Molecular Biotechnology
https://doi.org/10.1007/s12033-022-00627-5

REVIEW PAPER

Tobacco-Based Vaccines, Hopes, and Concerns: A Systematic Review

Mintu Mathew[1] · **Jaya Thomas**[1]

Received: 2 August 2022 / Accepted: 26 November 2022

Abstract

Emerging infectious diseases have vigorously devastated the global economy and health sector; cost-effective plant-based vaccines (PBV) can be the potential solution to withstand the current health economic crisis. The prominent role of tobacco as an efficient expression system for PBV has been well-established for decades, through this review we highlight the importance of tobacco-based vaccines (TBV) against evolving infectious diseases in humans. Studies focusing on the use of TBV for human infectious diseases were searched in PubMed, Google Scholar, and science direct from 1995 to 2021 using the keywords Tobacco-based vaccines OR transgenic tobacco OR *Nicotiana benthamiana* vaccines AND Infectious diseases or communicable diseases. We carried out a critical review of the articles and studies that fulfilled the eligibility criteria and were included in this review. Of 976 studies identified, only 63 studies fulfilling the eligibility criteria were included, which focused on either the in vitro, in vivo, or clinical studies on TBV for human infectious diseases. Around 43 in vitro studies of 23 different infectious pathogens expressed in tobacco-based systems were identified and 23 in vivo analysis studies were recognized to check the immunogenicity of vaccine candidates while only 10 of these were subjected to clinical trials. Viral infectious pathogens were studied more than bacterial pathogens. From our review, it was evident that TBV can be an effective health strategy to combat the emerging viral infectious diseases which are very difficult to manage with the current health facilities. The timely administration of cost-effective TBV can prevent the outburst of viral infections, thereby can protect the global healthcare system to a greater extent.

Keywords Transgenic · Tobacco-based vaccines (TBV) · Infectious diseases · Total soluble protein (TSP) · *Agrobacterium*-mediated

Abbreviations

PBV	Plant-based vaccines
TBV	Tobacco-based vaccines
VLPs	Virus-like particles
HBV	Hepatitis B virus
TSP	Total soluble protein
STD	Sexually transmitted diseases
E. coli	*Escherichia coli*
TB	Tuberculosis
MAbs	Monoclonal antibodies
HBsAG	Hepatitis B surface antigen
Ab	Antibodies
SC	Subcutaneous
IM	Intramuscular
CT-B	Cholera toxin B
LTB	Labile enterotoxin subunit B
HPV	Human papillomavirus
Ag85B	TB antigen 85B
ACR	α-Crystalline antigen
FW	Fresh weight
ELP	Elastin-like protein
TMV	Tobacco mosaic virus
PVX	Potato virus X
GP1	Glycoprotein
HPV	Human papilloma virus
HIV	Human immunodeficiency virus
CDC	Centre of disease control and prevention
VP	Viral proteins
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
p24-Nef	Negative regulatory protein
HA	Hemagglutinin

✉ Jaya Thomas
jayamarythomas@gmail.com

Mintu Mathew
mintumathew02@gmail.com

[1] Department of Pharmacology, Amrita School of Pharmacy, Kochi, Kerala, India

Published online: 17 December 2022

SARS-CoV	SARS Covid-19
C4V3 polypeptide	C4 peptide fused to the N terminus of V3 loop
HCMV	Human cytomegalovirus
SARS	Severe acute respiratory syndrome
PTGS	Post-transcriptional gene silencing
PVX	Potato virus X
CP	Coat protein
ELISA	Enzyme linked Immunosorbent assay
ppH5HA-I	Purified plant-derived H5 Hemagglutinin-1
FCA	Freund's complete adjuvant
Bm ALT-2	*Brugia malayi* Abundant Larval *Transcript-2*
BAT	British American tobacco
PBS	Phosphate buffer saline
RCT	Randomized controlled trial
HIV	Human immunodeficiency virus

Introduction

Vaccines are biological preparations that improve our immunity by stimulating the antibodies production in humans and animals. Its development played a crucial role in combating the largest crisis in the healthcare system caused by infectious diseases [1–3]. Edward Jenner developed the first vaccine for smallpox infection in 1796 [4]. Hait initiated the research on the plant-based vaccine (PBV) and late in the 1980s vaccine research focusing on edible vaccines began, laying the foundation for a new era of PBV which lies in the concept of converting edible food into potential vaccines [1, 3]. Despite this success of vaccine therapies, the emerging new infectious diseases especially the current pandemic COVID-19 have proven the glaring gaps in vaccine production capacities globally, which highlights the importance of massive expansion of vaccine research. The challenges faced by conventional vaccine production strategies can be overcome to a certain extent by the introduction of PBV. Research over the two decades has proven that plants can be engineered to act as bioreactors (Biopharming) offering an attractive and affordable platform for vaccine production eg: Chimeric human growth hormone produced via transgenic sunflower and tobacco by Barta in 1986 [5]. The escalating cost of vaccines is considered a challenging factor for conducting vaccination campaigns in developing countries. A published report by Kate et al. highlights the exponential increase in the total vaccination cost from $1.37 in 2001 (for 6 diseases) to $38 in 2011 (for 11 diseases) this might be due to the expensive and complex production techniques involving equipment like fermenters and bioreactors which can be replaced by providing the basic need of light, water and greenhouse to yield larger quantities of safe and cost-effective PBV [1, 4, 6, 7].

Tobacco—Precursor in Transgenic Research

The tobacco plant is a robust perennial, erect glandular-pubescent herb, indigenous to America, with over 70 species with varying biological activities. *Nicotiana benthamiana* contains chief chemical constituents such as Nicotine, nornicotine, anabasine, myosmine, anatabine, nitrate, sorbitol, nicotinic acid, nicotelline, nicotianine [8]. The detrimental use of tobacco has been renowned to all, considered as the leading cause of life-threatening diseases such as cancer, emphysema, and heart diseases which is of global concern for centuries. According to WHO more than 8 million people a year around the world were dying because of the tobacco misuse, of which more than 7 million due to direct tobacco use and 1,2 million due to passive smoking [8–10]. As per the records, the history of tobacco begins in 1492 when Christopher Columbus discovered an herb used by American Indians treating illness with herb leaves, which was unnoticed till then. After 1536, the sacred role of spiritual, emotional, mental, and physical uses was studied by several experts globally and the plant were taken to various countries across the world. Most Indigenous nations have their own stories regarding the introduction and cultivation of tobacco in their home country [8]. Tobacco production accounts for approximately 0.25% of cultivated land in India. In 1885, tobacco leaves took their position in the British Pharmacopoeia; later they got removed from medical practice historically. Several investigations highlighted the enormous healing property of tobacco such as antispasmodics, diuretics, antiemetics, expectorants, sedatives, and in rheumatic swellings, anesthetics, antiglaucoma activity, antioxidant, antibacterial, antiviral, antimicrobial, anthelmintic, anticonvulsants, and for antifungal activities of which the use for psychiatric and neurological disorders are quite commendable since decades [8, 11] In 1990 Arntzen and colleagues implemented the idea of the edible vaccine using 's' gene of hepatitis B virus (HBV) against hepatitis B antigen and Streptococcus mutants surface protein antigen A (0.02% recombinant protein) was obtained from total soluble leaf proteins of transgenic tobacco [12, 13].

In recent years adapting plants as expression systems of various biologics like vaccines, monoclonal antibodies, biosimilars, and biopolymers paved a greater opportunity to benefit human health especially for the rapid response to disease outbreaks [12–14]. Studies suggest that the beginners of plant-based biologics production were from stably transformed crop plants such as rice, maize, barley, safflower, later in the following decades of large varieties of plant kingdom including microalgae, rice moss, sundews, pitcher plants, lettuce, melon, barley, tomato, carrot, tobacco, corn,

wheat, soybean and sunflower has been explored, within a short period it got extended to edible vaccines research among them tobacco shows tremendous results and considered it as ideal plant model in transgenic research [5, 15]. Tobacco leaves and seeds are considered as a living factory for molecular farming, since they act as an excellent vehicle for the mass production of very complex molecules and recombinant protein [12–14]. The comparison of PBV with the conventional vaccine production is given in Table 1. The present systematic review was performed to assess the relevance of tobacco-based vaccines in fighting the human infectious diseases.

Expression Systems of Tobacco-Based Vaccines

The basic principle behind the TBV production involves the incorporation of transgene into the plant cells (tobacco leaves) through nuclear or plastid integration. The antigen of interest was integrated with the vector and later it is transferred to the expression system. Based on the location/ site where the transgene is inserted into the plant cell it can be stable or permanent transformation system, transient transformation system [1, 4].

Stable Expression System

The foreign gene is integrated into the cell's genome, either in the nucleus or the chloroplast, resulting in the permanent transformation of a genetic cell line that can be propagated through vegetative or sexual reproduction methods [13]. Nuclear transformation and chloroplast transformation are two types of antigen expression in transgenic plants. In nuclear transformation, genes are integrated into the plant cell nucleus via nonhomologous integration and the expressed proteins undergo eukaryotic post-translational modifications but with minimal accumulation of recombinant proteins which can be stored in various organelles like cytoplasm, nucleus, chloroplast, mitochondria depending upon the signalling of fused peptides [1, 16]. Different transformation methods such as electroporation, micro projection, particle bombardment, glass beads, polyethylene glycol and *Agrobacterium* mediated can be used of which *Agrobacterium* mediated is widely used since it provides an opportunity for multicomponent vaccine production [1, 13, 17].

In chloroplast transformation or plastid transformation, genes are directly injected into the tobacco chloroplast through homologous recombination which offers the safety, flexibility and potential to produce significant level of recombinant proteins with minimal prokaryotic post-translational processing [16–18]. Chloroplast transformation is widely used and has several advantages such as reduced chance of gene escape, coordinated expression of genes with the aid of single promoter, transgene containment with increased biosafety, whereas its inability to perform the glycosylation can led to stunted growth and male sterility in host plant as a result of polytropic effect which can affect the manufacture of pharmaceutical such as monoclonal antibodies, degradation of proteins and efficacy of vaccines [16–18].

Transient Expression System

Transient expression is used widely for the recombinant proteins production which includes the expression of foreign gene without being integrated into the genome. The genetic material is delivered into the cell by the viral vectors reports higher frequency of occurrence without the requirement of whole plant regeneration. Despite the need of technical support in whole process and difficulties in initiation, it is considered as an efficient system for the producing high yield of desired proteins within a short period of time [13, 16, 17]. Techniques like magnifection, alteration of virus codon usage and incorporation of eukaryotic introns and agrobacterium-mediated transfer can maximize efficacy of transient expression and improves the yield [16].

Methods of Gene Transfer

Direct Gene Delivery Method

This is the simplest method involving the direct integration of selected RNA or DNA into plant cell, which is coated with tungsten or gold which acts as a micro carrier. The coated DNA will penetrate into the cell with the aid of gene gum and under high pressure of helium gas. Biolistic method also known as micro-projectile bombardment or gene gun method is the most popular method, where the transfer of foreign DNA into variety of plant host cell is possible, even though it can damage the tissue cell and is a costly process [1, 4].

Indirect Gene Delivery

An ideal method for gene delivery where the genes are delivered through vector mediated gene delivery and proteins are produced in chosen plant cells infected using plant bacteria (*Agrobacterium* species) or plant virus. The gram – ive *Agrobacterium* is most widely since it provides stable integration of the antigen and transfer their genes (T-DNA) into the nucleus of plant by naturally infecting them. Based upon the plasmid they carry it is divided into two Ti-plasmid tumour-inducing plasmid and Ri-plasmid root-inducing plasmid that is *Agrobacterium tumefacians and Agrobacterium rhizogenes*, respectively [1, 4].

Another method is through the genetically engineered plant virus which act as a vector to generate the chimeric

Table 1 Comparison of plant-based vaccines with conventional vaccine production

	Plant-based vaccines	Conventional vaccines	Reference
Cost	Production and maintenance cost is very minimal, since mostly cultivation in field or greenhouse depends on the natural resources like sunlight, water for the growth	Requirement of expensive technique and equipment like fermenter and bioreactors makes its production costlier	[85]
Storage	Vaccine antigens can be lyophilized and stored at ambient temperature for many years, maintaining the efficacy of expressed protein	Proper storage under cold condition including cold chain process is essential for maintaining the potency	[3, 86]
Delivery	Oral delivery is possible for majority of the PBV preferably the uncooked form but that is not possible in cases of TBV	Most of the preparation are administered through the parenteral route and requires the skilled professionals	[2, 3, 86]
Coverage	Long lasting humoral and cellular responses, exhibits dual immunity by activating both mucosal and systemic immunity also have seroconversion in presence of maternal antibodies, which provides first-line defence against infecting pathogens invading through mucosa, such as Mycobacterium tuberculosis and agents causing diarrhoea, pneumonia, STDs, HIV, etc	Involves both humoral and cellular mediated response but depends upon the type of vaccine the immunity coverage will vary Eg: Polysaccharide vaccine are T-cell independent antigens	[13, 86–89]
Production process	The compatibility with various production systems, combined with the ease of genetic modification, allows for the simultaneous transfer of multiple genes with increased expression levels, which can aid in the large-scale production of a diverse range of therapeutic proteins with high biomass yield in a cost-effective manner. Continuous exposure to raw materials can cause oral tolerance and allergenicity in workers, and extracting and purifying industrial grade proteins is a difficult process	All processes must be carried out in a clean and aseptic environment with well-equipped facilities. It is difficult to maintain the stability of the product so optimum temperature should be maintained throughout the process. Despite after the lengthy and expensive process only low yield is obtained	[3, 5, 16, 26, 90, 91]
Contamination and Toxicity	The bioengineered plants can result in higher chance of transgene pollution or cross contamination and adverse effects which can be minimized by following the proper guidelines. Only at very high concentration it can be toxic. Since only limited clinical study results are available there exist discrepancy regarding proper dosage and duration	The changes of cross contamination are less but can be infectious (reversal to virulent form) with increased risk of allergic response as well as toxicity especially in immunocompromised patients	[18, 87, 92]

gene for viral coat protein. Within limited time high levels of desired peptides or proteins are expressed through viral replication activity and by altering the viral capsid proteins vaccine epitopes can be synthesised. Before vaccination the product obtained is purified from infected plant and simultaneously another plant is reinfected with recombinant virus for maintaining the continuity of vaccine production [1].

