[53]	Immunogenic in mice
4	Recombinant vaccinia virus B5	Smallpox	Tobacco and Collard	Agroinfiltration	[54]	
5	Rabies virus glycoprotein and nucleo protein	Rabies	Viral vectors in Spinach leaves	Plant virus (TMV)-based expression system	[55]	Immunogenic in mice and humans
			Maize (*Zea mays*)	Biolistic	[56]	Immunogenic in mice
			Viral vectors in tobacco leaves	Agroinfiltration	[57]	
			Lettuce	*A. tumefaciens*	[58]	
6	Hepatitis B surface antigen	Hepatitis B	Tomato	*A. tumefaciens*	[59]	
			Maize	*A. tumefaciens*	[60]	
7	Dengue virus glycoprotein	Dengue	Tobacco	*A. tumefaciens*	[61]	
8	Ebola immune complex	Ebola virus	*Nicotiana benthamiana*	Gemini viral vector	[62]	Immunogenic I mice
9	Monoclonal Antibody cocktail Zmapp	Ebola (2014)	Tobacco		Mapp Biopharmaceutical, 2014	Administered in 2 patients
10	Influenza vaccine	Influenza	Tobacco		Medicago Inc, 2018	Clinical trials
11	SARS-CoV-2 S protein	COVID-19	*N. benthamiana*	Viral vectors	Medicago Inc., 2020	Clinical trials
			Bacterial			
1	Cholera toxin B subunit protein	Cholera	Potato	*A. tumefaciens*	[63]	Immunogenic in mice
			Tomato	*A. tumefaciens*	[24]	
			Rice (MucoRice-CTB/Q)	*A. tumefaciens*	[64]	
2	*E. coli* heat-labile toxin B subunit	*Escherichia coli*	Potato tubers	*A. tumefaciens*	[65]	Immunogenic in mice
			Maize seeds	A. tumefaciens	[66]	
			Carrots	*A. tumefaciens*	[22]	Immunoprotective in mice
3	*Pseudomonas aeruginosa* membrane protein F epitope	*Pseudomonas aeruginosa*	Cowpea mosaic virus epitope display in cowpea Tobacco mosaic virus epitope display in tobacco	Plant virus-mediated	[67]	Immunogenic in mice

Continued

4	Yersinia pestis F1 and LcrV antigen	Plague	Tobacco (*N. benthamiana*)	Deconstructed TMV based system	[68]	Immunogenic in guinea pigs
			Tomato	*A. tumefaciens*	[69]	Immunogenic in monkeys
5	TonB protein	*Helicobacter pylori*	*Arabidopsis thaliana*	*A. tumefaciens*	[70]	
6	UreB subunit of *Helicobacter pylori*	*Helicobacter pylori*	Carrot	*A. tumefaciens*	[71]	
			Parasites			
1	Synthetic peptide vaccine	Cystiocercosis caused by *Taenia solium*	Papaya	Biolistic	[72]	Tested in Mice
			Genetic/Metabolic disorders			
1	Aβ42 gene Or Amyloid beta peptide	Alzheimer's disease	Rice	*A. tumefaciens*	[73]	
			Tomato	*A. tumefaciens*	[74]	
2	Taliglucerase alpha	Gaucher's disease	Carrot		[75]	Product commercialised

and cheaper. Plants are most economical and feasible production systems for vaccines or recombinant products. Replacement of fermenters and bioreactors with contained plant growth rooms or greenhouses with appropriate biological containment reduces manufacturing cost [6]. Production costs of a recombinant protein in transgenic plants are 10 - 50-fold lower than that by *E. coli* fermentation [77]. Plant vaccines can also be delivered orally, overcoming the cost and inconvenience of purification and injections [78].

2.5.2. High Yield

Use of plants as source of therapeutic proteins has a major advantage that production in large quantities is possible. Feasibility for scaling up and high expression level of recombinant genes/proteins are also high in plant systems. Also, large scale cultivation is possible, and this can be adopted in less developed or resource-poor nations which lack sophisticated facilities or infrastructure for production of life-saving drugs.

