



**SAPIENZA**  
UNIVERSITÀ DI ROMA

# **Vaccine Production using Plants as Alternative Expression System**

*Ph. D. Course in:*

Molecular design and characterization for the promotion  
of health and well-being: from drug to food

*Department:* Drug Chemistry and Technologies

*XXXVIII Cycle*

*Ph. D. Candidate:* Teresa Iucci

*Supervisor:* Prof.ssa Luisa Mannina

*Director of PhD Program:* Prof.ssa Giovanna Poce

A.A. 2025-2026



*A mamma e papà, per avermi dato la possibilità di sognare.*

*A Michi, per realizzare insieme a me quei sogni ogni giorno.*

*A Ade, il mio perfetto opposto.*

*A nonno Giannino.*





# TABLE OF CONTENTS

|                                                                                              |           |
|----------------------------------------------------------------------------------------------|-----------|
| <b>ABSTRACT .....</b>                                                                        | <b>7</b>  |
| <b>1. INTRODUCTION .....</b>                                                                 | <b>9</b>  |
| 1.1 The importance of vaccines .....                                                         | 9         |
| 1.2 Plant Molecular Farming .....                                                            | 11        |
| 1.3 Whole Plant vs Plant cells .....                                                         | 21        |
| 1.3.1 Whole Plants in PMF .....                                                              | 22        |
| 1.3.2 Plant Cell Cultures in PMF .....                                                       | 23        |
| 1.3.3 Comparative Analysis Between Whole Plants and Plant Cell Cultures .....                | 24        |
| 1.4 Future of Plant Molecular Farming .....                                                  | 25        |
| <b>2. AIMS and OBJECTIVES.....</b>                                                           | <b>27</b> |
| <b>3. MATERIALS AND METHODS .....</b>                                                        | <b>29</b> |
| 3.1 Methods from Chapter 4 .....                                                             | 29        |
| 3.2 Methods from Chapter 5 .....                                                             | 32        |
| 3.3 Methods from Chapter 6 .....                                                             | 38        |
| 3.4 Methods from Chapter 7 .....                                                             | 45        |
| <b>4. UPS – Upstream Process Optimization .....</b>                                          | <b>52</b> |
| 4.1 Introduction to Upstream Processing in Plant Molecular Farming .....                     | 52        |
| 4.2 Overview of the Host Plant: <i>Nicotiana benthamiana</i> .....                           | 53        |
| 4.3 Laboratory-Scale Upstream Processing: UCL Prototype System .....                         | 57        |
| 4.3.1 Lab-Scale Cultivation Conditions .....                                                 | 58        |
| 4.3.2 Optimization and Validation of the Lab-Scale Greenhouse .....                          | 64        |
| 4.3.3 <i>Agrobacterium tumefaciens</i> – Transformation and Agroinfection on Lab-scale ..... | 69        |
| 4.4 Industrial-Scale Upstream Processing: The SPL Case Study .....                           | 72        |
| 4.4.1 Industrial Scale Plant Cultivation System .....                                        | 73        |
| 4.4.2 Agroinfiltration at Industrial Scale .....                                             | 76        |
| 4.4.3 Optimization of the Agroinfiltration parameters .....                                  | 77        |
| 4.5 Comparative Analysis: Lab-Scale vs Industrial Systems .....                              | 80        |
| <b>5. THE TB002: A Recombinant Subunit Vaccine Produced in Plants .....</b>                  | <b>82</b> |

|       |                                                                   |            |
|-------|-------------------------------------------------------------------|------------|
| 5.1   | Introduction and Scientific Background .....                      | 82         |
| 5.2   | Experimental Design.....                                          | 83         |
| 5.3   | Downstream Process development and optimization .....             | 83         |
| 5.3.1 | Protein Extraction: Buffer Screening and Clarification .....      | 85         |
| 5.3.2 | Immobilized Metal Affinity Chromatography (IMAC) .....            | 87         |
| 5.3.3 | Intermediate Purification and Diafiltration .....                 | 91         |
| 5.3.4 | Thrombin Cleavage and His-tag Removal .....                       | 92         |
| 5.3.5 | Final Polishing and Formulation.....                              | 95         |
| 5.4   | Analytical Characterization .....                                 | 99         |
| 5.5   | Discussion and Future Directions .....                            | 100        |
| 6.    | <b>mRNA Vaccine Platform Produced in Plants .....</b>             | <b>102</b> |
| 6.1   | Introduction and Scientific Background .....                      | 102        |
| 6.2   | Experimental Design.....                                          | 103        |
| 6.3   | Extraction and Characterization of mRNA produced in plants.....   | 105        |
| 6.3.1 | pGreenII – Binary Plant Vector .....                              | 105        |
| 6.3.2 | <i>Agrobacterium tumefaciens</i> Transformation .....             | 107        |
| 6.3.3 | Agroinfiltration of <i>Nicotiana benthamiana</i> plants.....      | 108        |
| 6.3.4 | mRNA Expression Quantification.....                               | 109        |
| 6.4   | Conclusions .....                                                 | 122        |
| 7.    | <b>CCMV: a VLP Produced in Plants.....</b>                        | <b>124</b> |
| 7.1   | Introduction and Scientific Background .....                      | 124        |
| 7.2   | Experimental Design.....                                          | 125        |
| 7.3   | CCMV Expression and Purification from <i>E. coli</i> .....        | 126        |
| 7.4   | CCMV Expression and Purification from <i>N. benthamiana</i> ..... | 141        |
| 7.5   | Conclusions.....                                                  | 144        |
| 8.    | <b>CONCLUSIONS .....</b>                                          | <b>146</b> |
| 9.    | <b>BIBLIOGRAPHY .....</b>                                         | <b>149</b> |

## ABSTRACT

The COVID-19 pandemic and the increasing frequency of global health emergencies have emphasized the need for vaccine manufacturing platforms that are not only rapid and flexible, but also scalable, sustainable, and globally accessible. Conventional expression systems based on mammalian or microbial cells are constrained by high production costs, complex infrastructure, and limited scalability in low-resource settings. In this context, Plant Molecular Farming (PMF) has emerged as a compelling alternative, leveraging the ability of plants to produce complex biopharmaceuticals. PMF offers short production times, low capital investment, and the potential for decentralized biomanufacturing, thus addressing critical limitations of traditional vaccine supply chains. This PhD research explored PMF as an alternative platform for vaccine production, with a particular focus on downstream processing and purification, the key bottlenecks that remain against industrial translation. Two complementary vaccine strategies were investigated: recombinant protein vaccines and nucleic acid–based vaccines, enabling a comparative assessment of process feasibility and analytical challenges.

The project was structured into two complementary phases, one conducted in an industrial setting and the other in an academic environment, allowing to investigate both perspectives. The first phase was carried out at Special Product's Line (SPL, Anagni, Italy) and addressed the development of a downstream process for TB002, a recombinant subunit vaccine candidate against tuberculosis produced in *Nicotiana benthamiana*. Process optimization included buffer screening, immobilized metal affinity chromatography, thrombin-mediated tag removal, and final polishing. Despite challenges such as aggregation and protein loss during sterilization, a robust protocol was established, enabling the expression of a recombinant subunit vaccine suitable for preclinical validation. The second phase was conducted at University College London (UCL, London, UK) and focused on nucleic acid–based vaccine platforms. A model mRNA vaccine encoding GFP was adapted for plant expression using the pGreenII binary vector and delivered into *N. benthamiana* via *Agrobacterium*-mediated infiltration. Expression was confirmed in planta, and a range of extraction and purification methods were compared, including anion-exchange and oligo(dT) chromatography, magnetic beads capture, and commercial silica kits. While transcript recovery was achieved, yields and purity were strongly method-dependent, highlighting the technical complexity of plant-derived mRNA purification. In parallel, virus-like particles (VLPs) derived from Cowpea Chlorotic Mottle Virus (CCMV) were developed as protective nanocapsule for mRNA. The coat protein

was expressed in both *E. coli* and *N. benthamiana*, purified using a combination of chromatographic strategies, and tested for assembly/disassembly under pH-controlled conditions. These studies provided proof-of-concept for plant-based VLP production and their potential application in mRNA encapsulation. Overall, this PhD work demonstrates both the opportunities and bottlenecks of Plant Molecular Farming for producing vaccines. Industrial-scale efforts highlight the feasibility of GMP-compatible processes, while academic investigations expand the scope of PMF toward next-generation platforms integrating mRNA production and VLP-mediated delivery. Together, these findings support the potential of plants as versatile, sustainable, and scalable biofactories for future vaccine development.