Production Process Involved in Tobacco-Based Vaccines

The production of TBV involves various steps as follows:

1. Selection of antigens from pathogenic organism: The initial step in the production of TBV involves the identification and selection of immune protective antigen with the aid of assays such as phage display technology from pathogens. Genomics, bioinformatics and proteomics tools are used for the designing of immunogen and the transgene encoding the antigen whereas recombinant DNA technology was used for the synthesis [19].
2. Selection of plant expression vector: Based on the antigen of interest the specific expression vectors were selected, which are the plant virus having positive sense of RNA genomes [20]. Seed-specific promoters will aid in the selective expression of antigen protein in particular tissue or organ. Usually, the viral genetic sequence is placed into a plasmid which they clone and introduced into *Agrobacterium tumefaciens*, a bacterial vector that can transfer genetic material to plants only [14]. Tobacco mosaic virus (TMV), plum pox virus (PPV) potato virus X (PVX), alfalfa mosaic virus (A1MV) cowpea mosaic virus (CPMV) are the most commonly used vectors [19, 21].
3. Selection of plants: The selection of plants, depends upon on its compatibility of transformation, *in-vitro* regeneration, expression strategy, biomass yield, life-cycle, containment, production cost. *Nicotiana benthamiana* and *N. tabacum* were considered to be ideal fulfilling all these parameters and used extensively for the transgenic production [19]. Open-field cultivation or closed indoor cultivation involving either green house, bioreactor, vertical farming or hydroponics is preferred for the production of plant biomass. Plants are grown up to 4–6 weeks in suitable environment and healthy plants are subjected for the transformation later [5, 22].
4. Plant transformation: Stable and Transient transformation methods are mainly involved in the transformation of plants with antigen containing recombinant vector. This transformation occurs with the aid of either the direct or indirect gene delivery method, along with vaccum infiltration technique. Eg: *Nicotiana benthamiana*, plant's leaves were dipped upside down and submerged in bacterial solution of the modified *Agrobacterium*, and vacuum compresses the leaves to expel air from the plant cells. *Agrobacterium* transfers the multiple copies of the GOI (Gene of interest) into the leaf tissue when vacuum is broken [14, 23]. Later it is removed from the suspension transferred to the incubation room in greenhouse for 5–7 days period, where the cellular activity begins results in the generation of numerous recombinant proteins [1, 5, 24].
5. Regeneration of plants: Plants are incubated for 6–12 days from transformed cell under the suitable conditions such as plant growth regulators, culture condition like light, temperature, presence of selective agent. Two pathways such as somatic embryogenesis and organogenesis are mainly involved in plant regeneration, but in transient expression these steps are evaded since the whole plant is involved and DNA or transgene is not stably integrated, it's expressed temporarily in the host [19]. Depending upon the protein production, expression method and optimal plant maturation harvesting is done. In case of tobacco leaves are harvested, usually by 33–55 days the manufactured protein embedded into VLPs floats between the cell wall and plasma membrane [14, 23]. Later, they are subjected to homogenization and purification where the products are recovered by combination of filtration and chromatography techniques [5, 24].
6. Isolation, Characterisation and evaluation of immunogen or plant vaccine: The purification and isolation of expressed recombinant proteins or VLPs is a challenging process which can be performed by density gradient centrifugation techniques such as isopycnic centrifugation based upon the buoyant density of VLPs or rate -zonal centrifugation techniques based upon the size and sedimentation coefficient of VLPs. Iodixanol or sucrose is used in density gradient centrifugation, some VLPs get purified in iodixanol. Gel electrophoresis, electron microscopy analysis is performed in the post purification stage. The quantification of antigen or foreign in plant is evaluated using western blot assays and ELISA. Preclinical animal studies are performed for evaluating the immunogenicity of obtained TBV, where splenocyte proliferation assays and ELISA are used for estimation of the antibody levels and proliferation of specific immune cells [25]. Those vaccine candidates who proven the immunoprotected potential in preclinical studies are eligible for the clinical trials in human [5]. The production process of tobacco-based pharmaceuticals is illustrated in Fig. 1.

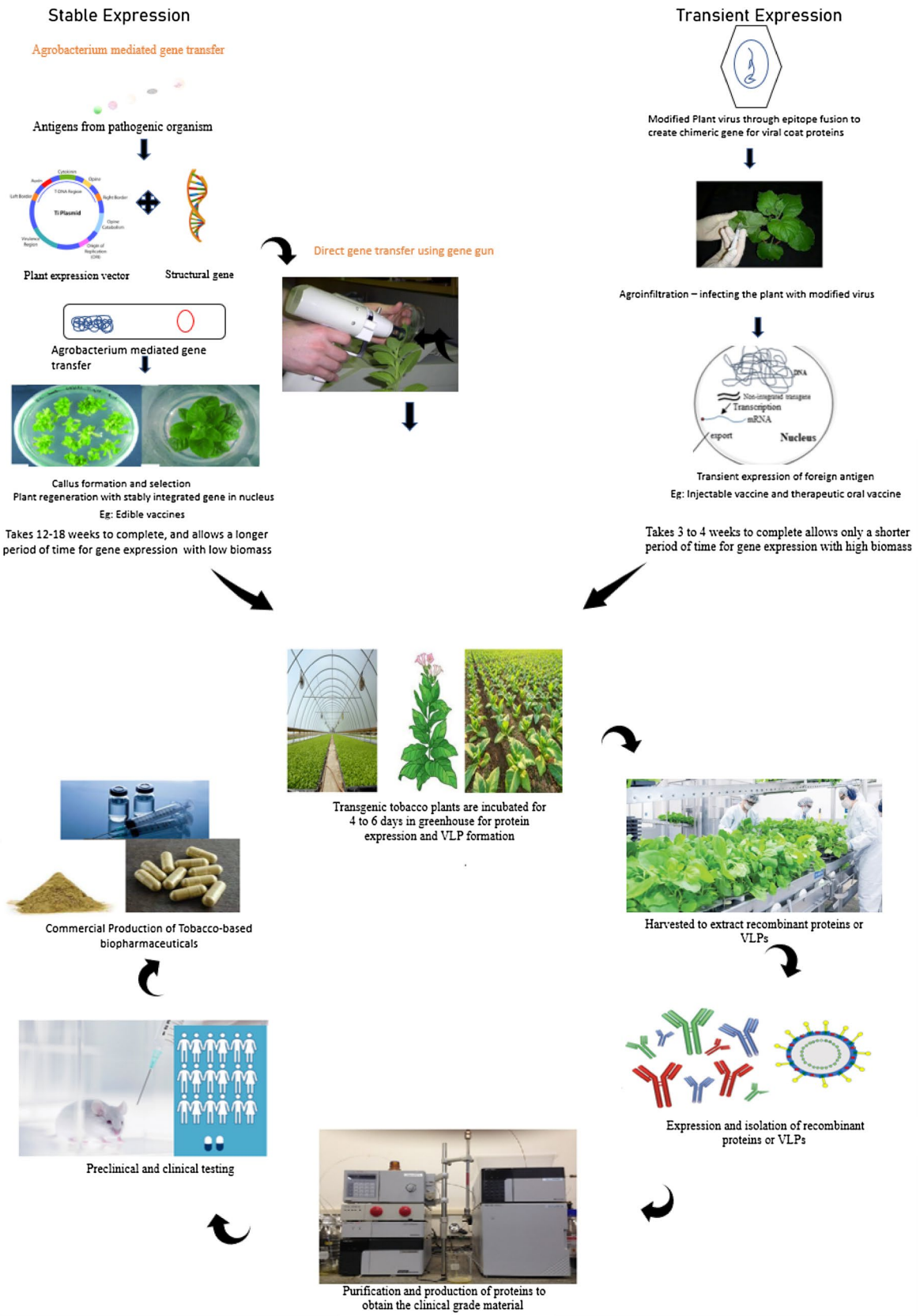

Fig. 1 Production of Tobacco-based biopharmaceuticals

Regulatory Considerations

During the process of PBV development, regulatory consideration act as a critical component for product approval, foremost it is mandatory to follow the guidelines especially the good manufacturing practices (GMP) and good laboratory Practices (GLP) issued by the regulatory agencies [24, 26]. Many health organisations, including the World Health Organization and International Council of harmonization (ICH) involving regions of the United States, Europe and Japan plays a vital role in the developing and implementing, monitoring various regulatory strategies for the manufacturing and distribution of safe, effective, and high-quality biopharmaceuticals for better public health outcomes [24, 27, 28]

According to the regulations of FDA in 2017 suggest that any tobacco-based drugs or devices will get approval only after fulfilling the MRTP (modified-risk tobacco products) authorization with the evidence of therapeutic indications claims of new product as well as the details about the product reducing the harm [29]. In terms of molecular farming strict rules and GMPs are implemented by specific country's regulatory boards such as USDA-APHIS in the USA and ECCC in Canada mainly focusing to avoid the environmental hazards associated with the cultivation [30]. Moreover, the slight variations in regulatory consideration guidelines are evident in different countries.

Methodology

For this systematic review, all eligible studies on the use of TBV for human infectious diseases published from 1995 up to October 2021 were systematically searched through electronic databases; Google Scholar, PubMed, Scopus, and Science direct using the keywords as follows: Tobacco-based vaccines OR transgenic tobacco OR *Nicotiana benthamiana* vaccines AND Infectious diseases OR communicable diseases. The studies published in the English language were considered. In addition, for identifying further pertinent articles using references from retrieved studies a manual search was conducted.

Eligibility Criteria

In this systematic review, only those studies which satisfy the inclusion criteria were included:

(i) The full-text publication written in the English language
(ii) Studies focusing on the use of transgenic tobacco/TBV for human infectious diseases
(iii) Both clinical and preclinical studies (in vitro and in vivo) evaluating the effect vaccine candidate expressed in transgenic tobacco

Exclusion criteria:

(i) Studies focusing on the usage of transgenic tobacco/tobacco-based vaccines for animal infectious diseases
(ii) Studies exploring the use of transgenic tobacco for the expression of other biologicals such monoclonal antibodies, biosimilars, and biopolymers
(iii) Studies focusing on the usage of transgenic tobacco involving the recombinant proteins expression for non-infectious human diseases such as autoimmune disease, lifestyle disease
(iv) Studies with insufficient data or not able to extract the information such as articles without full texts, duplicate articles, abstract, reviews, republished data, comments, editorials, and conference papers.

Article Screening and Data Extraction

All the abstracts and titles were screened for eligibility and those articles which fulfilled the inclusion criteria were subjected to full-text article review. The screening step focused solely on whether the article found any keywords searching related to tobacco-based vaccines for infectious diseases was performed by two investigators, disagreements were solved by discussion based on the eligibility criteria, later the included studies were subjected to full-text screening. After identifying the eligible studies, to enhance the critical appraisal and accuracy the following data were collected and documented in the standardized data collection form. The details include Title, first author name, the country where the study was conducted, year, journal details type of publication. Moreover, the critical analysis is based on the type of study including preclinical or clinical, animal or human infection, involving other biologicals, comparison studies. After the full-text review of the eligible studies, the studies were categorized into three tables representing the details about the in vitro, in vivo, and clinical studies information of TBV used for various human infectious diseases.

Results and Discussion

In the initial literature search in the databases, a total of 976 studies were found. Of which around 763 studies were excluded in the initial screening itself of which 209 review articles, 206 book chapters, 71 conference abstracts, 216

others like mini-reviews, short communications, patent reports, editorials, comments, discussions, etc., 55 duplicates, and 6 other language publications. About 213 articles were subjected for full-text reviewing in which only 63 articles were eligible for the current systematic review. We recognized about 74 TBV studies focusing on animal infectious diseases, adding hope to the veterinary society to limit the outburst of the epidemic, also about 55 studies focusing on the expression of several biologicals other than vaccines which can be used in the management of non-infectious diseases were identified. The detailed reasons for the exclusion of studies are given in the flow diagram represented in Fig. 2.

In this systematic review, the TBV studies were categorized into three: *In vitro* and *in vivo* (preclinical studies), and clinical studies focused on TBV against human infectious diseases. Most of the preclinical studies were conducted in the USA (17), followed by Korea (6), China (5), India (5), and the UK (4).

In Vitro Studies

The basic principle behind the in vitro production of TBV involves the integration of the transgene into the plant cells through nuclear or plastid integration, before the transformation to expression system (stable or transient systems) the target sequence of the selected antigen is integrated with the viral vector [1, 4]. Around 43 In vitro studies of 23 different infectious pathogens were recognized of which 15 viral infections were studied more, than 6 bacterial infections which highlights the scope of TBV in managing the emerging viral infectious disease where the proper pharmacological management is not well-established.

HIV (6), HPV (5), *M. tuberculosis* (4), and dengue virus (4) were the most studied infectious pathogens, maybe because of the high prevalence of diseases and easy availability of suitable plant expression systems [31–37].

Agrobacterium tumicfaciens mediated transient expression is considered as the most suitable platform due to its potential to accumulate significant level of recombinant