2.5.3. Easy to Prepare and Ease of Administration

Designing a recombinant vector, introduction and integration into plant system for production of antibodies, or other proteins of therapeutic value is relatively easy. The edible vaccines are easy to handle as well. When a new microbe or its antigen is evolved posing a threat to human health, it is easy to modify the synthesis of plant-based vaccines than animal-based ones. Edible vaccines are easy to deliver through oral administration and can be directly consumed without need for any injection. Edible vaccine is a needle-less vaccination method or a substitute of painful immunization procedures that require sophistication or trained manpower. It is also inexpensive, attractive to children, can be stored nearby the place of usage, harmless, and offers systemic and mucosal immunity.

Edible vaccines are safe oral-delivery vehicles wherein specific plant tissues such as grains, fruits, or leaves can be used as formulation of vaccines, without extensive purification and processing.

2.5.4. Post-Translational Modifications

Post-translational modifications such as glycosylation, folding and assembly are significant for a protein to be biologically active and function as a vaccine. Plants have machinery for expression, folding, assembly, and glycosylation, necessary for preservation of immunogenic activity of vaccines. In plants, the foreign or recombinant proteins of therapeutic value are glycosylated, accurately folded and the multimeric proteins assembled properly, to have structural integrity and biological activity for functioning as a vaccine [18]. Protein synthesis as well as post-translational modifications of proteins in plants is similar to that of animal cells, making it possible to use plants as bioreactors for animal proteins/pharmaceuticals.

2.5.5. Safety

The plantibodies or plant vaccines produced using plant-based systems are mostly safe and devoid of any toxic components [79]. Plant vaccines in edible plant parts can be directly consumed (edible vaccines). Plants do not host animal or human pathogens such as viruses or prions, as in the mammalian cell culture systems or transgenic animals, and hence do not transmit these. Chances of contamination with the pathogens during fermentation and extraction processes, is less in plant systems, Plant-based vaccines and therapeutics also have no biosafety and environmental issues as with other animal or microbial systems of production of vaccines, except that of transgene containment.

2.5.6. Stability, Storage and Transport

The plant products can be stored safely for long duration at room temperature, unlike the need for refrigeration in case of other animal-based vaccines. Edible or plant-based vaccines can also be easily produced by a freeze-dried process, leading to formulations with high stability under a cold chain-free distribution [80]. Proteins produced in plants such as seeds remain stable for years at ambient temperatures, without loss of activity.

2.6. Challenges or Constraints of Plant-Based Vaccines

Plant expression system has several advantages for human as well as veterinary vaccine production, however, only few of vaccine candidates are under clinical trials. Commercial human vaccines are not available due to low level of expression, relatively weak efficacy, and comparatively shallow knowledge on the characteristics of plant-made antigen and production system [49]. Some of the challenges or constraints in the plant-based vaccines are discussed.

2.6.1. Poor Immunogenicity

Immunogenic response depends on nature of the vaccine, route of administra-

tion and the delivery system. Many antigens are poor immunogens, recognized poorly by the immune system and are prone to degradation in the harsh environment of the digestive tract. Plant cells protect vaccine antigen and prevent degradation as it passes through the gut. Immunogen such as Cholera toxin B subunit (CTB), which can modify the cellular environment to present the antigen, can act as an efficient transmucosal carrier molecule and delivery system for plant-derived subunit vaccines and can overcome this problem.

2.6.2. Variability in Dosage

It is difficult to measure the effective dose for a mucosally delivered vaccine as it is exposed to the complex environment of the gastrointestinal tract. Further, oral vaccines may require co-administration with specific adjuvants to reach sufficient immunogenic activity [81]. An insufficient amount of antigen would not produce the immune response needed for protection against disease and inappropriate dosage could lead to tolerance to vaccine and ineffectiveness of vaccine.

2.6.3. Alteration in Glycosylation and Allergenicity

Many therapeutic proteins or N glycoproteins synthesised in plants differ in their glycosylation patterns from those derived from the mammalian systems. This may also induce increased allergenicity or reduced immunogenicity. The glycosylation pathways in plants can be altered for humanising the plant-derived vaccines or therapeutic proteins.

2.6.4. Degradation

Antigens delivered to the intestinal immune system is rapidly degraded within the harsh environment of the digestive tract, though plant cells provide protection and prevent degradation of the vaccine antigen, as it passes through the gut.

2.6.5. Spoilage

Edible vaccines such as fruits are perishable and cannot be stored for long time. They quickly spoil after ripening and the protein content is also very less.