# 1. INTRODUCTION

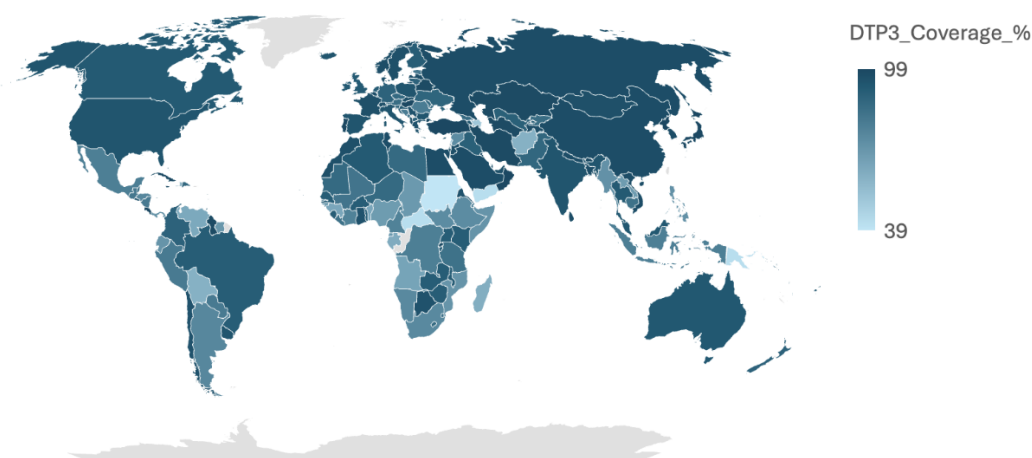
## *1.1 The importance of vaccines*

Vaccination is widely recognized as one of the most transformative and cost-effective interventions in modern medicine (1). Large-scale immunization campaigns have eradicated smallpox and led to dramatic declines in diseases such as polio, measles, and diphtheria (2,3). Over the past century, the science of vaccinology has evolved profoundly (4). Traditional vaccine platforms, including live attenuated and inactivated formulations, stimulate protective immunity by exposing the host to pathogen-derived components, thereby activating both humoral and cellular responses (5). While effective, these strategies often require adjuvants or repeated booster administrations and may not be suitable for all populations (6). In recent decades, the field has embraced biotechnological innovations such as recombinant subunit vaccines, virus-like particles (VLPs), DNA plasmids, viral vectors, and messenger RNA (mRNA) vaccines (7,8). Among these, mRNA platforms have emerged as particularly powerful tools for rapid response, as demonstrated during the COVID-19 pandemic (9,10). mRNA vaccines represent a breakthrough, enabling fast design, scalable production, and robust immune responses with favourable safety profiles. Unlike conventional vaccines, they do not require pathogen cultivation or recombinant protein expression and purification, thereby shortening development timelines. Once delivered, synthetic mRNA drives in situ antigen production within host cells, facilitating immune recognition through both MHC class I and II pathways (11). Advances such as codon optimization, self-amplifying RNA constructs, and lipid nanoparticle delivery systems have further improved their stability, translational efficiency, and immunogenicity (7, 12, 13). Another promising platform is represented by virus-like particles (VLPs), which mimic the external structure of viruses while lacking genetic material. Licensed VLP-based vaccines, such as those against human papillomavirus (HPV, e.g., Gardasil), demonstrate high immunogenicity owing to their repetitive, multivalent surface, while maintaining strong safety profiles (14). Importantly, VLPs can be manufactured in heterologous expression systems (e.g., yeast, plants, insect cells), facilitating large-scale production. DNA vaccines, although still in early stages for human application, have shown great promise in veterinary and preclinical studies (15). Their thermostability, low production cost, and ease of storage and transport render them especially attractive for deployment in resource-limited settings.

Beyond direct health benefits, immunization contributes significantly to socioeconomic stability. Vaccines reduce healthcare expenditures, improve productivity, and support

economic development (16,17). Indeed, vaccination programs are considered among the most cost-effective public health interventions. In low- and middle-income countries (LMICs), every dollar invested in childhood immunization is estimated to yield up to \$44 in economic returns through healthcare savings, increased productivity, and reduced long-term disability (18). Thus, vaccines represent not only biomedical tools but also instruments of equity and development (19). Despite the technological gains, vaccine access remains deeply unequal (20). High-income countries often secure priority access to doses, whereas LMICs experience shortages due to limited production capacity, stringent cold chain requirements, raw material bottlenecks, and fragmented regulatory systems (21,22). These barriers span production capacity, affordability, allocation, logistics, and confidence, with tangible health and economic consequences. In many LMICs, additional challenges include travel costs and time to reach clinics, weak infrastructure, conflict zones, and recurrent cold-chain failures. Humanitarian crises have aggravated this gap: in conflict-affected regions such as Sudan and Yemen, the number of zero-dose children has risen sharply. As of October 2021, high-income countries had administered approximately 125 vaccine doses per 100 population, nearly three times the rate in lower-middle-income countries (~45/100), and almost thirty times that in low-income countries (~4/100) (23).

As illustrated in Figure 1, coverage of essential childhood immunizations such as DTP3 remains highly variable between regions, with substantial gaps between high- and low-income countries (data from WHO/UNICEF WUENIC, 2024) (24).



**Figure 1.** Global coverage of essential childhood immunizations (DTP3) in 2024.

Data adapted from WHO/UNICEF WUENIC (2024).

Global vaccine supply chains are highly dependent on a limited number of critical suppliers, making them vulnerable to disruption, as seen during the COVID-19 pandemic. Establishing and validating GMP-compliant manufacturing facilities can require years and investments of hundreds of millions of dollars (25). Furthermore, the diverse and unique national regulatory frameworks often delay scale-up and distribution (26). Intellectual property (IP) restrictions have further complicated technology transfer, prompting debate around patent waivers and global IP pooling mechanisms (27–28). Although many countries and organizations advocated for temporary IP waivers, opposition from several high-income nations limited technology transfer, constraining local manufacturing capacity and deepening inequities. Nevertheless, safety, efficacy, and stability remain non-negotiable priorities for all vaccines (29). Vaccines must comply with rigorous standards set by agencies like the FDA, EMA, and WHO, ensuring batch-to-batch consistency in sterility, potency, and purity (30). Innovations in formulation, such as nucleoside modifications and thermal stabilization, have expanded the feasibility of broad and equitable distribution (31–32). Looking forward, scalable solutions such as modular manufacturing platforms, decentralized production hubs, and harmonized regulatory pathways are critical to strengthen global preparedness (33–34). In summary, vaccines are not only life-saving tools but also strategic assets for global resilience (35). Addressing access inequities, enhancing technological agility, and reinforcing manufacturing infrastructure must remain global priorities to ensure timely and equitable immunization against both endemic and emergent threats (36). The inequalities in vaccine access and production highlighted the urgent need for innovative, scalable, and cost-effective biomanufacturing platforms and to explore alternative expression platforms. Plant Molecular Farming (PMF) has emerged as one such approach, offering novel solutions to many of the challenges associated with traditional systems. The following section introduces the field of PMF, highlighting its evolution, technical foundations, and relevance to modern pharmaceutical manufacturing.

## ***1.2 Plant Molecular Farming***

Plant Molecular Farming is a rapidly evolving subfield of biotechnology that harnesses genetically modified plants to produce high-value recombinant proteins, including vaccines, monoclonal antibodies, enzymes, growth factors, and other therapeutic agents (37–38). This platform represents a transformative approach to pharmaceutical manufacturing due to its scalability, biosafety, cost-effectiveness, and flexibility. Unlike conventional production

systems such as bacterial fermentation or mammalian cell cultures, PMF offers a unique set of advantages. Plants are naturally free from human pathogens, thereby reducing the risk of contamination with agents such as viruses or prions (39). In addition, the infrastructure required for plant cultivation is considerably less expensive and technically less demanding than that needed for bioreactors and sterile fermenters (39–40). The potential of PMF has been highlighted during recent health crises. A prominent example is Medicago, a Canadian biopharmaceutical company that advanced a plant-derived virus-like particle (VLP) vaccine against COVID-19, produced in *Nicotiana benthamiana*, to clinical trials (41). The capacity of PMF to rapidly scale vaccine production in response to epidemics and pandemics has drawn significant attention from both academia and industry (42). Beyond vaccines, plant-based systems have been applied to produce antibodies (e.g., ZMapp for Ebola), enzymes such as glucocerebrosidase for Gaucher disease, and a variety of diagnostic reagents (43). Furthermore, edible vaccines expressed in crops such as potatoes and tomatoes represent a potential frontier in global immunization strategies, particularly in low-resource settings (44). As synthetic biology and genetic engineering tools advance, the customization of plant expression platforms becomes increasingly feasible. These developments enable higher yields, improved protein folding and glycosylation, and greater alignment with regulatory standards (45–46). Overall, PMF holds promise not only for therapeutic protein production but also for reshaping the global vaccine bioeconomy (46).

Having outlined the general scope and potential of Plant Molecular Farming, it is important to first trace its historical evolution and then examine the distinctive features that make this platform unique. The aim of this introduction is to provide the contextual framework for the experimental work described in the following chapters.