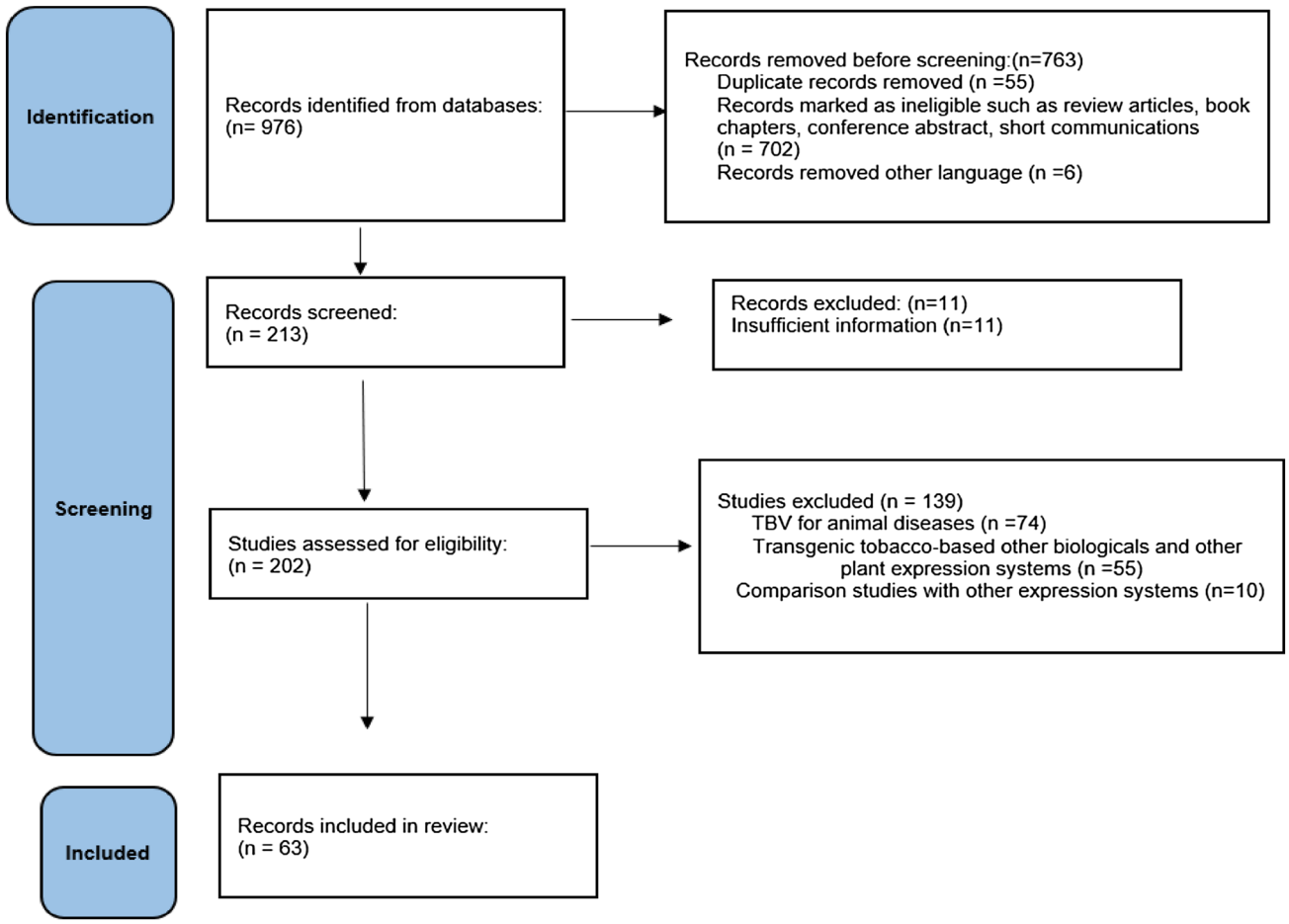

Fig. 2 The study selection process flow diagram

proteins, ease in handling technique which offers the transfer of larger segments of a gene with minimal rearrangement. Except the diseases like HIV, Tuberculosis, Cholera, Tetanus, EBV and Gastroenteritis around 22 different *in-vitro* studies used this platform. The diseases involving the high virulence pathogens like HIV and Tuberculosis tobacco chloroplast expression system is used due to the less chance of gene escape with transgene containment. Moreover, higher yield of proteins with more immunogenic properties was obtained eg: Study by Gonzalez et al. involving HIV p24 capsid protein (4–40% TSP) and Varsani et al. involving HPV-16 L1 capsid protein (24% TSP) [37–39]. In majority of studies, the confirmation of the transgene incorporation into the genome was assessed using southern blotting. The DNA and RNA were isolated and subjected to various analysis for the detection of proteins like western blotting and immunoassays like ELISA for the quantification of proteins. Finally, the active molecules like VLPs are purified and subjected to microscopic and other specific analytical parameters.

Since different types of pathogens were studied and different expression systems were used could not make an extensive summary out of it instead the details about the *in-vitro* studies including the details of the infectious pathogen or strain of interest, expression system involved, amount of total soluble protein, and its major findings are represented in Table 2.

In Vivo Studies

In vivo studies were performed to assess the immunogenicity profile of vaccine candidates in various animal species. Western blotting and ELISA were conducted to analyse the immunogenicity profiles and the majority of the studies reported that after immunization using the disease-specific vaccine candidates against infectious diseases in animal models exhibit high immunogenicity profile by inducing neutralizing antibodies within a short period of time. The most suitable animal species to study the antigenicity property was found to be BALB/c mice of 8-week-old and the frequently used route of administration were subcutaneous, intraperitoneal, and oral routes.

In vivo analysis, shows that HPV antigenic proteins immunogenicity was evaluated more compared to the other infectious species. Even though there exist 43 different in vitro studies discussed, only about 23 in vivo studies of 15 different vaccine candidates were identified. These sudden drop-down in vivo studies might be due to the low output of yield during in vitro studies analysis, expenses, and difficulty in conducting in multiple animal models moreover its undesirable antigenicity report of proteins derived. In vivo studies assessing the immunogenicity of different tobacco-based vaccine candidates shows the details about the dose of vaccine candidate or protein used, the animal involved with the route of administration, and their major reports about the immunogenicity against the specific infectious pathogen are represented in Table 3.

To date, out of the 15 viral pathogens studied except rotavirus, norovirus, Japanese encephalitis, and human cytomegalovirus (HCMV) all other TBV candidates were subjected to the in vivo analysis, but only 10 are under various phases of clinical trials to fight against emerging infectious diseases. The clinical trials were conducted in US and Canada. The eligibility for clinical trial might be based upon the possibility of better yield from the tobacco-based expression system in in vitro studies and high immunogenicity profiles of in vivo studies.

Poliovirus

Poliomyelitis is a viral disease at the brim of eradication with vaccines. Recently the use of subunit vaccines is hindered due to the property of pathogenic state revert and circulating vaccine-derived poliovirus transmission e.g.: The live-attenuated vaccine by the Sabin vaccine Institute was withdrawn from the market by WHO [40]. This can be overcome by the use of plant-made VLPs which are more stable, Daniell et al. prove that lettuce-derived oral booster vaccines free from viruses and cold chain provide long-term immunity against all three serotypes o poliovirus [41] similarly Bolaños-Martínez et al. in 2020 [42] report that the transgenic tobacco-made polio capsid proteins VP1, VP2, VP3, and VP4 show strong systemic and mucosal immunity with an increased level of neutralization titer among the immunized animals [42].

Human Papillomavirus (HPV)

HPV-associated cervical cancer is a highly prevalent sexually transmitted infection which can be prevented by the use of vaccines by GlaxoSmithKline's Cervarix and Merck's Gardasil which are efficacious but very costly [43, 44]. This can be resolved by the use of cost-effective tobacco-based chimeric VLPs. Even though several transgenic tobacco-based systems in vitro studies were conducted only 3 in vivo studies analyzed the immunogenicity of HpV16-L1 and LTB in mice, of which Hongli et al. and Millan et al. report strong systemic and mucosal immune responses administered through mucosal and intraperitoneal routes, respectively, whereas Biemelt et al. report that 40 ng of CsCI-purified VLPs doesn't show stronger immune response [31, 39].

Table 2 *In-vitro* studies on Tobacco expression of different antigenic epitopes as a vaccine candidate for human infectious diseases

SL No	Disease/Pathogen	Method of pathogenesis	Antigenic epitope	Expression level	Findings	Reference
1	Plasmodium Falciparum-Malaria	*Agrobacterium*-mediated transformation Transient expression	Pfs25-CP VLP Pfs25 bonded to coat protein (CP) of Alfalfa mosaic virus using TMV launch vector	50 mg/kg expressed at Day 7 of post-infiltration	Purified Pfs25-CP VLPs produced by incorporation of 20–30% of Pfs25 onto the VLP surface where size consistent	[82]
2	Vibrio cholera	Transplastomic (chloroplast) Biolistic method	Cholera toxin B-subunit (CT-B)	4.1% Total soluble protein (TSP)	Improved production of transmucosal carrier molecule and delivery system like CTB in transgenic chloroplast and makes the oral administration economical	[6]
3	Human papillomavirus (HPV)	Transplastomic (chloroplast) Biolistic method	HPV-16 capsid, L1	24% TSP	Matured plants exhibit the highest expression level of HPV L1 protein and its Conformation-specific epitopes gathered into VLP were displayed in transmission electron microscopy	[39, 37]
4		Freshly prepared RNA transcripts were inoculated with Tobacco mosaic virus-derived vector (TMV-L1 protein)	Human papillomavirus type 16 (HPV-16) L1 gene	Fresh leaf materials of 20–37 µg /kg	The extract concentrate contains L1 protein which was characterized using the neutralizing was antigenic and conformation-specific Monoclonal Antibodies (MAbs) H16:V5 and H16:E70 was bound to the protein produced by plant	[37]
5		*Agrobacterium*-mediated transformation Transient expression	HPV16 L1 protein	0.034–0.076% of TSP	TSP was capable to produce 55 nm VLP compatible with HPV VLP which are able to induce erythrocyte hemagglutination in mouse	[93]
6		*Agrobacterium*-mediated transformation Transient expression	HPV16L1 and LTB proteins HPV16L1 oral vaccine	TSP of 0.35–0.76% for LT-B and 0.22–0.31% for L1	HPV16L1 and LTB were highly expressed and identified by ELISA and electron microscopy which exhibit strong immunogenicity and biologically active	[31]
7		*Agrobacterium*-mediated transformation Transient expression	HPV major capsid protein 1	0.5% TSP	Conformation-specific epitopes of L1 protein was displayed and assembled into VLP	

Table 2 (continued)

SL No	Disease/Pathogen	Method of pathogenesis	Antigenic epitope	Expression level	Findings	Reference
8	Mycobacterium tuberculosis Tuberculosis	Chloroplast expression Biolistics-mediated transformation	M.tuberculosis 6 kDa early secretory antigenic target (ESAT-6)	1.2% TSP	Maximum expression of ESAT-6 is attained 10 days after ethanol induction	[35]
9		Potato virus X (PVX) vector derived Agroinfiltration	Free CP and ESAT-6 and ESAT-2A-CP fused	0.5% -1% TSP	PVX vector derived expression system is rapid, convenient and economical can be used further for the assessment of function and production of ESAT-6	[94]
10		Chloroplast expression Biolistic-mediated transformation	CTB or LipY fused ESAT6 and Mtb72F	7.5% and 1.2% TSP for CTBE-SAT-6 and CTB-Mtb72F, respectively	Partial haemolysis of red blood cells is shown in haemolysis assay of purified CTB-ESAT6 and in GM1-binding assay this fusion protein forms pentamers with the GM1-ganglioside receptor to bind	[95]
11		Nuclear expression *Agrobacterium*-mediated stable transformation	ACR antigens and Ag85B fused to a heavy chain Ab against ACR TB-recombinant immune complexes (RICs)	0.2% TSP	Purified TB-RICs were shown to be biologically active and able to bind to complement C1q component and antigen-presenting cells in the surface	[96]
12	Avian H5N1 influenza	*Agrobacterium*-mediated transformation Transient expression	Hemagglutinin (HA) protein of H5N1 pathogen Influenza HA-VLP	98–99% of proteins present in Viral H5 and H1 proteins	VLPs preparations are highly pure with no protein contamination, hyper sensibility and no induction of IgG or IgE directed against glycans	[51, 97]
13	H5N1 influenza virus (A/Indonesia/05/05 strain)	Transient expression	Purified plant-derived H5 Hemagglutinin-1 (ppH5HA-I)	ppH5HA-I was 60 mg/kg of fresh biomass approximately	Ferret anti-serum produced against A/Indonesia/05/05 strain was analysed using Western blotting, ELISA and SDS- PAGE	[52]

Table 2 (continued)

SL No	Disease/Pathogen	Method of pathogenesis	Antigenic epitope	Expression level	Findings	Reference
14	Human immunodeficiency Virus (HIV)	Stable nuclear expression Agroinfiltration	HIV p24 capsid Protein	3.5 mg/g TSP	Stably transmitted p24 gene using the plant expression system act as a feasible means of producing HIV diagnostic procedure as well as vaccine	[98]
15		Chloroplast expression (Transplastomic)Biolistics-mediated transformation	Negative regulatory protein Nef (p24-Nef) is fused with p24 HIV-1 p24-Nef	4 to 40% TSP	HIV-1 p24-Nef derived from c chloroplast efficient subunit vaccine candidate for oral boosting later subcutaneous priming of p24 and/or Nef injection	[38]
16		Chloroplast expression (Transplastomic) Biolistics-mediated transformation	C4V3 recombinant proteins	15 µg of C4V3 is presented in 50 mg of tobacco tissue freeze-dried upto 25 µg/g accumulation level estimated in fresh weight (FW) leaves	Both ELISA and western blotting shows significant antigenicity of C4V3 Transgene integration was verified using southern blot hybridization	[99]
17		Chloroplast expression (Transplastomic) Biolistics-mediated transformation	Multiepitopic protein (multi-HIV) obtained from both HIV envelope proteins of gp120 and gp41	16 µg multi-HIV/g of fresh tobacco biomass	PCR analysis and confocal microscopy and western blot analysis was used for the verification of developed multi-HIV gene and chloroplast containing localized recombinant, respectively	[63, 100]
18		TMV derived vector inoculation TMV-VPlHISc and TMV-p24-HISc plasmids	HIV-1 p24 using the TMV30B-HISc vector	6–8 µg/g VPl-His	From 25gm injected leaves of TMV-p24-HISc, 2.5 mg of p24-His isolated, and the recombinant protein was obtained. 10–15 times higher yield was obtained from p24-His than VPl-His	[34]
19		Chloroplast expression (Transplastomic) Biolistics-mediated plastid transformation	HIV-1 $Pr55^{gag}$ polyprotein	7–8% TSP, equivalent to 312–363 mg/kg FW	Transgenic plastids produce Gag proteins were capable to accumulate into particles similar to VLPs made in baculovirus/insect cells and E. coli systems	[36]

Table 2 (continued)

SL No	Disease/Pathogen	Method of pathogenesis	Antigenic epitope	Expression level	Findings	Reference
20	Hepatitis C virus (HCV)	Transient expression TMV mediated	Chimeric protein hypervariable region 1/cholera toxin subunit B (HVR1/CTB)	100 µg TSP obtained from leaf tissue of 12.5 mg	Subgenomic CTB/HVR1 message transcribed from recombinant viral RNA later translated to discrete polypeptides will accumulate into pentameric CTB particles, on the surface, they displayed HVR1 epitope, exhibit maximum expression level in RT-PCR protein analysis and ELISA assay	[66]
21	Hepatitis B	*Agrobacterium*-mediated transformation Transient expression	HBsAg	2 µg/g HBsAg FW	HBsAg expression maximum levels obtained from transformed tobacco cells reported by both ELISA and Western blot analysis. Also reported similar buoyant density 1.095 g/mL to human serum-derived ones. pHER100 vector transformed cells by ELISA shows 10 ng/mL of spent medium	[67]
22		AgroInfiltration MagnICON viral vectors Transient expression	HBsAg assembles into VLPs	295 µg/g leaf FW at post-infection day 10	Polyclonal enzyme-linked immunosorbent assay measures the HBsAg which gets accumulated by the disulphide-linked dimers into VLPs, antigen's conformational 'a' antigenic determinant was displayed	[101, 12]
23	Poliomyelitis	*Agrobacterium*-mediated transformation Transient expression	Poliovirus capsid proteins- VP1, VP2, VP3, and VP4 Polio antigens	0.31–16.85 µg/g of FW in leaf tissues	All candidate transgenic lines express the capsid proteins and dot blot analysis reports positive signal in the hyperimmune sera labelled with Anti-Poliomyelitis Virus 1 antiserum or anti-VP2, anti-VP3, or anti-VP4 under native condition	[42]
24	Measles	*Agrobacterium*-mediated transformation Transient expression	Measles virus hemagglutinin (H) protein	MAb-366 detected H protein in extracts of pBinH/KDEL 8B (T1) line with greater absorbance than pBin control transgenic	Plants converted with the pBinH construct accumulated tiny amounts of H protein, whereas those with the C-terminal KDEL sequence in the pBinH/KDEL construct accumulated more	[45]