2.6.6. Generation Time for Transgenics

Stable plant transformation to generate transgenics that express vaccine proteins takes much time, from months to years, depending on the plant species. Long time required for development or transformation, analysis of transgenics, selection and bulking up of producer line are some constraints.

2.6.7. Risks to the Environment and Human Health

Environmental issues of plant vaccines include gene transfer and exposure to antigens or selectable marker proteins, while risks to human health include oral tolerance, allergenicity, inconsistent dosage, worker exposure and unintended exposure to antigens or selectable marker proteins in the food chain. These risks are controllable through appropriate regulatory measures at all stages of production and distribution of a potential plant-made vaccine [45].

2.6.8. Transgene Escape and Containment

The potential and prospects of plant made pharmaceuticals is restricted by the potential of transgene spread from crops through outcross, challenges in transgene biocontainment, unpredictable impact of epigenetic events on transgene expression etc [14] [17]. Escape of foreign genes to weedy relatives through outcrossing is a concern. Plant cell culture bioreactors or greenhouses and use of plant virus expression systems to produce vaccine proteins in large quantities can be thought of as safer alternatives. The infamous escape of transgene in case of Prodigene and Starlink corn are examples. ProdiGene produced a transgenic corn that expressed a vaccine for preventing bacteria-induced diarrhoea in pigs, but in 2002, ProdiGene failed to eradicate plants that had seeded from their previous season's transgenic corn crop which contaminated non-transgenic soybeans. In 2003, the Animal and Plant Health Inspection Service (APHIS) of the US Department of Agriculture (USDA) made it mandatory for engineered plants producing pharmaceuticals to be grown under permit. Inefficient transgene biocontainment is a serious hurdle to commercialisation of molecular pharming using plants [82].

2.7. Current Status of Regulations on Plant Vaccines

Regulatory hurdles remain a barrier to molecular farming, further increasing the cost and time, which otherwise are major advantages of plant-made vaccines. Purification, quality controls for vaccine approval are major cost factors in (human) vaccine production [25]. Containment of the recombinant material is a concern which needs to be carefully monitored, to prevent these from entering the food chain and environment. The recombinant plant-based vaccines produced in transgenic plants must undergo a tight regulatory process before commercialisation. The paradigm of plant-made vaccines (PMVs) has evolved from vaccines consumed by world's poorest populations through fresh produce derived from their local farm, to eating engineered fruit or vegetables prescribed by a health care worker, to a plant product derived from batch processed, freeze-dried plant tissues prescribed by a health care worker to current paradigm that PMVs are not food materials that need to meet still-evolving regulations of national regulatory authorities for drug administration(FDA) and Department of Agriculture (USDA) [44]. Plants producing pharmaceuticals are regulated by USDA and the regulatory framework is developed by the FDA and Centre for Veterinary Medicine (CVM) [78]. The antigen present in edible vaccines is considered as a chemical, that does not comply with FDA rules concerning nutritional additives, but is recognized as non-GRAS (Generally Recognized As Safe). These vaccines, under the category of food, would be included as a genetically modified food and thus are not considered a high health risk. Due to this ambiguity, a legal void currently exists with respect to regulations for standardizing edible vaccine commercialization. It is not yet clear what part of the vaccine discharges the antigen itself, the transgenic, modified fruits or the transgenic seeds [49]. In the presence of this legal uncertainty, every country is

expected to evaluate whether the entrance of edible vaccines (or the plants producing them) is permitted [32]. In 2005, the World Health Organization (WHO) delivered a report on the implementation of good agricultural practices for the development of biopharmaceuticals. This report includes detailed information about methods of quality control for medicinal plants, testing to assess identity and purity, and recommended materials for plants in biopharmaceuticals

2.8. Future Prospects

Bio-farming or molecular farming is attractive because of its flexibility, scalability, low manufacturing cost, no toxicity or pathogenic contamination, but many projects are at various developmental stages and not many are yet available to the pharmaceutical industry. Optimization of lab protocols for up-scaling the production of therapeutics at commercial level is important for clinical use. Plant metabolic engineering is a highly significant technology for production of high-value pharmaceutical compounds. Fusion proteins for multicomponent vaccines against multiple diseases are a potential tool to incorporate into immunisation programmes. Unlike genetically engineered microbial systems such as viruses, which pose more risks to the environment and humans, and have more chances of escape, difficulty in controlling and monitoring such escapes or unintended presence, plants are immobile. Control, containment and monitoring of genetically engineered plants are easier and containment can be achieved by regulating pollen transmission. Transgenic mitigation through linking the transgene to genes that confer a selective disadvantage such as a dwarfing mitigator gene, or other mechanisms like cleistogamy in rice, total sterility in tubers and bulb-propagated crops, synthetic auxotrophy etc. could contain gene flow.