The concept of using plants as biofactories was first articulated in the early 1980s, following the advent of methods for stable transformation of plant genomes (47). A seminal milestone was the demonstration by Barta et al. (1986) of foreign gene expression in plants, which opened the way to the production of therapeutic proteins in crops such as tobacco and potato (48). Early applications included the expression of human serum albumin and other pharmaceutically relevant proteins. However, initial progress was constrained by low yields, lack of scalable production platforms, and limited regulatory acceptance. These challenges drove the improvement of transformation methodologies, including *Agrobacterium tumefaciens*-mediated gene transfer and the development of viral vectors, which enabled higher expression efficiency. The emergence of transient expression systems – most notably



through agroinfiltration in *Nicotiana benthamiana* – established the foundation of modern molecular farming (39, 41). During the 1990s, these advances were complemented by early proof-of-concept studies on oral vaccines produced in plants, targeting diseases such as hepatitis B and cholera (43). In the 2000s, the introduction of viral vector–based strategies, including the magnification technique developed by Gleba et al. (2005), revolutionized the field by achieving rapid and high-level transient expression (49). The relevance of PMF was further demonstrated during the 2014 Ebola outbreak, when the monoclonal antibody cocktail ZMapp, produced in glycoengineered tobacco, was administered in compassionate use cases (50–51). This episode not only validated the feasibility of plant-derived biotherapeutics but also highlighted the responsiveness of PMF platforms to urgent global health needs. The COVID-19 pandemic catalysed unprecedented investments and developments in PMF technologies. A landmark example was provided by Medicago (Canada), which advanced a VLP-based SARS-CoV-2 vaccine to Phase III clinical trials and received conditional regulatory approval in 2022 (52). Although later acquired and restructured, Medicago’s achievement demonstrated the industrial maturity of plant-based platforms. Alongside Medicago, several companies have established themselves as key players in the global PMF landscape. Kentucky BioProcessing (USA) has developed plant-based vaccines and monoclonal antibodies, while Icon Genetics (Germany) has pioneered vector design and proprietary expression technologies. Leaf Expression Systems (UK) has positioned itself as a contract development and manufacturing organization specialized in plant-made biologics, and Baiya Phytopharm (Thailand) has advanced a COVID-19 vaccine candidate into clinical trials using *N. benthamiana* (53). Other notable contributors include iBio (USA), which has developed plant-based antibody and vaccine platforms, and Nomad Bioscience (Germany), which has focused on innovative vector systems and biopharmaceutical applications. Together, these companies illustrate both the geographical diversification and the industrial relevance of PMF. Today, plant molecular farming stands at the intersection of biotechnology and synthetic biology. Advances in chassis engineering, glycoengineering, and expression optimization, combined with an evolving regulatory environment increasingly receptive to plant-derived biologics, have transformed PMF from an academic curiosity into a viable industrial platform (54,55). As a result, plants are now poised to play a central role in future pharmaceutical supply chains, supporting both routine biomanufacturing and rapid responses to global health emergencies. Understanding this historical trajectory (Figure 2) is essential to appreciate the biotechnological concepts that characterize Plant Molecular Farming and frame its role within modern pharmaceutical production.

| 1983                                                                                              | 1986                                    | 1990s                                                                                                                                                                                                                                                     | 2003-2009                                                                                                                                                                                                                 | 2014                                                                                                                              | 2012-2019                                                                                                                                                           | 2020-2022                                                                                                                                                                                                                                                                    | Present                                                                                               |
|---------------------------------------------------------------------------------------------------|-----------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|
| First demonstration of <i>Agrobacterium tumefaciens</i> -mediated stable gene transfer in plants. | Expression of a foreign gene in plants. | First reports of recombinant pharmaceuticals in plants: human serum albumin and antibodies in <i>N. tabacum</i> and potato<br><br>The concept of edible is vaccines introduced<br><br>Development of improved expression vectors and plant viral systems. | Introduction of magnification, a high-efficiency transient expression method using both <i>Agrobacterium</i> and viral vectors.<br><br>First large-scale demonstration of plant-based antibodies for infectious diseases. | Development of ZMapp, a cocktail of three monoclonal antibodies against Ebola produced in glycoengineered <i>N. benthamiana</i> . | Expansion of industrial PMF capacity: companies such as Medicago, Icon Genetics, Kentucky BioProcessing, iBio, and Nomad Bioscience establish industrial pipelines. | COVID-19 pandemic accelerates PMF. Medicago advances a plant-derived VLP-based SARS-CoV-2 vaccine to Phase III trials and obtains regulatory approval in Canada.<br><br>Baiyi Phytopharm (Thailand) initiates clinical trials with a plant-based COVID-19 vaccine candidate. | PMF enters the era of synthetic biology and glycoengineering, with increasing regulatory recognition. |

**Figure 2.** Historical milestones in the development of Plant Molecular Farming, from early demonstrations of gene expression in plants (1980s) to the emergence of industrial platforms and recent pandemic applications.

Plant Molecular Farming builds upon four decades of plant genetic engineering, where advances in transformation and expression technologies have progressively expanded the scope of recombinant protein production (56). The way in which a foreign gene is expressed in plants not only influences yield but also affects the accuracy of post-translational modifications and the suitability of the platform for regulatory approval. Broadly, two complementary approaches are employed: transient expression and stable expression. Both strategies enable the introduction and regulation of heterologous genes within plant systems, but they differ fundamentally in mechanism, timescale, and application scope (57), each with distinct advantages and limitations. Transient expression is widely recognized for its speed, flexibility, and capacity to achieve high protein levels within short production windows. The most established approach is agroinfiltration, in which *Agrobacterium tumefaciens* carrying the gene of interest is introduced into plant tissues, typically the leaves of *Nicotiana benthamiana*. Because this method bypasses genomic integration, it reduces regulatory complexity and enables rapid protein production within days (58). Transient expression has been especially impactful in pandemic preparedness, allowing fast deployment of vaccine candidates such as virus-like particles (VLPs) and monoclonal antibodies, as evidenced by Medicago's COVID-19 VLP vaccine (41). Yields can be further enhanced through magnification, which combines the efficiency of *Agrobacterium* with viral vectors, thereby boosting consistency and scalability (49–59). In addition to agroinfiltration, a wide range of plant viral vectors – including those derived from Tobacco mosaic virus (TMV) and bean yellow dwarf virus (BeYDV) – have been engineered to drive robust transient expression across different plant species (58). Stable expression involves the integration of

the target gene into the plant genome, either in the nuclear DNA or in the plastid genome. Nuclear transformation is well established and compatible with tissue-specific promoters, allowing complex expression patterns. It is particularly suited for long-term production and scalability through seed propagation, though yields can be affected by position effects and transgene silencing (60). In contrast, chloroplast transformation enables polycistronic expression and high protein accumulation. Its maternal inheritance in most species provides intrinsic gene containment, which is desirable from a biosafety perspective (61). However, plastids lack glycosylation machinery, limiting their use for therapeutic proteins that require human-like glycan structures (62). As a result, chloroplast engineering is mainly applied to antigens or enzymes where glycosylation is not required. The selection between the two expression strategies depends on product requirements, regulatory pathways, and production timeline (Table 1). For emergency responses, transient systems are unparalleled in speed. For sustained manufacturing, stable systems provide greater economy of scale and containment (63).

**Table 1.** Comparative overview of transient and stable expression strategies in Plant Molecular Farming. Each approach offers specific advantages and limitations that guide its application to pharmaceutical production.

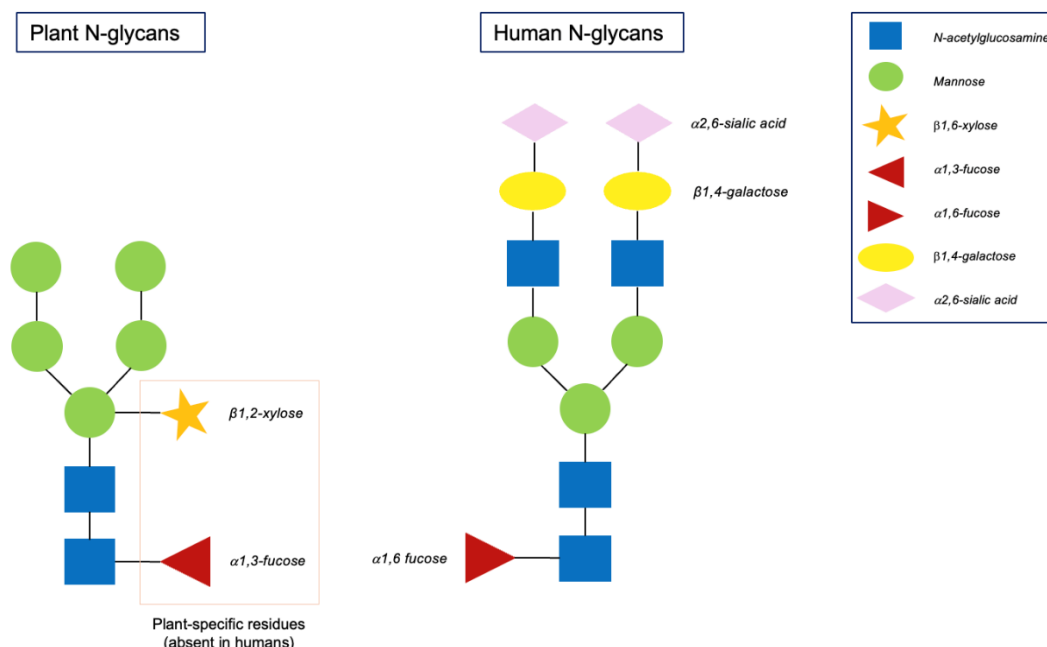
|                              | Transient Expression                                                                     | Stable Expression                                       |
|------------------------------|------------------------------------------------------------------------------------------|---------------------------------------------------------|
| <i>Time to Production</i>    | - Rapid (days to weeks)                                                                  | • Slow (months to years)                                |
| <i>Scalability</i>           | - High: suitable for medium/large-scale production                                       | • Very High: scalable once stable lines are established |
| <i>Yield</i>                 | - High, especially with viral vectors                                                    | • Variable, lower protein expression                    |
| <i>Limitations</i>           | - Batch-to-batch variability                                                             | • Long development times<br>• Limited flexibility       |
| <i>Regulatory Acceptance</i> | - Less standardized                                                                      | • More accepted due to genetic stability                |
| <i>Genomic Integration</i>   | - Temporary                                                                              | • Stable genomic integration                            |
| <i>Applications</i>          | - Rapid development of vaccines<br>- Proof-of-concept studies<br>- Antibodies production | • Commercial Scale productions                          |

Selecting the appropriate plant host is equally critical in PMF. Ideal hosts are those with high biomass yield, rapid growth, ease of transformation, and human-compatible post-translational modification systems (36). *Nicotiana benthamiana* and *N. tabacum* represent the dominant model species due to their well-characterized genomes, high transformation efficiencies, and

robust protein expression capacity (44). Cereal crops like rice and maize offer advantages in scalability and seed-based storage, but face challenges in glycosylation fidelity and public GMO acceptance. Aquatic plants, notably *Lemna minor* (duckweed), are gaining interest for their fast growth, compact cultivation systems, and suitability to containment, making them attractive for GMP-compliant facilities (64). Comparative analysis reveals that dicots, especially tobacco species, are superior in terms of glycoengineering potential, regulatory familiarity, and recombinant protein yield (65). Conversely, monocots provide biomass scalability, and aquatic plants offer biocontainment and lower infrastructure demands. Ultimately, the selection of plant species is determined by the nature of the target product, production scale, regulatory pathway, and downstream processing requirements. Advancements in genome editing technologies such as CRISPR/Cas9 and synthetic biology platforms now allow for tailored optimization of host plants, improving glycosylation pathways, reducing proteolytic degradation, and enhancing yield stability (66, 67). These tools are gradually expanding the repertoire of plants suitable for PMF, enhancing its commercial viability and therapeutic relevance.