Table 2 (continued)

SL No	Disease/Pathogen	Method of pathogenesis	Antigenic epitope	Expression level	Findings	Reference
25	Dengue Virus	*Agrobacterium*-mediated transformation Transient expression	Dengue envelope glycoprotein EIII with cholera toxin B-subunit protein (CTB–EIII)	0.019% TSP	Biological activity for intestinal epithelial cell membrane glycolipid receptor was reported in plants with CTB-EIII protein expressed. GM1-ganglioside was evident in GM1-ELISA	[102]
26		*Agrobacterium*-mediated transformation Transient expression	Type 2 Envelope glycoprotein E with immunogenic domain III of the dengue virus (EIII)	0.13 and 0.25% of TSP	Domain III dengue virus E glycoprotein (EIII) is able to prevent dengue infection	[33]
27		*Agrobacterium*-mediated transformation Transient expression	DENV (Dengue virus) VLPs	The 55 kDa TSP was detectable only after co-expression of *denv1-agronsp.*	Methods used to improve the expression of the protein complex prM-E were ineffective in increasing the yield of VLPs rather, it increases purification difficulties	[49]
28		Tobacco mosaic virus (TMV) Transient expression	Domain III of the dengue 2 envelope protein (D2EIII)	113.7 µg /mg TSP in plants inoculated with /SP/D2EIII/SEKDEL/His6	The N-terminal 5 untranslated region-omega sequence of D2EIII reports augmented protein formation which was responsive with anti-D2EIII polyclonal and anti-His tag antibodies	[50]
29	Helicobacter pylori (H. pylori)	*Agrobacterium*-mediated transformation Transient expression	HspA (heat-shock protein A)	0.09% TSP in tobacco lines T2 and T5, and 0.071% and 0.064% in T1 and T3 lines, respectively	Both in ELISA and western blot identified significant levels of HspA protein	[103, 73]
30	Rotavirus	*Agrobacterium*-mediated transformation Transient expression	Rotavirus capsid protein VP7, VP6, and VP2 of G1 genotype	The extract containing G1 RoVLPs was 5 times concentrated on a 750 kDa molecular weight after microfiltration 4.9 mg/kg TSP obtained	Aluminum hydroxide 2 doses adjuvanted G1 Ro-VLP (1 µg, 5 µg, or 30 µg), is capable of eliciting distinct immune responses with minimal toxicity	[73]
31	Norovirus	*Agrobacterium*-mediated transformation Transient expression	GI Norwalk capsid protein (NVCP)	~20% of TSP	Within a short span using the BeYDV plant expression system enriched one plant leaf is capable of producing milligram quantities of norovirus VLPs	[104]
32		*Agrobacterium*-mediated transformation Transient expression	Narita 104 virus capsid protein (NaVCP)	0.3 mg/g FW leaf at post-infection day 4	The presence of fully assembled VLPs is confirmed using transmission electron microscopy	[105]

Table 2 (continued)

SL No	Disease/Pathogen	Method of pathogenesis	Antigenic epitope	Expression level	Findings	Reference
33	Severe acute respiratory syndrome coronavirus (SARS-CoV)	*Agrobacterium*-mediated transformation Transient expression	SARS-CoV nucleocapsid (rN) protein	79 µg/g of fresh leaf	Expression of the recombinant SARS-CoV N protein expression was improved by PTGS suppressor p19 protein	[106]
34		*Agrobacterium*-mediated transformation Transient expression	SARS-CoV-S1 protein	79-kDa S1 protein	The expression of recombinant S1 protein is higher in distinct transgenic lines, according to Western blot examination utilizing particular antibodies	[107]
35	Japanese encephalitis	*Agrobacterium*-mediated transformation Transient expression	JEV cDNA encoding prM and E proteins	Transgenic plants carrying the TE-S cassette expressed a JEV E protein of ~44 kDa, (2 out of 7) whereas TLE-S expression of JEV Es protein fused to LT-B of ~58 kDa (1 out of 7)	Co-expression of JEV prM and E proteins was not possible, while E protein alone can be effectively expressed	[108]
36	Human cytomegalovirus (HCMV)	*Agrobacterium*-mediated transformation Transient expression	The immunodominant glycoprotein B complex of HCMV (gB, UL55)	1.3 ± 7.4 mg/ml that produce antigenic glycoprotein B ranging from 70 ± 146 ng/mg extracted from tobacco seeds protein	Native glycoprotein B produced was capable of inhibiting immunofluorescence on HCMV-infected human fibroblasts	[109]
37	Enterotoxigenic Escherichia coli Gastroenteritis	Chloroplast expression *Agrobacterium*-mediated transformation	Escherichia coli heat-labile toxin (LTB)	3.3% TSP	The LTB protein expression levels are increased by 3.3 percent in the F1 hybrid generation by crossing chloroplast-transformed and synthetic LTB transgenic lines, compared to 2.2 percent in the chloroplast-transformed line and 2.8 percent in the synthetic LTB gene line	[110]
38		Transplastomic (chloroplast) Biolistic method	Heat-labile enterotoxin (LT) of non-toxic subunit B (LTB)	2.5% TSP	Approximately 250-fold higher yield generated than nuclear transformation. LTB protein bound to GM1-ganglioside receptors indicated in GM1–ELISA binding assay	[111]

Table 2 (continued)

SL No	Disease/Pathogen	Method of pathogenesis	Antigenic epitope	Expression level	Findings	Reference
39	Lymphatic filariasis	*Agrobacterium*-mediated transformation Transient expression	*Brugia malayi* Abundant Larval *Transcript-2* (*Bm* ALT-2) filarial protein	50 to 90 ng/µg TSP	The recombinant P-ALT-2 is recognized by polyclonal antibodies against the E. coli-expressed filarial proteins which indicate the similarity between the plant-derived protein and *E.coli*-expressed protein	[112]
40	Tetanus	Transplastomic (chloroplast) Biolistic method	Tetanus toxin protein (TetC)	25% and 10% of TSP for the native and synthetic genes	Significant anti TetC antibodies accumulation was observed in both genes by ELISA proving the flexibility of plastids for expression of unmodified high-AT and high-GC genes	[113, 114]
41	Rabies	Agroinfiltration Stable expression	G protein fused with CTB	0.4% TSP	The fusion protein was a 403 kDa pentameric protein with a high affinity for the GM1 receptor compared to the original bacterial CtxB	[115]
42		*Agrobacterium*-mediated nuclear transformation	Surface glycoprotein (G protein) of rabies virus	0.38% TSP	At C-terminus of the G protein, an endoplasmic reticulum retention signal was involved. The presence of the G protein was analyzed using immunoblot analysis	[70]
43	Epstein-Barr virus (EBV)	Transplastomic (chloroplast) Biolistic method	EBV viral capsid antigen (VCA)	0.02–0.04 ng/mg (i.e., 0.002%–0.004%) of TSP	The Plastids can be the potential factories in the production of EBV VCA antigen. Even though it exhibits very minimal quantity, the transgene was actively transcribed and the RNA was translated to immunoreactive protein	[116]

Table 3 In Vivo studies assessing the immunogenicity of different tobacco-based vaccine candidates against human infectious diseases

Sl No	Disease	Vaccine candidate/ protein used with dose	Route of administration and Animal used	Immunogenicity findings	Reference
1	Poliomyelitis	Polio antigens of 0.8 µg of VP1, 1.40 µg of VP2, 0.43 µg of VP3, and 0.60 µg of VP4	BALB/c male mice of 7 to 8 week-old Subcutaneous mucosal	Polio VPs passed the immunogenicity assessment determined by ELISA capable of inducing local and systemic humoral responses	[42]
2	Human papillomavirus (HPV) Cervical Cancer	Chloroplast derived HPV-16 capsid, L1 transformed plants 0.2 ml leaf extract in Freund's complete adjuvant (FCA) or aluminium hydroxide gel	BALB/c mice 8-week-old Intraperitoneal	Anti-HPV-16 neutralizing antibodies were detected by ELISA in immunized mice	[37, 39]
3		0.38 µg HPV16L1 protein in 50 µg concentrated plant extract, 0.75 µg LT-B protein, or HPV16L1/LT-B mixture	6 to 8 week-old BALB/c mice female Mucosal	The L1/LTB-immunized group shows greater stimulation index (SI) of spleen as well as percentage of IFN-γ + / IL-4 + CD4 + T-cells than L1group and control	[31]
4		40 ng of CsCI-purified VLPs	BALB/c female mice Subcutaneous route	Stronger evidence of immune response is not observed even though feeding tubers from transgenic potato induces anti-L1 antibody response than tobacco	
5	Tuberculosis	BCG of 100 µL SC or with 10 µg TB-RICs preparation (in 30µL) intranasally, after isoflurane anaesthesia	6- to 8-week-old BALB/c mice female Subcutaneous intranasal	Mucosal boosting of BCG-immunized mice with TB-RICs elicited antigen-specific serum IgG antibody responses	[96]
6	Measles	Recombinant H protein injection and oral feed contain approximately 0.3–0.4 g of tobacco leaf extract and 1 mg of the CTB mucosal adjuvant	Female BALB/c mice 8-week-old Intraperitoneal mucosal	Administration of derived recombinant H protein in both routes can develop serum anti-H protein neutralizing antibodies	[45]

Table 3 (continued)

Sl No	Disease	Vaccine candidate/ protein used with dose	Route of administration and Animal used	Immunogenicity findings	Reference
7	AIDS	Purified recombinant p24-His 0.5 mg diluted in elution buffer and FCA	Female rabbits	Immune sera were able to detect the native p24 from diverse surfaces of HIV-1 infected by HUT78/ARV T-cell line after the first booster dosage, demonstrating a strong and specific humoral response to the p24-His	[34]
8		50 mg of freeze-dried leaf powder, 150 g of E. coli-derived C4V3 (group +), 50 mg of freeze-dried tobacco leaf powder from wild-type plants (group WT), and the vehicle alone provided approximately 15 g of C4V3 (group TT) (group PBS). On days 0, 7, 14, and 21, each group received four weekly doses by oral feeding	Female BALB/c mice 8- to 11-week-old Mucosal	CD4 + T-cell proliferation responses as well as both systemic and mucosal antibody response was observed in immunized mice	[117]
9		100 µg Multi-HIV protein derived from 50 mg of freeze-dried leaves plus 500 µl PBS	Female BALB/c mice 11 weeks of age Mucosal	With HIV peptide, orally inoculated mice stimulate the generation of specific interferon-gamma in both CD4 and CD8 + T cells, eliciting antibody responses against both the V3 loop of gp120 and the ELDKWA epitope of gp41	[100]
10	H5N1 influenza virus A/Indonesia/05/05 strain	Mice: Two different antigen doses 45 µg and 15 µg of ppH5HA-I were tested at 2 weeks interval along with 10 g of Quil A adjuvant. Ferrets: 90 or 45 µg doses of ppH5HA-I (or PBS as a control) plus 50 g of Quil A on days 0, 14, and 28	BALB/c mice 6-week-old and Male Fitch ferrets of 5- to 8-month-old In both Subcutaneous route	After Immunization both animals generated serum hemagglutinin-inhibiting antibodies	[52]
11	Dengue	In the first four immunizations on days 0, 21, 50, and 64, 10 g of pure D2EIII protein in PBS emulsified with Titer-Max Gold adjuvant was used, followed by 20 g in the following vaccination on days 80, 87, 101, 115, and 128	C3H female mice 5-week-old Intramuscular	Neutralizing activity for Type 2 dengue virus is exhibited by inducing the formation of anti-dengue virus antibody	[50]
12		VLPs/B-CLP groups, a 10 µg/dose was introduced, and 0.7 µg/dose for the EIII subunit	BALB/c mice 6- to 9-week-old	Immunoassay states that DENV1-SP + NSP VLPs elicit a stronger antibody response than DENV-E domain III	[49]
13	Peptic ulcer (H. Pylori)	Fed with 100 µg of HspA protein extracts emulsified (1:1) in FCA per mouse was administered weekly for 3 continuous weeks after an overnight fast	8 weeks old female BALB/c mice Mucosal	After Mucosal immunization of mice induce anti-HspA serum antibody, fused to the HspA purified bacterial protein	[103]

Table 3 (continued)

Sl No	Disease	Vaccine candidate/ protein used with dose	Route of administration and Animal used	Immunogenicity findings	Reference
14	Lymphatic filariasis	1:1 ratio of 25 µg of purified protein (ALT-2) in 100 µl PBS mixed with alum	Female BALB/c mice 6 to 8 week-old Intramuscular	Antibody isotype assay and titers showed that ALT-2 protein immunized mice can elicit noticeable IgG1, whereas IgG3 is induced by recombinant ALT-2 produced by E.coli	[112]
15	Malaria	Pfs25-CP VLP equivalent to 1.0, 0.1 or 0.01 µg of Pfs25 with or without 0.3% AlhydrogelH on days 0 and 21	Female BALB/c mice 6 to 8 week-old Intramuscular	The use of Alhydrogel to immunize mice with 1 or 2 doses of Pfs25-CP VLPs resulted in serum antibodies with 100%, 99.5% and 98.8% transmission-blocking activity was observed for doses of less than 5.0, 1.0 and 0.2 mg, respectively	[82]
16	Hepatitis C virus (HCV)	Recombinant immunogen HVR1/CTB protein of 30 µl dose, 15 µl on each nostril on days 0, 7, 14, 21, and 69	8 weeks old C57B1/6 female mice Intranasally	AntiCTB serum antibody and anti-HVR1 serum antibody, specifically fused to HCV VLP were generated after the intranasal immunization with recombinant HVR1/CTB protein in mice	[66]
17	Hepatitis B	HBsAg 1 µ g per dose) at weeks 0, 1 and 2	Mice Intraperitoneal	HBsAg-specific antibodies are induced in mice after immunization with partially purified HBsAg	[101]
18	Tetanus	Concentrated protein solution contains 100 µg of plant TetC with 50 µg CT	BALB/c female mice Intranasal and mucosal	Protective levels of TetC antibodies are generated after the mucosal immunization in mice, suggesting the safe nasal and oral route of administration	[113, 114]
19	Rabies	10 µg of the glycoprotein in FCA at 1 week and in Freund's incomplete adjuvant (FIA) at 3 weeks	Six to nine-week-old male C57BL/6 J mice	Immunization with glycoprotein induces neutralizing antibodies	[72, 71]
20		25 µg of the purified plant-derived G protein with Freund's adjuvant	BALB/c mice, intraperitoneal	Complete protective immunity against the intracerebral rabies virus is evident after the 3rd booster dose of G protein, immunization in mice which shows apparently higher immunogenicity than commercial viral vaccine	[70]

Table 3 (continued)

Sl No	Disease	Vaccine candidate/ protein used with dose	Route of administration and Animal used	Immunogenicity findings	Reference
21	Severe acute respiratory syndrome (SARS)	From tobacco roots, 50 mg of powdered Fed obtained is reconstituted in saline and administered by gastric intubation	Female BALB/c mice 6 to 8 week-old, Parenteral	Both in Western blot and ELISA analysis SARS-CoV-specific IgG was detected	[107]
22		Soluble plant extract obtained from fresh tobacco leaves 500 mg or 10 µg of the purified sN protein from E. coli per dose, emulsified with Freund's adjuvant	Female BALB/c mice 6 to 8 week-old Intraperitoneal	The presence of antibodies of the subclasses IgG1 and IgG2a in mice sera following immunization with the rN protein-specific IgG suggests successful B-cell maturation and differentiation in mice after immunization with the rN protein-specific IgG. During the immunization, the expression of IFN- and IL-10 was up-regulated in splenocytes at different times	[106]
23	Norovirus	Partially purified NaVCP VLPs (25 µg) day 0 and 21	Female BALB/c mice 5-week-old, Intranasal	Immunized mice show significant antibody responses in mucosa and serum	[105]

Measles

Measles is an systemic infectious viral disease caused by the negative sense RNA paramyxovirus of the genus *Morbillivirus* [43]. Several plant-based systems were explored for obtaining the PBV for measles, of which tobacco was the beginner. In 2001 Huang et al. investigated the immunogenic property of immunodominant recombinant measles virus hemagglutinin (MVH) protein and found to be immunogenic in mice [45]. After the administration of DNA vaccine and tobacco leaves derived antigen oral boosters significant systemic and mucosal immune response was observed [46].