Low-cost technologies for the production of biopharmaceuticals using plant systems should be used in cases of unprecedented public health crisis such as that caused by COVID-19. Since the production processes and systems of production or sophisticated expression platforms for vaccines and therapeutics are already established, it is possible to rapidly generate the vaccines with higher yield under cGMP practices. *N. benthamiana* plants can be transiently transformed with target vaccine gene and the leaf biomass harvested and processed to purify the antigen or vaccine. In addition to transient expression systems, plant biomass propagation with plant cell suspension cultures should be refined.

The Canada-based biopharmaceutical company Medicago Inc. is into transient expression of SARS-CoV-2 virus S protein, using a virus-like particle (VLP) grown in *Nicotiana benthamiana* to develop a potential vaccine against the coronavirus disease that has now reached a global pandemic level. Universities and Institutes from several countries including the US, Germany, UK, South Africa, South Korea, Mexico and Thailand are working in the molecular farming field, investing efforts and establishing partnerships and collaborations for treatment or vaccine for COVID-19. Transient transformation approaches are rapid (expression within a week) while regeneration of stably transformed plant takes up to 3 months, which is not suitable for addressing a fatal, exponen-

tially-growing pandemic such as COVID-19.Though concept and methodology are concise, there are only limited number of edible vaccines which are approved, tested and commercialised. The COVID-19 pandemic outbreak warrants a deep investigation and introspection into the technology status, application and progress to address the problem [83]. Transient expression in plant cell suspension cultures seem to be a feasible strategy to produce plant-based vaccine for deadly pandemic outbreak of COVID-19.

3. Conclusion

Plant-based vaccine production systems can highly reduce the manufacturing costs involved in conventional vaccine production systems and can rapidly increase the scale of production. These two major advantages are a boon to vaccine development in developing or resource-poor countries, to reduce morbidity and mortality due to infectious diseases, and other orphan diseases which are poorly-funded in terms of research and development. Also, the plant-based vaccines can be administered orally, skilled administration is not required, can be produced in food and non-food crops, and easily commercialised due to possibility of growth in controlled environmental conditions as in cell suspension culture. There is also an element of flexibility of production in plant-based systems. But a major drawback is the tight regulatory systems which takes a long while to release the product. A vaccine or curative drug is the only way and means to prevent the spread and mounting death toll caused by novel deadly pandemics such as the novel coronavirus SARS-CoV-2. The anticipated cure or vaccine can be developed by 2021 by 12-18 months, when the solution to the problem of pandemic will lose its significance due to the alarming fatality rate. In the wake of the global calamity of the pandemic COVID-19 caused by SARS-CoV-2, can the regulatory processes be revisited and amended or reframed for development of vaccines and approving the strategies of vaccine production, which are in the pipeline? Mechanisms and strategies already existing to prevent them from entering food chain should be incorporated. The production of the vaccines can be stopped after achieving a tight grip or control over the disease or cure for the disease. In the long run, we should be better equipped and prepared to face the deadly epidemic or pandemic outbreaks with vaccines, which are the weapons in the war against the pathogens. Beyond COVID-19 as well, there will always be a need to immediately respond to new strains of viruses and pandemics that may emerge in the future. In an emergency or crisis, what is required is solution to the problem than solution unrealised by further problems of regulations. Regulatory processes are for risk management and for maintaining quality standards of production, for the successful use of vaccines and not to completely freeze the use of the most economical and feasible promising vaccine development technology for resource-poor nations. Plant-based vaccines are one of the affordable, powerful ammunitions for developing nations, against pandemic outbreaks and can be used as a potential weapon to save mankind.

Disclaimer

The views expressed if any are of the author and not of the organisation in which the author is associated.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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