PMF combines advantages of scalability, reduced production costs, and low contamination risks, positioning it as a compelling alternative to traditional protein production systems (50). Economically, PMF requires substantially lower infrastructure investments than mammalian cell culture platforms. Whereas conventional biopharmaceutical manufacturing relies on sterile facilities and costly bioreactors, plant-based systems can be operated in greenhouses or even open-field cultivation, thereby reducing both capital and operational costs (68). A critical asset of dicotyledonous plants is their ability to perform complex post-translational modifications, which enhances the therapeutic potential of plant-derived biologics (55). In this context, glycoengineering has emerged as a pivotal advancement, addressing one of the most significant limitations of PMF: the intrinsic differences between plant and human glycosylation patterns (69). Plant N-glycans typically contain  $\beta(1,2)$ -xylose and  $\alpha(1,3)$ -fucose residues, absent in humans, which may reduce therapeutic efficacy or trigger immunogenic responses (70) (Figure 3). To overcome this, plant glycosylation pathways have been extensively engineered. Strategies include the suppression of endogenous glycosyltransferases and the introduction of human genes encoding enzymes such as  $\beta(1,4)$ -galactosyltransferase and  $\alpha(2,6)$ -sialyltransferase (55, 71). These modifications enable the production of recombinant proteins with human-like glycan profiles, improving pharmacokinetics, extending serum half-life, and reducing immunogenicity (72). The impact of glycoengineering has been particularly evident in the development of therapeutic

monoclonal antibodies (mAbs). For instance, anti-Ebola and anti-HIV antibodies expressed in glycoengineered *Nicotiana benthamiana* exhibited enhanced Fcγ receptor binding and increased antibody-dependent cellular cytotoxicity (ADCC), features critical for therapeutic efficacy (73). In the vaccine field, tailoring glycan structures can modulate antigen recognition, stability, and immune processing, thereby enhancing immunogenicity in a controlled more beneficial way (74). Looking ahead, future developments in PMF are likely to include fully synthetic glycosylation pathways customized for specific therapeutic applications, as well as streamlined glycoengineering workflows enabled by automated genome editing technologies such as CRISPR/Cas9 (69). A major frontier remains the incorporation of sialylated N-glycans, which is currently limited by the absence of endogenous sialylation machinery in plants (75). The convergence of glycoengineering with synthetic biology and modular expression systems is expected to further expand the applicability of PMF, enabling the production of biologics that demand highly specific glycoforms. By resolving the mismatch in glycan structures, glycoengineering has transformed plant systems from proof-of-concept platforms into competitive candidates for the commercial production of complex biologics. It stands as a cornerstone in the evolution of PMF, enhancing both its therapeutic relevance and industrial viability.



**Figure 3.** Comparison between plant-specific and human N-glycosylation profiles. Plants naturally produce N-glycans carrying β(1,2)-xylose and α(1,3)-fucose residues, which are absent in humans and may induce immunogenicity.

While microbial and mammalian systems remain the dominant hosts for recombinant protein production, plant-based platforms are increasingly positioned as viable alternatives. Each

system carries distinct strengths and limitations in terms of product quality, scalability, biosafety, and economics. Mammalian cell cultures, particularly Chinese hamster ovary (CHO) cells, have long been considered the gold standard for complex biologics due to their ability to perform authentic human-like post-translational modifications, including the glycosylation. However, these facilities require expensive infrastructure, strict biosafety measures, and energy-intensive operations, resulting in high capital and operating expenditures (76). Microbial hosts, such as *E. coli* and *Pichia pastoris*, provide unmatched speed and simplicity in upstream processing. They offer rapid growth and low-cost fermentation infrastructure, making them attractive for certain proteins (77). Yet, their inability to perform complex glycosylation and frequent reliance on refolding from inclusion bodies limit their application for many therapeutic biologics (78). Plant systems occupy a distinctive middle ground. As discussed in the previous section, native glycosylation patterns differ from humans, but advances in glycoengineering now enable the production of proteins with humanized glycan profiles (79). In parallel, techno-economic analyses (TEAs) have demonstrated that PMF can achieve highly competitive costs of goods (COGs) (80). Commercial-scale TEAs for antimicrobial proteins in plants, for instance, have reported unit production costs in the range of \$3.00–6.88 per gram, underscoring the economic feasibility of PMF when upstream titres and streamlined downstream processing (DSP) are achieved. Similar analyses for monoclonal antibodies (mAbs) produced via transient expression indicate costs of goods of approximately \$121/g, while highlighting markedly lower capital expenditures for greenhouse or vertical farm-based facilities compared with mammalian bioreactors at equivalent throughput (81). However, there is an aspect that must be emphasized when interpreting such cross-platform benchmarks. Downstream processing (DSP) remains the dominant cost driver across all systems, since without tailored extraction, capture, and polishing strategies, upstream advantages are often negated. The analyses indicate that PMF can achieve cost profiles competitive with microbial and mammalian systems, particularly when coupled with high transient expression and optimized plant-specific DSP (82). The relatively low infrastructure and consumable costs of greenhouse-scale PMF platforms make them especially attractive for early-stage studies and rapid prototyping. Especially when combined with glycoengineering and host optimization, PMF has the potential to outperform traditional platforms in both cost and quality, while offering additional benefits of sustainability and biosafety (83) (Table 2).

**Table 2.** Comparative overview of microbial, mammalian, and plant-based biomanufacturing platforms in terms of product quality, economics, and sustainability.

|                               | Microbial systems<br>( <i>E. coli</i> , <i>Pichia pastoris</i> )                                                                  | Mammalian systems<br>(CHO cells)                                                                       | Plant-based systems<br>( <i>Nicotiana benthamiana</i> )                                                             |
|-------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------|
| <i>Time to Product</i>        | • Rapid (days)                                                                                                                    | • Moderate (weeks)                                                                                     | • Moderate (weeks to months)                                                                                        |
| <i>Product Quality</i>        | • No post-translational modifications                                                                                             | • Complex biologics<br>• Authentic human-like glycosylation and post-translational modifications       | • Post-translational modifications<br>• Different native plant glycans from humans                                  |
| <i>Scalability</i>            | • Moderate (fermenters)                                                                                                           | • Moderate (expensive bioreactors)                                                                     | • High (greenhouses and vertical farms)                                                                             |
| <i>Infrastructure Costs</i>   | • Moderate                                                                                                                        | • Very High                                                                                            | • Low                                                                                                               |
| <i>Operating Costs</i>        | • Low                                                                                                                             | • High                                                                                                 | • Low                                                                                                               |
| <i>Biosafety Requirements</i> | • Moderate                                                                                                                        | • Very Strict                                                                                          | • Low                                                                                                               |
| <i>Sustainability</i>         | • Low (resource-intensive fermenters)                                                                                             | • Low (high energy and resource demanding)                                                             | • High                                                                                                              |
| <i>Main Limitations</i>       | <ul style="list-style-type: none"> <li>○ Lack of glycosylation</li> <li>○ Application to limited group of therapeutics</li> </ul> | <ul style="list-style-type: none"> <li>○ Expensive infrastructures</li> <li>○ Long timeline</li> </ul> | <ul style="list-style-type: none"> <li>○ Differ in native glycans</li> <li>○ Uncertain regulatory patter</li> </ul> |

Beyond technical performance, PMF facilities are closely aligned with the principles of sustainability, which represents one of the strongest comparative advantages over conventional biomanufacturing platforms (37, 84). By leveraging renewable resources, minimizing waste, and enabling valorisation of residual biomass, PMF integrates naturally into the framework of the circular bioeconomy (85). Unlike mammalian cell cultures, which require highly controlled and energy-intensive bioreactors, PMF relies on plants that grow using sunlight, water, and atmospheric CO<sub>2</sub>, resulting in lower carbon emissions and reduced resource demands (86). Non-food crops such as *Nicotiana tabacum* can be cultivated on marginal land, thereby mitigating potential concerns about food security (87). Furthermore, residual biomass can be reused for bioenergy generation or agricultural inputs, reinforcing the contribution of PMF to circular economy models. Plants also carry intrinsic biosafety advantages. They present a lower risk of contamination by human or animal pathogens, reducing the need for stringent sterilization and biosafety measures and decreasing the likelihood of zoonotic cross-contamination (88). This not only enhances product safety but also lowers the energy footprint typically associated with containment in mammalian and microbial systems. In addition, the use of biodegradable growth substrates, reusable greenhouse infrastructure, and the absence of animal-derived components further position PMF within the objectives of green biotechnology. Overall, the integration of PMF into sustainable and circular economy frameworks strengthens its appeal as a next generation

biomanufacturing strategy. Through efficient land use, valorisation of biomass, minimized ecological impact, and reduced biosafety requirements, PMF emerges as a platform that combines technological innovation with environmental responsibility.