Dengue Virus

Dengue is a tropical endemic disease caused by mosquitoes *Aedes aegypti* and *Aedes albopictus* exhibiting one of four serotypes of dengue virus (DENV-1 to DENV-4) [47]. Usually disease prevention is practiced by proper vector control methods recently a tetravalent live-attenuated vaccine (Dengvaxia) is found to be an effective and on market now [48]. Studies proven that tobacco, lettuce, rice, potato are hopeful platform for dengue antibody and vaccine production among them the tobacco produces higher yield of recombinant proteins such as non-structural protein 1 (NS1) and envelope protein domain III (cEDIII) the former from DENV-2 has high sensitivity and lower specificity [33]. Both are found to be immunogenic in mice and in order to enhance the oral tolerance, cEDIII was fused to CTB which reports higher affinity for GM1 Ganglioside [33, 49, 50].

Influenza

Influenza is a highly spreadable viral infection caused by a single-stranded RNA virus belongs to orthomyxovirus family transmitted by sneezing and coughing. Globally, various strains of influenza had caused a different outburst of disease with variable intensities. Three types (Type A, B, C) of influenza virus can affect human of which B is commonly affecting children [43, 44]. The antigenic drift caused by the genetic mutation will alter the strains of the virus and so the existing vaccines as well as treatment strategy will not be effective, so the development of new vaccines is vital for the prevention of the sudden outbursts. Studies proven that tobacco-based systems made significant contributions as a cost-effective platform of vaccine and antibody generators against the various strains of influenza in different outburst [44]. The *in-vitro* studies with transient expression results in the very high yield viral H5 and H1 proteins as well as ppH5HA-I, followed by the in vivo analysis in mice and ferrets proves high immunogenicity [51, 52].

The effectiveness of the vaccine was evaluated based on the findings in the hemagglutination-inhibition and virus

microneutralization antibody titers. The first clinical study for influenza-based TBV was conducted in 2010 for evaluating the safety, immunogenicity and tolerability of the H5 VLP Influenza vaccine two successive doses at 21 days separately in 255 healthy adults. Three dose levels such as 20 µg, 30 µg and 45 µg combined Alhydrogel 1%, or 45 µg without Alhydrogel were administered. HI antibody titers are used for evaluating the immunogenicity but the study results were not published [53]. Later, Landry et al. conducted a preclinical and clinical development study on hemagglutinin (HA) protein VLPs of H5N1 influenza is capable of exhibiting cross-reactive antibodies in ferrets.

Phase I safety, reactogenicity and immunogenicity study of different strains of influenza vaccine was conducted at different time period, includes the study by Landry et al. involving 48 healthy adults who received alum-adjuvanted H5 VLP vaccine 2 doses-5, 10 or 20 µg or placebo (alum) 21 days apart [54]. Chichester et al. performed a study in 100 healthy adults of 18–49-year-old administered two HAI-05 vaccines of 3 dose-escalating levels (15, 45 and 90 µg) adjuvanted with or without (90 µg) Alhydrogel® through intramuscularly 3 weeks apart [55]. In 2014, Cummings et al. conducted a dose-escalation study of HAC1, recombinant hemagglutinin-based subunit influenza vaccine of pandemic A/California/04/2009 (H1N1) strain, with and without Alhydrogel (15 µg, 45 µg and 90 µg) in 80 subjects were allotted into six vaccine study groups [56]. All these study shows promising dose-dependent immunogenicity results that exhibit inhibition of hemagglutination and microneutralization antibody titers exhibit low humoral immune responses, while 90 µg unadjuvanted group elicit the highest responses which are safe, and well-tolerated with maximum seroconversion rates [54–56].

To combat the strain of A/Indonesia/05/2005 H5N1 influenza, hemagglutinin-based VLP H5N1 vaccine was developed and Pillet et al. conducted a Phase II dose-ranging RCT for evaluating the cell-mediated immune (CMI) and humoral responses with and without alum and GLA-SE adjuvants. Two intramuscular doses of VLP along with Alhydrogel® or with a glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) were administered 21 days apart in 390 subjects. The hemagglutininination inhibition (HI) response was observed, low doses (3.75 or 7.5 µg H5VLP) of GLA-SE-adjuvanted vaccines report the HI inhibition whereas Alhydrogel adjuvanted vaccines 3 doses (10, 15 and 20 µg) fail to show the inhibition. All vaccinated groups especially groups with low dose GLA-SE adjuvanted H5VLP show better immune response, all exhibit polyfunctional and cross-reactive HA-specific $CD4^+$ T cell response [57].

Ward et al. evaluated the lot-to-lot consistency Quadrivalent VLP Influenza Vaccine through Phase III RCT. This study includes 1200 healthy adults of 18–49 years and received 3 consecutive lots of 0.5 mL QVLP (30 µg HA/strain) injection and observed for 21 days after vaccination for local and systemic reactions as well as blood was collected during pre- and post-immunization to analyse hemagglutination-inhibition (HI) antibodies which supports the early finding of safety and immunogenicity of QVLP [58, 59].

Wang et al. investigated the immunogenicity and safety of a non-adjuvanted influenza vaccine (IIV4) quadrivalent inactivated subunit. About 320 participants were registered, divided into four age groups, and two groups were given two doses of IM injection on days 0 and 28. In 6–35 months old toddlers, both the full dose subunit non-adjuvanted IIV4 split-virion (FD-subunit NAIIV4) and the half-dose (HD-subunit NAIIV4) were studied. In 3 years, cohorts, split-virion NAIIV4 active control group reported greater ADR. After vaccination, FD-subunit NAIIV4 has similar seroprotection and safety to the active control split-virion NAIIV4 as well as the half dosage in the 6–35-month toddlers' group. The results of this study show that the effective and safe FD-subunit NAIIV4 provides protection against circulating influenza viruses during the 2018–2019 flu season [60].

Human Immunodeficiency Virus

Acquired immunodeficiency syndrome (AIDS) is a mounting fatal infectious diseases caused by the HIV retrovirus directly affecting the immune system (CD4 + T cells, macrophages and dendritic cells). The prevalence of this global infectious diseases is uprising but still researchers are working to find a proper cure of this disease, currently the antiretroviral therapy (HAART) can limit the viral load alone. The economically poor regions of the world like eastern and southern Africa were affected more where this multiple drug regimen HAART therapy can be unaffordable and precautionary measures are not maintained well [61, 62]. To tackle the current scenario, numerous plant-based expression systems are found to be a compatible for the expression of HIV proteins as a potential vaccine candidate especially from soyabean seeds [44]. Out of the various *in-vitro* analysis conducted using chloroplast expression system high expression level of p24 capsid protein with the negative regulatory protein Nef of is obtained [38]. The multi- HIV protein obtained from both HIV envelope proteins gp120 and gp41 shows high immunogenic property in tested mice [63]. Even though numerous *in-vitro* expression studies were sucessful, but only very few preclinical studies were conducted which shows promising results.

Hepatitis C and Hepatitis B Virus

Hepatitis is an inflammation of the liver associated with either 5 core hepatitis viruses A to E, of which hepatitis B virus is small, double-stranded DNA virus belong to family Hepadnaviridae [43]. The prevalence of various types of hepatitis will vary in different region of the world, as per 2019 report of CDC, globally around 296 million and 58 million people are living with hepatitis B and C, respectively [121]. Studies proves that Hepatitis A and hepatitis B can be well managed using the vaccination, but vaccine research is still limiting in case of hepatitis C, D and E [64]. The beginning of PBV production starts in 1990, against the HBV surface antigen (HBsAg) in transgenic tobacco later various subunit vaccines core antigens HCV (HCcAg) and HBV (HBcAg) as well as antibodies against HBsAg were expressed in different plants [12, 65–67]. Most of the preclinical studies shows significant HBsAg-specific antibodies and AntiCTB serum antibody and anti-HVR1 serum antibody in tested mice [66, 67]. Even though phase 1 clinical trials conducted to assess the immunogenicity profile in humans through the oral route involving the transgenic potato and lettuce expressing HBsAg was sucessful, latter phases of clinical trial are hindered, but still the research in transgenic tobacco is under pipeline [44, 68].

Rabies Virus

Rabies is an infection transmitted by lyssaviruses through animal bites which can be managed very well by the timely administration of the post-exposure prophylaxis vaccination of recombinant immunoglobulin (RIG) around the wound in human and pre exposure prophylaxis in suspected animal species [69]. The expenses with the production cost of these animal cell culture vaccine is a challenge for the health economy of developing countries, which can be overcome by the use of plant-based rabies virus vaccines. The anti-rabies monoclonal antibodies E559 and 62-71-3, a rabies glycoprotein fusion to the ricin toxin B chain (rgp-rtxB), rabies-specific single-chain antibodies and fusion proteins, and the rabies virus surface glycoprotein and nucleoprotein are the proteins expressed in transgenic spinach, tobacco, maize [68, 69]. The efficacy of rabies virus glycoprotein derived from tobacco were found to be effective in mice shows cross-protection with significant neutralizing antibody [70–72]

Rotavirus

Rotavirus belong to the Reoviridae family, is transmitted through the fecal-oral route affecting the epithelium of small intestine and causing diarrhoea. Rotavirus vaccine is administered at infancy and provides 70–80% protection against gastroenteritis [43]. Parenteral non- replicating rotavirus VLP (Ro-VLP) vaccine consists of rotavirus G1 genotype surface proteins VP7, VP6 and VP2 and helper proteins NSP4 is obtained by transient expression of *N. benthamiana*. During the preclinical study the rats immunized with 2 doses of VLPs with aluminium hydroxide shows significant level of neutralising antibodies [68, 73]. Later, Kurokawa et al. in conducted the clinical trial among two population cohorts, where in cohort I involving 10 Australian adults received a single IM injection of either one intensifying dose levels of Ro-VLP (7 µg or 21 µg) or placebo whereas cohort II of 10 South African's received a single injection of 21 µg dose or placebo. Similarly in toddlers and infant's cohort group received placebo or one injection each from 2 doses (7 µg or 21 µg) and 3 doses (2.5 µg, 7 µg or 21 µg of Ro-VLP vaccine, respectively, 28 days apart. This study reports that Ro-VLP vaccine was well-tolerated irrespective to the doses levels it elicits homotypic immune response in infants (IgG and neutralizing antibody responses against anti-G1P [42] rotavirus indicating that can be a promising vaccine against rotavirus [74].

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2)

SARS-CoV-2 is encapsulated single-strand positive sense RNA virus belongs to large family coronavirus which are highly contagious causative agent of the pandemic 'coronavirus disease 2019' (COVID-19) characterised by the acute respiratory disease. Despite the fact that various strains of coronavirus SARS-CoV 2012 (Middle East respiratory syndrome-associated coronavirus, MERS-CoV) have infected people worldwide over the last 20 years, the current pandemic outbreak caused by SARS-CoV-2 had broken the global economy and healthcare system [75, 44]. From 2005 onwards, the SARS-CoV spike (S) protein was expressed in different plant system, but technologies was not well-established to make it to large scale [44].

Currently, the emergence of various SARS-CoV-2 variants and the high transmission rate highlight the need for immediate cost-effective approaches to preventing the massive spread, of which vaccination is regarded as an effective tool in managing the current health crisis (Mahmood et al. 2020); [60]. To meet the high demand for vaccines, the vaccine industry must undergo revolutionary changes. In response, rapid global efforts have resulted in an unprecedented number of vaccine candidates entering clinical trials beginning in 2020 [60]. Although recombinant DNA technology-based production has dominated among vaccine development systems, in order to meet the global population demand for vaccines, low-cost, high-output production systems are required, which was met by plant-based vaccines.

Two plant-based COVID-19 vaccine candidates, coronavirus virus-like particle (CoVLP) and by Medicago and Kentucky Bioprocessing (KBP)-201 by Kentucky BioProcessing Inc. are currently under clinical trials [58–60]. Medicago, a Canadian-based biopharmaceutical company considered the pioneer in plant-based research against various infectious diseases, as per its report's states that PBV covers protection against a larger population through the readily available in the market during the sudden outburst of the pandemic. Eg: After getting the genetic sequence production of the swine flu vaccine took only 19 days while conventional vaccine requires 6 months for the arrival in the market [76–78]. Based on the production principles used for the generation of plant-based influenza VLPs currently Medicago is focused on the development of the VLP-based SARS-CoV-2 vaccine through the insertion of the SARS-CoV-2 spike protein gene sequence into *Agrobacterium* trailed by the infection of *Nicotiana benthamiana* plants [79, 120]

Medicago conducted the Phase I Randomized controlled trial (RCT) of CoVLP shows promising safety and immunogenicity results in adults after the administration of two doses at 21 days apart (3.75 µg, 7.5 µg, and 15 µg), alone or adjuvanted with AS03 or CpG1018. High safety and efficacy were reported in Compared to convalescent patients' plasma adjuvanted CoVLP vaccine shows 10 times more neutralizing antibody responses [53, 80]. Thus, for improving immune response Medicago starts partnering with GlaxoSmithKline (GSK) to incorporate vaccine adjuvant even with lower doses of vaccines. This is now under the final phase of clinical trials and is soon expected to hit the global markets [15, 76].