Beyond environmental and economic considerations, the successful implementation of Plant Molecular Farming (PMF) also depends on overcoming societal and political barriers. While sustainability and biosafety provide compelling advantages, public perception, regulatory acceptance, and policy frameworks remain critical determinants of the feasibility and adoption of plant-based biomanufacturing (90). Societal acceptance continues to shape regulatory decisions, particularly in the European Union, where debates around biotechnology and genetically modified organisms (GMOs) remain influential (91,92). Although scientific consensus supports the safety of genetically modified plants, consumer acceptance lags. Transparency, stakeholder engagement, and education are key to building confidence in plant-based technologies (93). Public scepticism, often driven by limited understanding or persistent mistrust of genetic engineering, can slow the adoption of PMF-derived products despite their proven health and economic benefits. Several other challenges continue to limit the scalability and commercial viability of PMF. Expression yields remain variable, influenced by environmental conditions, transformation methods, and genetic stability (50). Stable systems often require extensive screening and optimization to achieve consistent yields. Downstream processing adds another layer of complexity: plant tissues are rich in secondary metabolites such as alkaloids and polyphenols, which can compromise protein integrity and interfere with purification (37). Achieving GMP-compliant purification protocols is therefore technically demanding and cost-intensive. Regulatory considerations present further obstacles. Current guidelines were largely designed around microbial and mammalian systems and do not fully capture the specificities of plant-based biopharmaceuticals (94, 95). The absence of harmonized standard protocols can delay approval and market entry, undermining the speed and flexibility that PMF otherwise offers. Addressing these gaps requires not only harmonized international standards but also more effective science communication and public engagement. Transparency in production methods, clear labelling policies, and participatory platforms where citizens, scientists, and policymakers co-develop guidelines could improve legitimacy and trust. At the same time, policy responses to recent crises suggest that acceptance can evolve rapidly. Countries such as Canada have shown openness to plant-derived biologics, particularly in the context of COVID-19, demonstrating how pandemic preparedness and sustainability agendas can accelerate regulatory adaptation



(96). Moving forward, the path to wider implementation of PMF will depend on proactive policies that balance innovation with safety, as well as coordinated efforts in risk–benefit communication and international regulatory harmonization (97).

While PMF offers clear economic, environmental, and technical advantages in specific contexts, its full potential will only be realized through continued progress in biotechnology, regulatory adaptation, and public engagement. Since its conceptualization in the 1980s, PMF has evolved from a niche research domain into a commercially relevant biotechnology platform, with early proof-of-concept studies demonstrating the ability of plants to express functional recombinant proteins (98). Over the past three decades, the field has reached notable milestones, particularly in the production of vaccines, monoclonal antibodies (mAbs), and enzymes (99). From the early use of tobacco plants for recombinant protein expression to the recent deployment of vaccine manufacturing, PMF has consistently demonstrated efficacy, scalability, and adaptability (100). Today, the platform stands at the intersection of innovation and challenge. With sustained investment from both public and private sectors, there is a real possibility for PMF to become fundamental in the next generation biomanufacturing, offering environmentally responsible and globally accessible solutions to pressing health challenges.

### **1.3 *Whole Plant vs Plant cells***

Building on the strategic and technological perspectives outlined in Sections 1.1 and 1.2, this section compares the two major plant-based platforms for biomanufacturing: whole plants and plant cell cultures. While whole plants offer remarkable flexibility and scalability, plant cell cultures provide distinct advantages in terms of standardization, containment, and regulatory compliance – critical requirements for pharmaceutical-grade production (101). Plant Molecular Farming (PMF) thus relies on two complementary approaches that differ in complexity, scalability, and regulatory suitability, yet both represent credible alternatives to microbial and mammalian expression platforms (40, 102). Historically, whole plants have dominated the field exhibiting low cultivation costs and high biomass yields (57). More recently, however, advances in plant cell culture technologies – including optimized suspension cultures, single-use bioreactors, and refined media formulations – have opened new possibilities for producing complex biologics such as monoclonal antibodies and therapeutic enzymes under standardized, GMP-compliant conditions (103). In whole-plant

systems, genetically engineered plants – most commonly *Nicotiana benthamiana* or *Nicotiana tabacum* – serve as biofactories for recombinant proteins. Expression can be achieved through both stable and transient strategies, with transient expression enabling rapid, high-yield production particularly suited for vaccines and antibodies (65, 66). Cultivation may occur in soil-based or hydroponic systems, ranging from greenhouses to vertical farms, thereby supporting scalability and flexible manufacturing (104). In contrast, plant cell culture systems are derived from dedifferentiated plant cells maintained in sterile, controlled bioreactor environments. Originating from callus or suspension cultures, they enable highly uniform and tightly regulated expression profiles, aligning more readily with GMP requirements (100). These systems are particularly valuable for sensitive biologics that require homogeneous glycosylation and consistent post-translational modifications.

Having introduced the two principal strategies, the following section examines whole-plant systems and plant cell cultures in greater detail, highlighting their respective strengths, limitations, and areas of application to enable a direct comparison.

### 1.3.1 Whole Plants in PMF

As discussed in the previous section, whole-plant systems have historically dominated Plant Molecular Farming owing to their scalability, flexibility, and relatively low infrastructure demands. Recent technological progress has further enhanced their performance. As already discussed in Section 1.2, transient expression strategies, particularly those based on deconstructed viral vectors, enable rapid turnaround, reducing the time from gene design to protein harvest to only a few weeks (60). From an economic perspective, whole-plant systems benefit from low input costs and the possibility of decentralized production (83, 105). Even simple greenhouse setups can be implemented in low-resource settings with minimal equipment, supporting distributed vaccine manufacturing – an especially relevant feature for pandemic preparedness (106). Technologies for cultivating whole plants range from traditional greenhouse systems to advanced vertical farming setups. Greenhouses offer scalability and environmental control, whereas vertical farming enhances space efficiency and biosafety by enabling production in confined areas with artificial lighting and climate regulation (107). Automated greenhouse infrastructure and LED-regulated vertical farms now allow protein production under tightly controlled conditions of light, temperature, and humidity (108, 109). In parallel, precision agriculture tools such as AI-driven irrigation, fertigation, and plant health monitoring are increasingly applied to improve scalability and consistency (110,

111, 112). However, whole plant systems present several limitations. The variability of expression levels due to differences in plant physiology, environmental conditions, and cultivation cycles can result in inconsistent yields. Moreover, biomanufacturing in plants is often complicated by the production of host-specific secondary metabolites. Tobacco-derived compounds such as nicotine, polyphenols, and alkaloids can complicate protein purification (113), requiring multiple chromatography steps or additional clarification techniques, thus impacting cost-efficiency. Efforts in metabolic engineering and genome editing are underway to develop low-nicotine or metabolite-free *Nicotiana* lines to mitigate these concerns (67). Whole-plant systems introduce product heterogeneity due to non-clonal propagation, necessitating stringent characterization of master seed stocks, environmental controls, and robust traceability systems for batch consistency (99).

### 1.3.2 Plant Cell Cultures in PMF

Plant cell cultures, especially suspension cultures derived from *Nicotiana tabacum* BY-2 or carrot cells, have emerged as promising platforms for the production of complex biologics (114). Cultivated under controlled and sterile conditions, these systems operate in bioreactors that facilitate compliance with GMP standards and reduce variability associated with environmental factors (54). A major advantage of cell cultures is their ability to support precise process control, enabling reproducible yields and simplifying downstream processing (115). The regulatory relevance of this approach was demonstrated by Protalix Biotherapeutics with the commercialization of Taliglucerase alfa (Elelyso), the first FDA- and EMA-approved plant cell-derived therapeutic protein, produced in carrot suspension cells (116). Plant cell cultures are typically established from dedifferentiated cells derived from callus or explant material and maintained in suspension (97). Bioprocessing strategies include stirred-tank reactors, wave-mixed bioreactors, and single-use disposable systems, all of which allow aseptic conditions and fine control over pH, temperature, and nutrient supply. Single-use bioreactors reduce contamination risks and simplify cleaning validation, aligning with cGMP requirements (117). Advances in plant cell fermentation have improved bioreactor performance and cell density, enabling higher protein yields. In addition to suspension cultures, plant hairy root cultures – induced by *Agrobacterium rhizogenes* – offer a stable and scalable platform for producing complex secondary metabolites and recombinant proteins (118). Compared to whole plants, cell cultures offer a more uniform production environment, which is particularly important for high-value biopharmaceuticals requiring consistent post-translational

modifications. Their enclosed and sterile operation also facilitates regulatory acceptance, as illustrated by the successful approval of Taliglucerase alfa (116). Despite these advantages, important limitations remain. Cell cultures display slower growth rates and lower biomass accumulation than whole plants, which constrains scalability (119). Bioreactor-based systems are capital-intensive, require constant monitoring, and have longer production timelines compared to transient expression platforms (120). Scale-up is technically demanding since plant cells are sensitive to shear stress and often require specialized reactor designs such as airlift or bubble-column systems. Optimized media formulations and oxygen transfer strategies are also essential to maintain productivity and viability during large-scale cultivation (121). As a result, plant cell cultures are generally more suitable for low-volume, high-value products, where standardization and regulatory alignment outweigh the disadvantages of higher costs and limited scalability (122).

### 1.3.3 Comparative Analysis Between Whole Plants and Plant Cell Cultures

The following Table 3 provides a side-by-side comparison of whole plant systems and plant cell cultures.

**Table 3.** Comparative overview of whole-plant and plant cell culture systems for molecular farming.

|                               | Whole Plant Systems                                              | Plant Cell Cultures                  |
|-------------------------------|------------------------------------------------------------------|--------------------------------------|
| <i>Production Platform</i>    | Greenhouses and Vertical farms<br>(non sterile environment)      | Bioreactors<br>(controlled, aseptic) |
| <i>Production Speed</i>       | Fast (weeks)                                                     | Slower (weeks to months)             |
| <i>Production Scalability</i> | High                                                             | Moderate                             |
| <i>Contamination Risk</i>     | Low                                                              | Low                                  |
| <i>Yield variability</i>      | High                                                             | Low                                  |
| <i>Regulatory Acceptance</i>  | Challenging                                                      | High                                 |
| <i>Infrastructure Cost</i>    | Low                                                              | High                                 |
| <i>Operating Cost</i>         | Low / Moderate                                                   | High                                 |
| <i>Therapeutic Fit</i>        | Rapid response to emergency, fast<br>scale up and large volumes. | High value biologics.                |

Whole-plant systems and plant cell cultures differ markedly in production cost, scalability, contamination risk, and regulatory suitability. Whole plants offer lower infrastructure costs and rapid scalability, making them ideal for surge capacity during health crises. By contrast, plant

cell cultures provide greater process control, regulatory alignment, and product consistency under sterile bioreactor conditions. The choice between the two platforms ultimately depends on product type, regulatory pathway, and production timelines.

The contrast is well illustrated by two landmark case studies. Taliglucerase alfa (Elelyso), produced in genetically engineered carrot cells by Protalix Biotherapeutics, was approved by the FDA in 2012 as the first plant cell–derived therapeutic protein. Its manufacturing exemplifies the strengths of cell culture platforms: controlled environments, reproducible yields, and clear regulatory acceptance (123). In contrast, Medicago’s influenza virus-like particle (VLP) vaccine, produced via transient expression in *Nicotiana benthamiana*, demonstrates the speed and scalability of whole-plant systems. Through agroinfiltration, Medicago achieved rapid VLP assembly and mass expression, scaling production with vertical farming and automated greenhouse facilities to reach Phase 3 trials and obtaining Health Canada approval (124). Together, these examples underscore the trade-off between precision and scalability in Plant Molecular Farming. Plant cell cultures are particularly suited for production that requires tightly regulated production, whereas whole plants excel in rapid-response scenarios and mass immunization. Both systems therefore play complementary roles within the broader landscape of plant-based biomanufacturing.

#### **1.4 Future of Plant Molecular Farming**

The future of Plant Molecular Farming lies in the strategic integration of whole-plant systems and plant cell cultures. Hybrid approaches aim to combine the scalability and cost-effectiveness of whole plants with the precision, sterility, and GMP compliance offered by cell cultures (81). Such complementarity would enable a flexible manufacturing pipeline capable of addressing diverse therapeutic needs, from vaccines and diagnostic reagents to monoclonal antibodies and enzyme therapeutics. The global market for plant-derived biologics is projected to expand significantly, driven by demand for affordable vaccines, rapid production cycles, and sustainable production methods (125). Decentralized PMF facilities may be particularly impactful in Asia, Latin America, and Africa, where local manufacturing could reduce reliance on global supply chains and enhance regional biosecurity (126). Plants offer a safe, cost-effective, and scalable platform for vaccine production. They reduce contamination risks associated with mammalian pathogens and can be rapidly reprogrammed for new antigens. The use of transient expression systems in *Nicotiana benthamiana* allows production cycles as short as three weeks (127). Additionally, PMF avoids the use of animal-derived components, aligning with global trends toward ethical and sustainable production

practices. Several companies have brought PMF-based products to clinical trials or even market. Medicago's plant-derived influenza vaccine, produced using transient expression in *N. benthamiana*, successfully completed Phase 3 trials and received regulatory approval in Canada. Protalix BioTherapeutics Taliglucerase alfa, manufactured in carrot cell suspension cultures, is FDA-approved for Gaucher's disease treatment (116). Greenovation Biotech has developed therapeutic glycoproteins in moss cell cultures (128). Baiya Phytopharm in Thailand advanced its COVID-19 vaccine candidate into clinical trials, showcasing regional innovation (129). The convergence of PMF with synthetic biology, CRISPR-based genome editing, and AI-guided metabolic engineering opens new frontiers in custom-designed biofactories (130). Companies like Leaf Expression Systems (UK), Icon Genetics (Germany), and Kentucky BioProcessing (USA) are advancing modular expression systems for rapid prototyping of biologics (131). These platforms aim to produce not only vaccines but also diagnostics, growth factors, and enzymes with enhanced stability and targeted glycosylation profiles. Sustainability remains a defining driver of PMF's long-term relevance: valorisation of residual biomass, integration into circular economy models, and replacement of energy-intensive mammalian cell systems with solar-powered greenhouses or hydroponic facilities all reinforce its environmental compatibility. Despite this progress, regulatory and societal challenges continue to shape the trajectory of PMF. In particular within the European Union, strict approval processes for genetically modified organisms (GMOs) and persistent public scepticism pose barriers to wider adoption (132). Addressing these challenges will require the development of plant-specific regulatory guidelines, robust risk–benefit assessment frameworks, and proactive public engagement strategies that distinguish pharmaceutical PMF from agricultural GMOs.

In conclusion, PMF is poised to become a central pillar of next generation biomanufacturing. Its flexibility, biosafety, and sustainability make it uniquely suited for both emergency response and routine healthcare. The pace at which PMF transitions from an alternative platform to a mainstream component of the global bioeconomy will ultimately depend on continued technological refinement, strategic investment, international regulatory harmonization, and effective public communication.

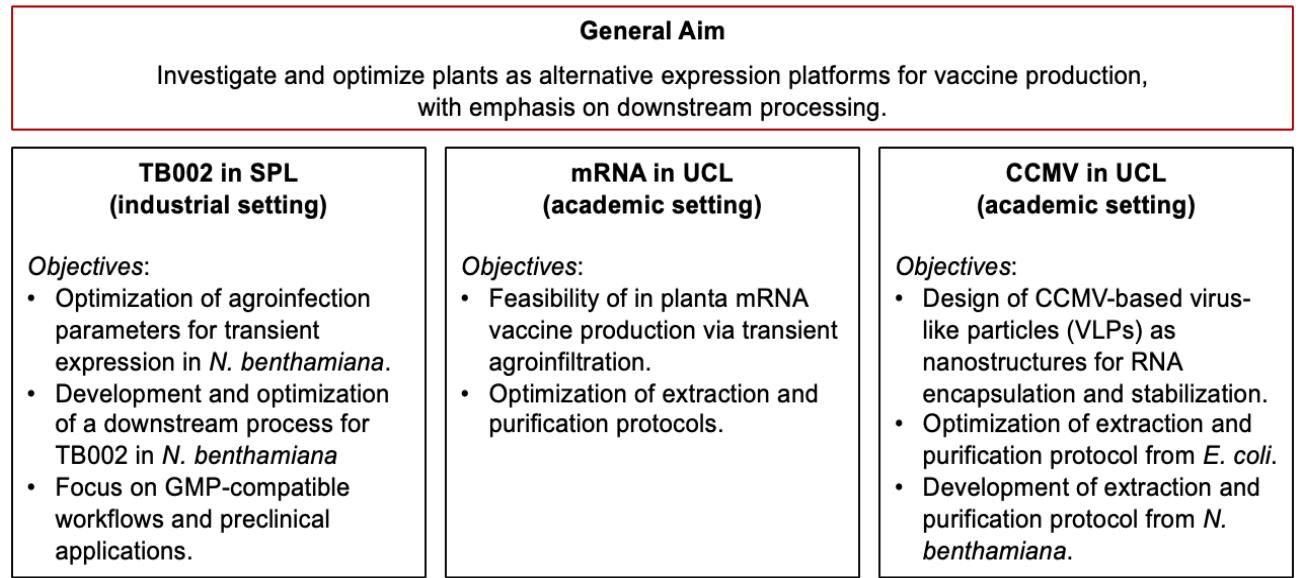
## 2. AIMS and OBJECTIVES

The aim of this PhD project was to investigate and optimize plants as alternative expression platforms for the production of vaccines, with particular emphasis on downstream processing strategies. In light of the growing global demand for cost-effective and scalable biomanufacturing systems, plant molecular farming offers significant potential. However, critical technical bottlenecks, especially during the purification stages, still limit the clinical and industrial translation of plant-derived biologics.

To address these challenges, the project was structured into two experimental phases, each conducted in a distinct setting and focused on different biopharmaceutical targets. The first phase took place at Special Product's Line (SPL), a pharmaceutical company based in Anagni (Italy). The industrial work focused on the development and optimization of the downstream process for a recombinant subunit vaccine candidate against Tuberculosis (TB) produced in *Nicotiana benthamiana*. This phase aimed to establish a purification workflow compatible with preclinical development and adaptable to GMP requirements, providing exposure to the constraints, quality standards, and process decisions typical of an industrial setting. The second phase took place at University College London (UCL), within the Department of Biochemical Engineering, and focused on two complementary research lines. The first investigated the expression and purification of a model mRNA vaccine produced in *Nicotiana benthamiana* via transient agroinfiltration. This work explored the feasibility of in planta mRNA production and addressed the challenges associated with its extraction and purification. Conducted in a more flexible laboratory environment, this work allowed the testing of novel protocols and extensive troubleshooting, including the comparison of chromatographic and non-chromatographic purification strategies. In parallel, a second line of research was dedicated to the development of virus-like particles (VLPs) derived from Cowpea Chlorotic Mottle Virus (CCMV) as a novel strategy for RNA encapsulation and protection. This work included cloning and expression of the CCMV coat protein in *E. coli* and in *N. benthamiana*, optimization of purification strategies and assembly/disassembly assays. Together, these activities provided a broader experimental platform to address both the production of mRNA and the design of plant-derived nanostructures for its stabilization and delivery.