Kentucky BioProcessing Inc. (KBP) is a member of the British American Tobacco (BAT) Group popular for the production of various tobacco-based biologicals located in Kentucky, United States [76]. (KBP)-201 is the TBV under clinical trial produced by *A. tumefaciens* agroinfiltration technique to enable the production of the SARS-CoV-2 protein subunits [119]. The highly glycosylated S1 polypeptide and these glycans help to produce the whole S1 and receptor binding domain with signal peptides to secrete proteins at their N-termini. BAT had proven its ability to produce 10 million influenzas vaccine doses in a month, using that same strategy they claim the potential of producing 1–3 million Covid19 doses per week [79]. In the preclinical trials, this vaccine candidate (BAT 2020) which is stable at room temperature even within a single dose shows an efficient immune response. After the promising results of Phase I trials, it had entered the Phase II trials with adjuvant and high dose of KBP-COVID-19 awaiting the US FDA approval for marketing [119].

Malaria

Malaria is a protozoal infectious disease caused by the bite of female Anopheles mosquito belong to plasmodium. Sometimes this infection can be life-threatening associated with severe anaemia, metabolic acidosis and multi-organ failure [81]. Malaria drug therapy has been found to be both expensive and susceptible to resistance. Furthermore, it has been found to be effective in combination form, which may increase the economic burden of the patient [69, 81]. Studies have proven that vector control method and vaccination therapy can be effective tools for the prevention but the production of economical vaccine that covers multiple parasite strains and stages is challenging. Various *P. falciparum* and *P. vivax surface proteins such as* Pfs25, Pf38, Pfs230, PfGAP50, MSP19, MSP142 and AMA1 are expressed in different plant systems such as arabidopsis, lettuce, tobacco and rice of which the high yield of Pfs25-CP VLPS obtained by the transient expression in tobacco leaves using TMV-based hybrid vector [82]. Later the immunogenicity study in the BALB/c mice reports significant IgG response as well as transmission-blocking activity even after 5–6 months after immunization [82]. In a study by Semiromi et al. emphasis the chloroplast -derived vaccine antigens property of dual immunity against malaria and cholera when (CTBAMA and CTB-MSP1) were fused together along with high yield by plastid transformation than the nuclear transformation that is up to 600 and 400-fold from tobacco and lettuce, respectively [83].

A Phase I dose-escalation study of Pfs25 VLP as a vaccine blocking the transmission against malaria was conducted by Chichester et al. in which the tolerability, safety, immunogenicity and reactogenicity of the Pfs25 VLP-FhCMB vaccine combined with Alhydrogel® adjuvant in 50 mM sodium phosphate aqueous solution containing total protein of 400 µg/mL was administrated IM at doses of 2, 10, 30 and 100 µg. About 44 healthy adults have participated in the study demonstrated acceptable safety and good antibody responses for doses greater than 30 µg but weak transmission reducing activity (TRA) with can be resolved by the altering the adjuvant formulation [84].

Details clinical trial status of vaccine candidate, company involved with the clinical trial registry identifier is given in Table 4.

Conclusion

Tobacco has been identified as a promising expression system for PBV production due to its compatibility, large-scale production at low-cost, and ability to limit the spread of infectious diseases in animals; however, extensive clinical trials have been hampered due to challenging safety

Table 4 Example of Tobacco plant-derived vaccines for human infectious diseases under clinical trial

Sl No:	Disease	Product name & Clinical trial status	Sponsor & CT registry Identifier	Reference
1	SARS-CoV-2	SARS-CoV-2 spike glycoprotein (VLP) Phase III (2021)	Medicago Inc. Quebec, Canada NCT05040789	[58, 59]
2		KBP-201 Phase I (2020)	Kentucky Bioprocessing, USA NCT04473690	[119, 60]
3	Rotavirus	Ro-VLP vaccine Phase I completed (2019)	Mitsubishi Tanabe Pharma Corporation, Japan NCT03507738	[74]
4	Malaria	Vaccine Pfs25 VLP *Plasmodium falciparum* Phase I (2017)	Fraunhofer, Center for Molecular Biotechnology, USA NCT02013687	[84, 82]
5	Indonesia/05/2005 (H5N1) strain	HAI-05 Influenza vaccine Phase I (2011)	Fraunhofer, Center for Molecular Biotechnology, Plymouth, USA NCT01250795	[55]
6	H1N1 Flu	HAC1 Vaccine Phase I (2014)	Fraunhofer, Center for Molecular Biotechnology, USA Walter Reed Army Institute of Research (WRAIR) Defence Advanced Research Projects Agency NCT01177202	[56]
7	H5N1 influenza A subtype infection	H5 VLP + GLA-SE Vaccine Phase II Completed (2014)	Medicago Inc. Quebec, Canada Syneos Health McGill University Health Centre Montreal, QC NCT01991561	[57]
8	Seasonal influenza virus	Influenza Quadrivalent VLP Vaccine Phase III (2020)	Medicago Inc. Quebec, Canada NCT03321968	[58, 59, 80]
9	Avian H5N1 influenza	H5 VLP vaccine with or without Alhydrogel Phase II (2010)	Medicago Inc. Quebec, Canada Syneos Health NCT01244867 NCT00984945	[54–56]
10	Pandemic Influenza (H1N1) 2009	Adjuvanted influenza H1N1 split-virion vaccine Phase I (2019)	University of Bergen, Norway Haukeland University Hospital NCT01003288	[118]

and regulatory constraints. The unexpected pandemic outbreak demonstrated the devastating impact of infectious diseases on our global economy, owing to a lack of proper treatment modalities, an increase in medication costs, and an increased burden of antibiotic resistance. Our review shows that timely administration of low-cost TBV can be an effective health strategy for combating emerging viral infectious diseases that are difficult to manage due to a lack of disease-specific antiviral therapy.

Acknowledgements We thank Amrita School of Pharmacy for providing library facilities for data extraction. Also, extent grateful to Muhammed Rashid P, Manipal College of Pharmaceutical Science, for the suggestions during the data evaluation.

Funding None

Data availability The data relating to the work is not deposited in any repository. It will be available from the corresponding author on the appropriate request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Laere, E., Ling, A. P. K., Wong, Y. P., Koh, R. Y., Lila, M. A. M., & Hussein, S. (2016). Plant-based vaccines: Production and challenges. *Journal of Botany, 2016*, 1–11. https://doi.org/10.1155/2016/4928637
2. Oyston, P., & Robinson, K. (2012). The current challenges for vaccine development. *Journal of Medical Microbiology, 61*(7), 889–894. https://doi.org/10.1099/JMM.0.039180-0
3. Shahid, N., & Daniell, H. (2016). Plant-based oral vaccines against zoonotic and non-zoonotic diseases. *Plant Biotechnology Journal, 14*(11), 2079–2099. https://doi.org/10.1111/PBI.12604

4. Kurup, V. M., & Thomas, J. (2020). Edible vaccines: Promises and challenges. *Molecular Biotechnology, 62*(2), 79–90. https://doi.org/10.1007/S12033-019-00222-1
5. Zacharie, L., Waterhouse, P., & Bally, J. (2020). Plant-based vaccines: The way ahead? *Viruses*. https://doi.org/10.3390/v13010005
6. Henry, D., Lee, S.-B., Panchal, T., & Wiebe, P. O. (2001). Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *Journal of Molecular Biology, 311*(5), 1001–1009. https://doi.org/10.1006/JMBI.2001.4921
7. Kate, E., & Cohn, J. (2013). Vaccines in developing countries: Why the high prices?—Speaking of medicine and health. Retrieved from https://speakingofmedicine.plos.org/2013/04/23/vaccines-in-developing-countries-why-the-high-prices/.
8. Kishore, K. (2014). Monograph of tobacco (*Nicotiana tabacum*). *Indian Journal of Drugs, 2*(21), 5–23.
9. Ilana, B., Torres, E. G., Walt, H., Wolf, U., Maake, C., & Martin-Soelch, C. (2020). 'Tobacco is the chief medicinal plant in my work': Therapeutic uses of tobacco in Peruvian Amazonian medicine exemplified by the work of a maestro Tabaquero. *Frontiers in Pharmacology*. https://doi.org/10.3389/FPHAR.2020.594591
10. Tobacco. (2021). WHO statistics. Retrieved from. https://www.who.int/news-room/fact-sheets/detail/tobacco.
11. Binorkar, S., & Jani, D. (2012). Traditional medicinal usage of tobacco: A review. *Journal on Complementary Medicine and Drug Discovery, 2*(2), 127. https://doi.org/10.5455/SPATULA.20120423103016
12. Mason, H. S., Lam, D. M., & Arntzen, C. J. (1992). Expression of hepatitis B surface antigen in transgenic plants. *Proceedings of the National Academy of Sciences of the United States of America, 89*(24), 11745–11749. https://doi.org/10.1073/PNAS.89.24.11745
13. Saxena, J., & Rawat, S. (2014). Edible vaccines. *Advances in Biotechnology, 12*, 207–226. https://doi.org/10.1007/978-81-322-1554-7_12
14. Potera, C. (2012). Vaccine manufacturing gets boost from tobacco plants. *Genetic Engineering and Biotechnology News, 32*(6), 8–10. https://doi.org/10.1089/GEN.32.6.02
15. Craig, J. (2021). Your next vaccine could be grown in a tobacco plant. *National Geographics*, July 8, 2021. Retrieved from https://www.nationalgeographic.com/science/article/your-next-vaccine-could-be-grown-in-a-tobacco-plant.
16. Tremblay, R., Wang, D., Jevnikar, A. M., & Ma, S. (2010). Tobacco, a highly efficient green bioreactor for production of therapeutic proteins. *Biotechnology Advances, 28*(2), 214–221. https://doi.org/10.1016/J.BIOTECHADV.2009.11.008
17. Dubey, K. K., Luke, G. A., Knox, C., Kumar, P., Pletschke, B. I., Singh, P. K., & Shukla, P. (2018). Vaccine and antibody production in plants: Developments and computational tools. *Briefings in Functional Genomics, 17*(5), 295–307. https://doi.org/10.1093/BFGP/ELY020
18. Jube, S., & Borthakur, D. (2007). Expression of bacterial genes in transgenic tobacco: Methods, applications and future prospects. *Electronic Journal of Biotechnology, 10*(3), 452. https://doi.org/10.2225/VOL10-ISSUE3-FULLTEXT-4
19. Parvathy, S. T. (2020). Engineering plants as platforms for production of vaccines. *American Journal of Plant Sciences, 11*(5), 707–735. https://doi.org/10.4236/AJPS.2020.115052
20. Cañizares, M. C., Nicholson, L., & Lomonossoff, G. P. (2005). Use of viral vectors for vaccine production in plants. *Immunology and Cell Biology, 83*(3), 263. https://doi.org/10.1111/J.1440-1711.2005.01339.X
21. Yusibov, V., Rabindran, S., Commandeur, U., Twyman, R. M., & Fischer, R. (2006). The potential of plant virus vectors for vaccine production. *Drugs in R and D, 7*(4), 203–217. https://doi.org/10.2165/00126839-200607040-00001/FIGURES/5
22. Moon, K. B., Park, J. S., Park, Y. I., Song, I. J., Lee, H. J., Cho, H. S., Jeon, J. H., & Kim, H. S. (2020). Development of systems for the production of plant-derived biopharmaceuticals. *Plants*. https://doi.org/10.3390/PLANTS9010030
23. Biopharma Dealmakers. (2018). Plant-derived vaccines. Retrieved from https://www.nature.com/articles/d43747-020-00537-y.
24. Tusé, D., Nandi, S., McDonald, K. A., & Buyel, J. F. (2020). The emergency response capacity of plant-based biopharmaceutical manufacturing-what it is and what it could be. *Frontiers in Plant Science, 11*(October), 1573. https://doi.org/10.3389/FPLS.2020.594019/BIBTEX
25. Van Zyl, A. R., & Hitzeroth, I. I. (2016). Purification of virus-like particles (VLPs) from plants. *Methods in Molecular Biology, 1404*(April), 569–579. https://doi.org/10.1007/978-1-4939-3389-1_37
26. Kirk, D. D., Kim, M., Walmsley, A. M., & Peterson, R. K. D. (2005). Risk analysis for plant-made vaccines. *Transgenic Research, 14*, 449–462. https://doi.org/10.1007/s11248-005-5697-3
27. European Medicine Agency. (2008). Guideline on the quality of biological active substances produced by stable transgene expression in higher plants draft agreed by BWP. Retrieved from http://www.emea.europa.eu.
28. WHO. (2005). WHO informal consultation on the scientific basis for regulatory evaluation of candidate human vaccines from plants. Retrieved from https://cdn.who.int/media/docs/default-source/biologicals/vaccine-quality/who-informal-consultation-on-scientific-basis-for-regulatory-evaluation-of-candidate-human-vaccines-from-plants0e0e04bd-5580-41c6-8957-7c4be9e065a7.pdf?sfvrsn=91d6f170_1&download=true.
29. Food and Drug Administration, HHS. (2017). Clarification of when products made or derived from tobacco are regulated as drugs, devices, or combination products; amendments to regulations regarding 'intended uses'. Final rule - PubMed. *Federal Registry, 82*(5), 2193–2217.
30. Hundleby, P. A. C., D'Aoust, M. A., Finkle, C., Atkins, J., & Twyman, R. M. (2022). Regulation of molecular farming products. *Methods in Molecular Biology, 2480*, 313–333. https://doi.org/10.1007/978-1-0716-2241-4_17/COVER
31. Hongli, L., Xukui, Li., Ting, L., Wensheng, Li., Lusheng, Si., & Jin, Z. (2013). Transgenic tobacco expressed HPV16-L1 and LT-B combined immunization induces strong mucosal and systemic immune responses in mice. *Human Vaccines & Immunotherapeutics, 9*(1), 83. https://doi.org/10.4161/HV.22292
32. Ma, J. K., Drossard, J., Lewis, D., Altmann, F., Boyle, J., Christou, P., Cole, T., et al. (2015). Regulatory approval and a first-in-human phase I clinical trial of a monoclonal antibody produced in transgenic tobacco plants. *Plant Biotechnology Journal, 13*(8), 1106–1120. https://doi.org/10.1111/PBI.12416
33. Kim, M.-Y., Yang, M.-S., & Kim, T.-G. (2010). Expression of dengue virus E glycoprotein domain III in non-nicotine transgenic tobacco plants. *Biotechnology and Bioprocess Engineering, 14*(6), 725–730. https://doi.org/10.1007/S12257-009-3011-6
34. Pérez-Filgueira, D. M., Brayfield, B. P., Phiri, S., Borca, M. V., Wood, C., & Morris, T. J. (2004). Preserved antigenicity of HIV-1 P24 produced and purified in high yields from plants inoculated with a tobacco mosaic virus (TMV)-derived vector. *Journal of Virological Methods, 121*(2), 201–208. https://doi.org/10.1016/J.JVIROMET.2004.06.022
35. Saba, K., Gottschamel, J., Younus, I., Syed, T., Gull, K., Lössl, A. G., Mirza, B., & Waheed, M. T. (2019). Chloroplast-based inducible expression of ESAT-6 antigen for development of a