The dual structure of this project – combining industrial and academic approaches – enabled a comprehensive analysis of PMF systems for both protein-based and RNA-based vaccine candidates. Particular attention was dedicated to overcoming the technical limitations related

to solubility, aggregation, impurity removal, and product stability, as well as to evaluating the broader implications of using plant-based systems, including their scalability, regulatory compliance, and suitability for deployment in real-world scenarios. The work aimed to critically examine the regulatory, economic, and logistical considerations surrounding the use of PMF-derived vaccines, leveraging insights gained from both an industrial and a laboratory setting. Ultimately, this work aimed to critically assess the feasibility of PMF for next-generation vaccine development and to contribute practical and conceptual insights into the positioning of plant-based platforms within the global biopharmaceutical landscape.



**Figure 4.** Schematic overview of the general aim and of the specific objectives of this PhD project.



### 3. MATERIALS AND METHODS

#### 3.1 Methods from Chapter 4

##### 3.1.1 Greenhouse Nutrition Regimen

*Nicotiana benthamiana* plants were cultivated using a staged hydroponic nutrition plan under controlled greenhouse conditions. A liquid nutrient solution was prepared weekly with varying concentrations of ionic, silicon, and oxygen-based additives (Growth Technology, UK), adjusted according to the developmental stage of the plants. Electrical conductivity (EC) values were monitored and maintained between 0.8 and 3.6 mS/cm over the six weeks of growth.

The nutrient schedule was as follows:

Week 1 (EC: 0.8): 3 mL/L Ionic Solution, 0.1 mL/L Silicon Solution, 0.2 mL/L Oxygen Solution.

Week 2 (EC: 1.2): 4 mL/L Ionic Solution, 0.2 mL/L Silicon Solution, 0.2 mL/L Oxygen Solution.

Week 3 (EC: 1.5): 5 mL/L Ionic Solution, 0.35 mL/L Silicon Solution, 0.2 mL/L Oxygen Solution.

Week 4 (EC: 2.5): 9 mL/L Ionic Solution, 0.5 mL/L Silicon Solution, 0.2 mL/L Oxygen Solution.

Week 5 (EC: 3.6): 13 mL/L Ionic Solution, 0.7 mL/L Silicon Solution, 0.2 mL/L Oxygen Solution.

Week 6 (post-agroinfiltration): same as Week 5.

The pH of the solution was maintained between 5.7 and 5.8. Oxygen solution was supplemented daily to maintain solution cleanliness. Prior to transplantation, rockwool substrates were conditioned by overnight immersion in an acid solution (pH 3–4), rinsed with deionized water, and dried to minimize alkaline buffering impact.

##### 3.1.2 *Nicotiana benthamiana* optimized growth condition (Lab Scale)

*Nicotiana benthamiana* plants were cultivated in a controlled-environment growth chamber of own design and equipped with LED light (Koscheal, UK) providing a photosynthetically active radiation (PAR) of  $\sim 125 \mu\text{mol m}^{-2} \text{s}^{-1}$  at canopy level. Light spectrum was centred on blue ( $\sim 450 \text{ nm}$ ) and red ( $\sim 660 \text{ nm}$ ) bands. A 16 h light / 8 h dark photoperiod was maintained throughout the growth cycle. Temperature and relative humidity were controlled at 25 °C and  $\sim 60\%$  RH, respectively. Environmental probes from Elitech (UK) logged temperature and humidity inside the unit and in the nutrient tank. A room humidifier (Bear Electric Appliance, UK) was employed to stabilize temperature RH following initial fluctuations.

Plants were grown on rockwool cubes (Cutilene, Netherlands) previously conditioned by overnight immersion in an acid solution (pH 3–4), rinsed with deionized water, and dried prior to be placed on a modular grid over a recirculating nutrition solution tray. Nutrient solution was replaced every 7–10 days; an oxygen solution was added daily to limit algae growth and to maintain clarity. Nutrient solution was applied according to the staged regimen described in Section 3.1.1. Electrical conductivity was maintained between 0.8 and 3.6 mS/cm according to growth stage, while pH was kept in the range of 5.7–5.8. A submersible pump 600L/H 8W (Decdeal, China) ensured continuous solution recirculation and root aeration. The unit operated within a filter hood to ensure controlled airflow and minimize external light contamination.

### **3.1.3 Electrocompetent Cells of *Agrobacterium tumefaciens***

A 50  $\mu$ L glycerol stock of *A. tumefaciens* strain LBA4404 was inoculated into 50 mL of antibiotic-free LB medium and incubated overnight at 30 °C with agitation at 250 rpm in a 200 mL baffled Erlenmeyer flask to enhance aeration. Cultures were chilled on ice for 20 min and harvested by centrifugation at 4,000  $\times$  g for 15 min at 4 °C. The supernatant was discarded, and the pellet was washed four times with ice-cold 10% (v/v) sterile glycerol. At each washing step, the resuspension volume was progressively reduced. The final pellet was resuspended in 0.5 mL of 10% (v/v) sterile glycerol, aliquoted (50–100  $\mu$ L), and stored at –80 °C until use.

### **3.1.4 Transformation of *Agrobacterium tumefaciens* Cells**

Electroporation was used to transform *A. tumefaciens* strain LBA4404, employing both in-house prepared and commercially obtained (GoldBio, USA) electrocompetent cells. Electroporation was performed using a Bio-Rad Gene Pulser electroporator (Bio-Rad, USA). Parameters were optimized with the control plasmid pCAMBIA (GoldBio, USA), and the final conditions were set as follows:

- Electroporation cuvette (Bio-Rad, USA) gap width: 0.2 cm
- Voltage: 2.5 kV
- Capacitance: 25  $\mu$ F
- Resistance: 400  $\Omega$

All materials (cuvettes, tubes, SOC medium) were pre-chilled on ice. A 50  $\mu$ L aliquot of electrocompetent cells was gently mixed with 2  $\mu$ L (200–500 ng) of plasmid DNA and transferred into a 0.2 cm ice-cold electroporation cuvette. A single pulse was applied, yielding

a time constant of 7–8 ms, indicating successful shock delivery. Immediately after electroporation, 200  $\mu$ L of chilled SOC medium was added to the cuvette under sterile conditions. The suspension was transferred into a sterile 15 mL Falcon tube containing an additional 800  $\mu$ L sterile SOC medium and incubated horizontally at 26 °C with shaking at 200 rpm for 4 h to promote cell recovery. After recovery, dilutions ranging from 50 to 100  $\mu$ L were plated on LB agar plates supplemented with the appropriate antibiotic. Plates were incubated at 29 °C for 48–72 h, and colonies were visible after 3 days.

### **3.1.5 Agroinfection via Vacuum Infiltration (Lab-Scale)**

Vacuum agroinfiltration was carried out using an 8 litres plastic vacuum desiccator (Cole-Parmer, UK) connected to a Microsart ® E-Jet vacuum pump (Sartorius). *Nicotiana benthamiana* plants were inverted and their aerial tissues immersed in the *Agrobacterium tumefaciens* suspension prepared at an optical density of OD<sub>600</sub> = 0.4 in infiltration buffer (10 mM MES, 10 mM MgCl<sub>2</sub>, pH 5.6). A negative pressure of -600 mbar was applied for 3 minutes to facilitate infiltration of bacterial cells into the leaf intercellular spaces. The pressure was then rapidly released to atmospheric level, ensuring uniform penetration of the suspension throughout the lamina. After infiltration, plants were gently rinsed with sterile deionized water to remove excess bacterial suspension from the leaf surface and returned to the controlled-environment greenhouse under the same conditions described in Section 3.1.2 for subsequent expression of the gene of interest.

### **3.1.6 *Nicotiana benthamiana* optimized growth conditions (Industrial Scale)**

At the Special Product's Line (SPL) facility, *Nicotiana benthamiana* plants were cultivated in a hydroponic vertical farming system under controlled environmental conditions. Plants were grown on rockwool cubes (Cutilene, Netherlands) arranged in stacked layers to maximize space utilization. Automated fertigation lines supplied nutrient solution (Growth Technology, UK) according to a staged program, with electrical conductivity (EC) gradually increased from 0.8 to 3.6 mS/cm and pH maintained between 5.7 and 5.8. Lighting was provided by high-intensity LED panels (Skylark ZK3-TB14-VE03/A), delivering a photosynthetically active radiation (PAR) of  $\sim 125 \mu\text{mol m}^{-2} \text{s}^{-1}$  at canopy level, with a 16 h light / 8 h dark photoperiod. Air temperature was controlled at  $25 \pm 1$  °C during the light phase and  $22 \pm 1$  °C during the dark phase, with relative humidity maintained between 55–65%. Environmental control was achieved through an integrated monitoring system, continuously logging temperature, humidity, pH, EC, and light intensity.