plant-based vaccine against tuberculosis. *Journal of Biotechnology, 305*(November), 1–10. https://doi.org/10.1016/J.JBIOTEC.2019.08.016
36. Scotti, N., Alagna, F., Ferraiolo, E., Formisano, G., Sannino, L., Buonaguro, L., De Stradis, A., et al. (2009). High-level expression of the HIV-1 Pr55gag polyprotein in transgenic tobacco chloroplasts. *Planta, 229*(5), 1109–1122. https://doi.org/10.1007/S00425-009-0898-2
37. Varsani, A., Williamson, A. L., Stewart, D., & Rybicki, E. P. (2006). Transient expression of human papillomavirus type 16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector. *Virus Research, 120*(1–2), 91–96. https://doi.org/10.1016/J.VIRUSRES.2006.01.022
38. Gonzalez-Rabade, N., McGowan, E. G., Zhou, F., McCabe, M. S., Bock, R., Dix, P. J., Gray, J. C., & Ma, J.-C. (2011). Immunogenicity of chloroplast-derived HIV-1 P24 and a P24-Nef fusion protein following subcutaneous and oral administration in mice. *Plant Biotechnology Journal, 9*(6), 629–638. https://doi.org/10.1111/J.1467-7652.2011.00609.X
39. Millán, A.-S., Ortigosa, S. M., Hervás-Stubbs, S., Corral-Martínez, P., Seguí-Simarro, J. M., Gaétan, J., Coursaget, P., & Veramendi, J. (2008). Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. *Plant Biotechnology Journal, 6*(5), 427–441. https://doi.org/10.1111/J.1467-7652.2008.00338.X
40. Minor, P. (2009). Vaccine-derived poliovirus (VDPV): Impact on poliomyelitis eradication. *Vaccine, 27*(20), 2649–2652. https://doi.org/10.1016/J.VACCINE.2009.02.071
41. Daniell, H., Rai, V., & Xiao, Y. (2019). Cold chain and virus-free oral polio booster vaccine made in lettuce chloroplasts confers protection against all three poliovirus serotypes. *Plant Biotechnology Journal, 17*(7), 1357–1368. https://doi.org/10.1111/PBI.13060
42. Bolaños-Martínez, O. C., Govea-Alonso, D. O., Cervantes-Torres, J., Hernández, M., Fragoso, G., Sciutto-Conde, E., & Rosales-Mendoza, S. (2020). Expression of immunogenic poliovirus sabin type 1 VP proteins in transgenic tobacco. *Journal of Biotechnology, 322*(October), 10–20. https://doi.org/10.1016/J.JBIOTEC.2020.07.007
43. CDC. (2021). *Pinkbook-epidemiology of vaccine preventable diseases | CDC* (14th edn). Retrieved from https://www.cdc.gov/vaccines/pubs/pinkbook/hpv.html.
44. Gómez, L., Maria, X. H., Alvarez, D., He, W., Baysal, C., Zhu, C., Armario-Najera, V., et al. (2021). Contributions of the international plant science community to the fight against human infectious diseases: part 1: Epidemic and pandemic diseases. *Plant Biotechnology Journal, 19*(10), 1901–1920. https://doi.org/10.1111/pbi.13657
45. Huang, Z., Dry, I., Webster, D., Strugnell, R., & Wesselingh, S. (2001). Plant-derived measles virus hemagglutinin protein induces neutralizing antibodies in mice. *Vaccine, 19*(15–16), 2163–2171. https://doi.org/10.1016/S0264-410X(00)00390-X
46. Webster, D. E., Cooney, M. L., Huang, Z., Drew, D. R., Ramshaw, I. A., Dry, I. B., Strugnell, R. A., Martin, J. L., & Wesselingh, S. L. (2002). Successful boosting of a DNA measles immunization with an oral plant-derived measles virus vaccine. *Journal of Virology, 76*(15), 7910–7912. https://doi.org/10.1128/JVI.76.15.7910-7912.2002
47. Pang, T., Mak, T. K., & Gubler, D. J. (2017). Prevention and control of dengue-the light at the end of the tunnel. *The Lancet Infectious Diseases, 17*(3), e79-87. https://doi.org/10.1016/S1473-3099(16)30471-6
48. FDA. (2021). DENGVAXIA | FDA. Retrieved from https://www.fda.gov/vaccines-blood-biologics/dengvaxia.
49. Ponndorf, D., Meshcheriakova, Y., Thuenemann, E. C., Alonso, A. D., Overman, R., Holton, N., Dowall, S., et al. (2021). Plant-made dengue virus-like particles produced by co-expression of structural and non-structural proteins induce a humoral immune response in mice. *Plant Biotechnology Journal, 19*(4), 745–756. https://doi.org/10.1111/PBI.13501
50. Saejung, W., Fujiyama, K., Takasaki, T., Ito, M., Hori, K., Malasit, P., Watanabe, Y., Kurane, I., & Seki, T. (2007). Production of dengue 2 envelope domain III in plant using TMV-based vector system. *Vaccine, 25*(36), 6646–6654. https://doi.org/10.1016/J.VACCINE.2007.06.029
51. Le Mauff, F., Mercier, G., Chan, P., Burel, C., Vaudry, D., Bardor, M., Vézina, L. P., Couture, M., Lerouge, P., & Landry, N. (2015). Biochemical composition of haemagglutinin-based influenza virus-like particle vaccine produced by transient expression in tobacco plants. *Plant Biotechnology Journal, 13*(5), 717–725. https://doi.org/10.1111/PBI.12301
52. Shoji, Y., Bi, H., Musiychuk, K., Rhee, A., Horsey, A., Roy, G., Green, B., et al. (2009). Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. *Vaccine, 27*(7), 1087–1092. https://doi.org/10.1016/J.VACCINE.2008.11.108
53. Medicago. (2012). Immunogenicity, safety, tolerability of a plant-made H5 VLP influenza vaccine. *Medicago*. Retrieved from https://clinicaltrials.gov/ct2/show/NCT01244867.
54. Landry, N., Ward, B. J., Trépanier, S., Montomoli, E., Dargis, M., Lapini, G., & Vézina, L. P. (2010). Preclinical and clinical development of plant-made virus-like particle vaccine against Avian H5N1 influenza. *PLoS ONE*. https://doi.org/10.1371/JOURNAL.PONE.0015559
55. Chichester, J. A., Mark Jones, R., Green, B. J., Stow, M., Miao, F., Moonsammy, G., Streatfield, S. J., & Yusibov, V. (2012). Safety and immunogenicity of a plant-produced recombinant hemagglutinin-based influenza vaccine (HAI-05) derived from A/Indonesia/05/2005 (H5N1) influenza virus: A phase 1 randomized, double-blind, placebo-controlled, dose-escalation study in healthy adult. *Viruses, 4*(11), 3227. https://doi.org/10.3390/V4113227
56. Cummings, J. F., Guerrero, M. L., Moon, J. E., Waterman, P., Nielsen, R. K., Jefferson, S., Gross, F. L., Hancock, K., Katz, J. M., & Yusibov, V. (2014). Safety and immunogenicity of a plant-produced recombinant monomer hemagglutinin-based influenza vaccine derived from influenza A (H1N1)Pdm09 virus: A phase 1 dose-escalation study in healthy adults. *Vaccine, 32*(19), 2251–2259. https://doi.org/10.1016/J.VACCINE.2013.10.017
57. Pillet, S., Aubin, É., Trépanier, S., Poulin, J.-F., Yassine-Diab, B., ter Meulen, J., Ward, B. J., & Landry, N. (2018). Humoral and cell-mediated immune responses to H5N1 plant-made virus-like particle vaccine are differentially impacted by alum and GLA-SE adjuvants in a phase 2 clinical trial. *Vaccines, 3*(1), 1–9. https://doi.org/10.1038/s41541-017-0043-3
58. Ward, B. J., Gobeil, P., Séguin, A., Atkins, J., Boulay, I., Charbonneau, P.-Y., Couture, M., et al. (2021). Phase 1 randomized trial of a plant-derived virus-like particle vaccine for COVID-19. *Nature Medicine, 27*(6), 1071–1078. https://doi.org/10.1038/s41591-021-01370-1
59. Ward, B. J., Séguin, A., Couillard, J., Trépanier, S., & Landry, N. (2021). Phase III: Randomized observer-blind trial to evaluate lot-to-lot consistency of a new plant-derived quadrivalent virus like particle influenza vaccine in adults 18–49 years of age. *Vaccine, 39*(10), 1528–1533. https://doi.org/10.1016/J.VACCINE.2021.01.004
60. Puna-Maya, M., & Choe, S. (2021). Plant-based COVID-19 vaccines: Current status, design, and development strategies of candidate vaccines. *Vaccines, 9*(9), 992. https://doi.org/10.3390/VACCINES9090992

61. UN. (2020a). Global HIV & AIDS statistics—Fact sheet | UNAIDS. Retrieved from https://www.unaids.org/en/resources/fact-sheet.
62. UN. (2020b). HIV and AIDS—Basic facts | UNAIDS. Retrieved from https://www.unaids.org/en/frequently-asked-questions-about-hiv-and-aids.
63. Sergio, R.-M., Rubio-Infante, N., Monreal-Escalante, E., Govea-Alonso, D. O., García-Hernández, A. L., Salazar-González, J. A., González-Ortega, O., Paz-Maldonado, L. M. T., & Moreno-Fierros, L. (2014). Chloroplast expression of an HIV envelop-derived multiepitope protein: Towards a multivalent plant-based vaccine. *Plant Cell, Tissue and Organ Culture, 116*, 111–123. https://doi.org/10.1007/s11240-013-0387-y
64. Ogholikhan, S., & Schwarz, K. B. (2016). Hepatitis vaccines. *Vaccines*. https://doi.org/10.3390/VACCINES4010006
65. Hernández-Velázquez, A., López-Quesada, A., Ceballo-Cámara, Y., Cabrera-Herrera, G., Tiel-González, K., Mirabal-Ortega, L., Pérez-Martínez, M., et al. (2015). Tobacco seeds as efficient production platform for a biologically active anti-HBsAg monoclonal antibody. *Transgenic Research, 24*(5), 897–909. https://doi.org/10.1007/S11248-015-9890-8
66. Nemchinov, L. G., Liang, T. J., Rifaat, M. M., Mazyad, H. M., Hadidi, A., & Keith, J. M. (2000). Development of a plant-derived subunit vaccine candidate against hepatitis C virus. *Archives of Virology, 145*(12), 2557–2573. https://doi.org/10.1007/S007050070008
67. Sunil Kumar, G. B., Ganapathi, T. R., Revathi, C. J., Prasad, K. S. N., & Bapat, V. A. (2003). Expression of hepatitis B surface antigen in tobacco cell suspension cultures. *Protein Expression and Purification, 32*(1), 10–17. https://doi.org/10.1016/J.PEP.2003.07.004
68. Stander, J., Mbewana, S., & Meyers, A. E. (2022). Plant-derived human vaccines: Recent developments. *BioDrugs, 36*(5), 573–589. https://doi.org/10.1007/S40259-022-00544-8
69. He, W., Baysal, C., Gómez, M. L., Huang, X., Alvarez, D., Zhu, C., Armario-Najera, V., et al. (2021). Contributions of the international plant science community to the fight against infectious diseases in humans-part 2: Affordable drugs in edible plants for endemic and re-emerging diseases. *Plant Biotechnology Journal, 19*(10), 1921–1936. https://doi.org/10.1111/PBI.13658
70. Ashraf, S., Singh, P. K., Yadav, D. K., Shahnawaz, Md., Mishra, S., Sawant, S. V., & Tuli, R. (2005). High level expression of surface glycoprotein of rabies virus in tobacco leaves and its immunoprotective activity in mice. *Journal of Biotechnology, 119*(1), 1–14. https://doi.org/10.1016/J.JBIOTEC.2005.06.009
71. PereaArango, I., Loza Rubio, E., Rojas Anaya, E., Olivera Flores, T., Gonzalez de la Vara, L., & Gómez Lim, M. A. (2008). Expression of the rabies virus nucleoprotein in plants at high-levels and evaluation of immune responses in mice. *Plant Cell Reports, 27*(4), 677–685. https://doi.org/10.1007/S00299-007-0324-9
72. Park, Y., Kang, H., Min, K., Kim, N. H., Park, M., Ouh, I.-O., Kim, H.-H., et al. (2021). Rabies virus glycoprotein produced in nicotiana benthamiana is an immunogenic antigen in mice. *Czech Journal of Genetics and Plant Breeding, 57*(1), 26–35.
73. Kurokawa, N., Lavoie, P. O., D'Aoust, M. A., Couture, M. M., Dargis, M., Trépanier, S., Hoshino, S., Koike, T., Arai, M., & Tsutsui, N. (2021). Development and characterization of a plant-derived rotavirus-like particle vaccine. *Vaccine, 39*(35), 4979–4987. https://doi.org/10.1016/J.VACCINE.2021.07.039
74. Kurokawa, N., Robinson, M. K., Bernard, C., Kawaguchi, Y., Koujin, Y., Koen, A., Madhi, S., et al. (2021). Safety and immunogenicity of a plant-derived rotavirus-like particle vaccine in adults, toddlers and infants. *Vaccine, 39*(39), 5513–5523. https://doi.org/10.1016/J.VACCINE.2021.08.052
75. Hu, B., Hua, G., Peng, Z., & Zheng, L. S. (2020). Characteristics of SARS-CoV-2 and COVID-19. *Nature Reviews Microbiology, 19*(3), 141–154. https://doi.org/10.1038/s41579-020-00459-7
76. Kumar, A. U., Kadiresen, K., Gan, W. C., & Ling, A. P. K. (2021). Current updates and research on plant-based vaccines for coronavirus disease 2019. *Clinical and Experimental Vaccine Research, 10*(1), 13. https://doi.org/10.7774/CEVR.2021.10.1.13
77. Rosales-Mendoza, S., Márquez-Escobar, V. A., González-Ortega, O., Nieto-Gómez, R., & Arévalo-Villalobos, J. I. (2020). What does plant-based vaccine technology offer to the fight against COVID-19? *Vaccines*. https://doi.org/10.3390/VACCINES8020183
78. Pipeline: Influenza, rotavirus, norovirus and coronavirus. (2021). Retrieved from https://www.medicago.com/en/pipeline/.
79. Venkataraman, S., Kathleen, H., Abdullah, M., & Mounir, A. (2021). Combating human viral diseases: Will plant-based vaccines be the answer? *Vaccines, 9*(7), 761. https://doi.org/10.3390/VACCINES9070761
80. Ward, B. J., Makarkov, A., Séguin, A., Pillet, S., Trépanier, S., Dhaliwall, J., Libman, M. D., Vesikari, T., & Landry, N. (2020). Efficacy, immunogenicity, and safety of a plant-derived, quadrivalent, virus-like particle influenza vaccine in adults (18–64 years) and older adults (≥65 years): Two multicentre, randomised phase 3 trials. *Lancet (London, England), 396*(10261), 1491–1503. https://doi.org/10.1016/S0140-6736(20)32014-6
81. Talapko, J., Škrlec, I., Alebić, T., Jukić, M., & Včev, A. (2019). Malaria: The past and the present. *Microorganisms*. https://doi.org/10.3390/MICROORGANISMS7060179
82. Jones, R. M., Chichester, J. A., Mett, V., Jaje, J., Tottey, S., Manceva, S., Casta, L. J., et al. (2013). A plant-produced Pfs25 VLP malaria vaccine candidate induces persistent transmission blocking antibodies against plasmodium falciparum in immunized mice. *PLoS ONE, 8*(11), e79538. https://doi.org/10.1371/JOURNAL.PONE.0079538
83. Davoodi-Semiromi, A., Schreiber, M., Nalapalli, S., Verma, D., Singh, N. D., Banks, R. K., Chakrabarti, D., & Daniell, H. (2010). Chloroplast-derived vaccine antigens confer dual immunity against cholera and malaria by oral or injectable delivery. *Plant Biotechnology Journal, 8*(2), 223. https://doi.org/10.1111/J.1467-7652.2009.00479.X
84. Chichester, J. A., Green, B. J., Jones, R. M., Shoji, Y., Miura, K., Long, C. A., Lee, C. K., et al. (2018). Safety and immunogenicity of a plant-produced Pfs25 virus-like particle as a transmission blocking vaccine against malaria: A phase 1 dose-escalation study in healthy adults. *Vaccine, 36*(39), 5865–5871. https://doi.org/10.1016/J.VACCINE.2018.08.033
85. Daniell, H., Singh, N. D., Mason, H., & Streatfield, S. J. (2009). Plant-made vaccine antigens and biopharmaceuticals. *Trends in Plant Science, 14*(12), 669–679. https://doi.org/10.1016/j.tplants.2009.09.009
86. Kwon, K. C., Verma, D., Singh, N. D., Herzog, R., & Daniell, H. (2013). Oral delivery of human biopharmaceuticals, autoantigens and vaccine antigens bioencapsulated in plant cells. *Advanced Drug Delivery Reviews, 65*(6), 782–799. https://doi.org/10.1016/J.ADDR.2012.10.005
87. Gomez, P. L., & Robinson, J. M. (2018). Vaccine manufacturing. *Plotkin's Vaccines*. https://doi.org/10.1016/B978-0-323-35761-6.00005-5
88. Penney, C. A., Thomas, D. R., Deen, S. S., & Walmsley, A. M. (2011). Plant-made vaccines in support of the millennium development goals. *Plant Cell Reports, 30*(5), 789. https://doi.org/10.1007/S00299-010-0995-5
89. Pollard, A. J., & Bijker, E. M. (2020). A guide to vaccinology: From basic principles to new developments. *Nature Reviews Immunology, 21*(2), 83–100. https://doi.org/10.1038/s41577-020-00479-7

90. Gumusoglu, S. B., Chilukuri, A. S. S., Santillan, D. A., Santillan, M. K., & Stevens, H. E. (2020). Neurodevelopmental outcomes of prenatal preeclampsia exposure. *Trends in Neurosciences, 43*(4), 253–268. https://doi.org/10.1016/j.tins.2020.02.003
91. Vaccine knowledge projects. (2021). Types of vaccine | Vaccine knowledge. Retrieved from https://vk.ovg.ox.ac.uk/vk/types-of-vaccine.
92. Tariq, H., Batool, S., Asif, S., Ali, M., & Abbasi, B. H. (2022). Virus-like particles: Revolutionary platforms for developing vaccines against emerging infectious diseases. *Frontiers in Microbiology, 12*(January), 4137. https://doi.org/10.3389/FMICB.2021.790121/BIBTEX
93. Liu, H.-L., Li, W.-S., Lei, T., Zheng, J., Zhang, Z., Yan, X.-F., Wang, Z.-Z., Wang, Y.-L., & Si, L.-S. (2005). Expression of human papillomavirus type 16 L1 protein in transgenic tobacco plants. *Acta Biochimica et Biophysica Sinica, 37*(3), 153–158. https://doi.org/10.1093/ABBS/37.3.153
94. Zelada, A. M., Calamante, G., de la Paz, M., Santangelo, F. B., Verna, F., Mentaberry, A., & Cataldi, Á. (2006). Expression of tuberculosis antigen ESAT-6 in *Nicotiana tabacum* using a potato virus X-based vector. *Tuberculosis, 86*(3–4), 263–267. https://doi.org/10.1016/J.TUBE.2006.01.003
95. Lakshmi, P. S., Verma, D., Yang, X., Lloyd, B., & Daniell, H. (2013). Low cost tuberculosis vaccine antigens in capsules: Expression in chloroplasts, bio-encapsulation, stability and functional evaluation in vitro. *PLoS ONE*. https://doi.org/10.1371/JOURNAL.PONE.0054708
96. Pepponi, I., Diogo, G. R., Stylianou, E., van Dolleweerd, C. J., Drake, P. M. W., Paul, M. J., Sibley, L., Ma, J.-C., & Reljic, R. (2014). Plant-derived recombinant immune complexes as self-adjuvanting TB immunogens for mucosal boosting of BCG. *Plant Biotechnology Journal, 12*(7), 840–850. https://doi.org/10.1111/PBI.12185
97. Yusibov, V., Streatfield, S. J., & Kushnir, N. (2011). Clinical development of plant-produced recombinant pharmaceuticals: Vaccines, antibodies and beyond. *Human Vaccines, 7*(3), 313–321. https://doi.org/10.4161/HV.7.3.14207
98. Zhang, G. G., Rodrigues, L., Rovinski, B., & White, K. A. (2002). Production of HIV-1 P24 protein in transgenic tobacco plants. *Molecular Biotechnology, 20*(2), 131–136. https://doi.org/10.1385/MB:20:2:131
99. Rubio-Infante, N., Govea-Alonso, D. O., Alpuche-Solís, Á. G., García-Hernández, A. L., Soria-Guerra, R. E., Paz-Maldonado, L. M., Ilhuicatzi-Alvarado, D., et al. (2012). A chloroplast-derived C4V3 polypeptide from the human immunodeficiency virus (HIV) is orally immunogenic in mice. *Plant Molecular Biology, 78*(4–5), 337–349. https://doi.org/10.1007/S11103-011-9870-1
100. Rubio-Infante, N., Govea-Alonso, D. O., Romero-Maldonado, A., García-Hernández, A. L., Ilhuicatzi-Alvarado, D., Salazar-González, J. A., Korban, S. S., Rosales-Mendoza, S., & Moreno-Fierros, L. (2015). A plant-derived multi-HIV antigen induces broad immune responses in orally immunized mice. *Molecular Biotechnology, 57*(7), 662–674. https://doi.org/10.1007/S12033-015-9856-3
101. Huang, Z., LePore, K., Elkin, G., Thanavala, Y., & Mason, H. S. (2008). High-yield rapid production of hepatitis B surface antigen in plant leaf by a viral expression system. *Plant Biotechnology Journal, 6*(2), 202–209. https://doi.org/10.1111/J.1467-7652.2007.00316.X
102. Kim, T.-G., & Yang, M.-S. (2010). Current Trends in Edible Vaccine Development Using Transgenic Plants. *Biotechnology and Bioprocess Engineering, 15*(1), 61–65. https://doi.org/10.1007/S12257-009-3084-2
103. Zhang, H., Zhang, X., Liu, M., Zhang, J., Li, Y., & Zheng, C.-C. (2006). Expression and characterization of helicobacter pylori heat-shock protein A (HspA) protein in transgenic tobacco (*Nicotiana tabacum*) plants. *Biotechnology and Applied Biochemistry, 43*(1), 33–38. https://doi.org/10.1042/BA20050135
104. Diamos, A. G., & Mason, H. S. (2018). High-level expression and enrichment of norovirus virus-like particles in plants using modified geminiviral vectors. *Protein Expression and Purification, 151*(November), 86–92. https://doi.org/10.1016/J.PEP.2018.06.011
105. Mathew, L. G., Herbst-Kralovetz, M. M., & Mason, H. S. (2014). Norovirus narita 104 virus-like particles expressed in *Nicotiana benthamiana* induce serum and mucosal immune responses. *BioMed Research International*. https://doi.org/10.1155/2014/807539
106. Zheng, N., Xia, R., Yang, C., Yin, B., Li, Y., Duan, C., Liang, L., Guo, H., & Xie, Qi. (2009). Boosted expression of the SARS-CoV nucleocapsid protein in tobacco and its immunogenicity in mice. *Vaccine, 27*(36), 5001–5007. https://doi.org/10.1016/J.VACCINE.2009.05.073
107. Pogrebnyak, N., Golovkin, M., Andrianov, V., Spitsin, S., Smirnov, Y., Egolf, R., & Koprowski, H. (2005). Severe acute respiratory syndrome (SARS) S protein production in plants: Development of recombinant vaccine. *Proceedings of the National Academy of Sciences, 102*(25), 9062–9067. https://doi.org/10.1073/PNAS.0503760102
108. Appaiahgari, M. B., Abdin, M. Z., Bansal, K. C., & Vrati, S. (2009). Expression of Japanese encephalitis virus envelope protein in transgenic tobacco plants. *Journal of Virological Methods, 162*(1–2), 22–29. https://doi.org/10.1016/J.JVIROMET.2009.07.002
109. Tackaberry, E. S., Dudani, A. K., Prior, F., Tocchi, M., Sardana, R., Altosaar, I., & Ganz, P. R. (1999). Development of biopharmaceuticals in plant expression systems: Cloning, expression and immunological reactivity of human cytomegalovirus glycoprotein B (UL55) in seeds of transgenic tobacco. *Vaccine, 17*(23–24), 3020–3029. https://doi.org/10.1016/S0264-410X(99)00150-4
110. Kim, B. M., & Kang, T. J. (2019). Expression of B subunit of *E. coli* heat-labile enterotoxin in the progenies of transgenic tobacco bred by crossing nuclear- and chloroplast-transgenic lines. *Protein Expression and Purification, 155*(March), 54–58. https://doi.org/10.1016/J.PEP.2018.11.005
111. Kang, T. J., Loc, N. H., Jang, M. O., Jang, Y. S., Kim, Y. S., Seo, J. E., & Yang, M. S. (2003). Expression of the B subunit of *E. Coli* heat-labile enterotoxin in the chloroplasts of plants and its characterization. *Transgenic Research, 12*(6), 683–691. https://doi.org/10.1023/B:TRAG.0000005114.23991.BC
112. Ganapathy, M., Perumal, A., Mohan, C., Palaniswamy, H., & Perumal, K. (2013). Immunogenicity of Brugia Malayi abundant larval transcript-2, a potential filarial vaccine candidate expressed in tobacco. *Plant Cell Reports, 33*(1), 179–188. https://doi.org/10.1007/S00299-013-1521-3
113. Tregoning, J. S., Nixon, P., Kuroda, H., Svab, Z., Clare, S., Bowe, F., Fairweather, N., et al. (2003). Expression of tetanus toxin fragment C in tobacco chloroplasts. *Nucleic Acids Research, 31*(4), 1174–1179. https://doi.org/10.1093/NAR/GKG221
114. Tregoning, J. S., Clare, S., Bowe, F., Edwards, L., Fairweather, N., Qazi, O., Nixon, P. J., Maliga, P., Dougan, G., & Hussell, T. (2005). Protection against tetanus toxin using a plant-based vaccine. *European Journal of Immunology, 35*(4), 1320–1326. https://doi.org/10.1002/EJI.200425453
115. Roy, S., Tyagi, A., Tiwari, S., Singh, A., Sawant, S. V., Singh, P. K., & Tuli, R. (2010). Rabies glycoprotein fused with B subunit of cholera toxin expressed in tobacco plants folds into biologically active pentameric protein. *Protein Expression and Purification, 70*(2), 184–190. https://doi.org/10.1016/j.pep.2009.10.002

116. Lee, M. Y. T., Zhou, Y., Lung, R. W. M., Chye, M. L., Yip, W. K., Zee, S. Y., & Lam, E. (2006). Expression of viral capsid protein antigen against Epstein-Barr virus in plastids of *Nicotiana tabacum* Cv. SR1. *Biotechnology and Bioengineering, 94*(6), 1129–1137. https://doi.org/10.1002/BIT.20948
117. Thirthalli, J., & Chand, P. K. (2009). The implications of medication development in the treatment of substance use disorders in developing countries. *Current Opinion in Psychiatry, 22*(3), 274–280. https://doi.org/10.1097/YCO.0b013e32832a1dc0
118. Jul-Larsen, Å., Madhun, A. S., Brokstad, K. A., Montomoli, E., Yusibov, V., & Cox, R. J. (2012). The human potential of a recombinant pandemic influenza vaccine produced in tobacco plants. *Human Vaccines & Immunotherapeutics, 8*(5), 653. https://doi.org/10.4161/HV.19503
119. Kentucky BioProcessing, Inc. (2021). KBP-201 COVID-19 vaccine trial in healthy volunteers—full text view—ClinicalTrials.Gov. Retrieved from https://clinicaltrials.gov/ct2/show/NCT04473690.
120. Medicago Newsroom. (n.d.). Retrieved October 3, 2021 from https://www.medicago.com/en/publications/.
121. WHO. (2021). Global progress report on HIV, viral sexually transmitted infections. *World Health Organization*. Retrieved from https://www.who.int/publications/i/item/9789240027077.