### **3.1.7 Agroinfection via Vacuum Infiltration (Industrial Scale)**

Agroinfiltration at industrial scale at Special Product's Line (SPL) was performed using a custom-designed, PLC-controlled vacuum infiltration system (MO.S.A.I.C, Italy). *Nicotiana benthamiana* plants aged 35 days were inverted and immersed into a 16 L stainless-steel infiltration tank, with trays processed in pairs. The bacterial suspension was prepared from *A. tumefaciens* GV3101 cultures carrying the expression construct, harvested and resuspended in infiltration buffer (10 mM MES, 10 mM MgCl<sub>2</sub>, 100 µM acetosyringone, pH 5.7) to a final OD<sub>600</sub> of 0.3–0.4. A vacuum pressure of -700mbar was applied for 4 minutes, followed by rapid restoration to atmospheric pressure. This transient differential facilitated displacement of intercellular air through stomata and penetration of the bacterial suspension into the mesophyll. Optimization studies confirmed that exposure times shorter than 4 minutes resulted in incomplete infiltration, while durations longer induced tissue collapse and necrosis. After infiltration, plants were rinsed with sterile water, returned upright, and transferred to a dedicated post-infiltration chamber under controlled environmental conditions (22–25 °C, 16 h light/8 h dark photoperiod, 60% relative humidity). Infiltrated plants were physically separated from non-infiltrated plants to ensure biosafety. Leaves were harvested at 5–7 days post infiltration, portioned into 2 kg bags, and stored at -80 °C pending downstream processing.

## **3.2 Methods from Chapter 5**

### **3.2.1 Protein Extraction and Buffer Screening**

Harvested *Nicotiana benthamiana* leaves were homogenized in extraction buffer at a ratio of 1:2 (w/v; g of tissue : mL of buffer) using T 25 Digital Ultra-Turrax™ Immersion Blender (IKA, Germany) under cold conditions. Multiple extraction buffers were initially screened to reduce aggregation and improve solubility of TB002. The final optimized buffer composition was: 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, 5 mM TCEP, 7 M urea, 1 mM PMSF, pH adjusted to 7.4. Polyvinylpolypyrrolidone (PVPP) was included in early trials to reduce interference from phenolic compounds but was omitted in the final protocol following evaluation. Following homogenization, extracts were clarified by centrifugation at 15,000 × g for 30 min at 4 °C (model Avanti JXN-30, Beckman Coulter Life Sciences, USA) and the supernatant was recovered. A second centrifugation step under identical conditions was applied to ensure removal of residual particulates prior to downstream purification.

### **3.2.2 Diafiltration and Concentration**

Extracted and clarified samples were processed by tangential flow filtration (TFF) using a VivaFlow 200 system (Sartorius, Germany) fitted with PES membranes. Two MWCO were evaluated (100 kDa and 50 kDa): the 50 kDa PES cassette was selected for routine processing based on superior retention of TB002 (~56 kDa). The cassette was connected to a peristaltic pump (VWR International, USA) providing continuous circulation of the feed. Diafiltration was conducted at 4°C with the peristaltic pump operated at ~1.5 bar; the feed flow was adjusted to maintain optimal transmembrane pressure. The diafiltration buffer consisted of 20 mM sodium phosphate, 500 mM NaCl, 5 mM TCEP, pH 7.4. A 5-fold buffer exchange was applied, reducing the working volume from 150–250 mL to ~30–50 mL. The retentate was then concentrated to ~10–15 mL. Process intermediates were analysed by SDS-PAGE, Western blot (anti-His) and UHPLC-SEC to verify integrity and confirm the absence of significant aggregation.

### **3.2.3 Immobilized Metal Affinity Chromatography (IMAC)**

Clarified extracts were subjected to affinity purification using immobilized metal affinity chromatography (IMAC). All steps were performed on an ÄKTA Pure system (Cytiva, Sweden). Columns were packed in-house with IMAC Sepharose HP resin (Cytiva, Sweden) in XK 26/20 (Cytiva, Sweden) housings. The method sequence was optimized as shown in Table 4.

**Table 4.** Optimized IMAC purification method, detailing equilibration, loading, washing, stepwise elution and strip conditions.

| Step                  | Conditions / Buffer composition                                                                        |
|-----------------------|--------------------------------------------------------------------------------------------------------|
| <i>Equilibration</i>  | 5 CV binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, 5 mM TCEP, 7 M urea, pH 7.4) |
| <i>Sample loading</i> | Clarified extract applied at 2 mL/min                                                                  |
| <i>Wash 1</i>         | 10 CV binding buffer to remove unbound proteins                                                        |
| <i>Wash 2</i>         | 5 CV wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 50 mM imidazole, 5 mM TCEP, 7 M urea, pH 7.4)    |
| <i>Elution</i>        | Stepwise elution: 5 CV 100 mM imidazole; 5 CV 250 mM imidazole.                                        |
| <i>Strip</i>          | 5 CV buffer containing 500 mM imidazole                                                                |
| <i>Equilibration</i>  | 5 CV binding buffer prior to the next run                                                              |

The IMAC eluates containing TB002 were pooled and immediately processed by desalting chromatography to remove urea and imidazole prior to further purification. Fractions were collected at each step and analysed by SDS-PAGE, Western blot (anti-His) and UHPLC-SEC.

### **3.2.4 Desalting Chromatography**

IMAC eluates containing TB002 were desalted on an ÄKTA Pure system (Cytiva, Sweden) using in-house packed Sephadex G-25 Fine columns (Cytiva, Sweden) mounted in XK 26/20 or XK 50 housings (Cytiva, Sweden), depending on batch scale. The column was equilibrated with desalting buffer consisting of 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4. In some runs, residual urea (0.1–2 M) was included to improve protein stability. The maximum sample load per run corresponded to one third of the column bed volume. Samples were applied at a linear velocity <60 cm/h (corresponding to ~2–3 mL/min for XK 26/20 and 10–15 mL/min for XK 50), and proteins were eluted isocratically with equilibration buffer. UV280-positive fractions were collected and pooled. The desalting step served to exchange the protein solution into a low-salt, urea-free buffer compatible with subsequent enzymatic reaction. Pooled fractions were immediately processed to avoid precipitation.

### **3.2.5 Thrombin Cleavage**

Following desalting, TB002 was subjected to enzymatic cleavage to remove the N-terminal His-tag, using thrombin protease (Cytiva 27-0846-01). Cleavage reactions were performed in solution at room temperature (22 °C) under gentle agitation. The optimized enzyme-to-substrate ratio was calculated at 10–20 U thrombin per mg of TB002. The reaction was carried out for 18h in diafiltration buffer (20 mM sodium phosphate, 0.1 M urea, pH 7.4), which ensured protein solubility and enzymatic activity. Following incubation, the sample was immediately processed by secondary IMAC to separate tag-free TB002 from uncleaved protein, His-tag fragments, and thrombin.

### **3.2.6 Secondary IMAC (Post-Cleavage Clean-Up)**

Following thrombin digestion, samples were applied on columns packed in-house with IMAC Sepharose HP resin (Cytiva, Sweden) connected to an ÄKTA Pure 150 system (Cytiva, Sweden). The column was equilibrated with a binding buffer that consisted of 20 mM sodium phosphate, 0.1 M urea, pH 7.5. The step was performed as shown in Table 5.

**Table 5.** Sequence of the optimized IMAC purification for thrombin-digested TB002, highlighting flow-through recovery of tag-free protein and elution of His-tagged species.

| Step                  | Conditions / Buffer composition                                                                                                                 |
|-----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Sample loading</i> | Thrombin-digested protein solution, flow rate 2 mL/min                                                                                          |
| <i>Flow-through</i>   | Collection of fractions containing tag-free TB002                                                                                               |
| <i>Elution</i>        | Strip directly with 100% elution buffer (20 mM sodium phosphate, 0.1 M urea, 0.5 M imidazole, pH 7.5) to elute His-tagged proteins and thrombin |

Flow-through fractions were collected and analysed by SDS-PAGE and Western blot (anti-TB002) and UHPLC-SEC. The FT fractions contained TB002 free of the His-tag, whereas His-tagged species and thrombin remained bound to the column and eluted with the strip step.

### **3.2.7 Size Exclusion Chromatography (SEC)**

The size exclusion step was performed on an ÄKTA Pure 150 system (Cytiva, Sweden) using a Superdex 200 HiLoad 26/100 column (Cytiva, Sweden). The column specifications were bed volume of 530 mL, bed height of 100 cm, and cross-sectional area of 5.3 cm<sup>2</sup>. The column was equilibrated with formulation buffer consisting of 20 mM sodium phosphate, 150 mM NaCl, 1% glycerol, pH 7.4–7.5. In some experiments, residual urea (50–100 mM) was included to stabilize TB002. Samples of maximum 20 mL were loaded, and proteins were eluted isocratically at a flow rate of 4–5 mL/min, corresponding to a linear velocity of <60 cm/h. Fractions across the main protein peak were collected and analysed by SDS-PAGE and Western blot. The SEC step was employed as a polishing and formulation step, enabling buffer exchange into the final formulation conditions.

### **3.2.8 Anion Exchange Chromatography (AEX)**

Three main AEX trials were conducted to evaluate the potential of Q-Sepharose Fast Flow (Cytiva, Sweden) for TB002 purification. Different urea concentrations and gradient strategies were tested. Anion-exchange chromatography (AEX) trials were performed on an ÄKTA Pure 150 system (Cytiva, Sweden) using Q-Sepharose Fast Flow resin (Cytiva, Sweden) packed in an XK 16/50 housing (bed volume 5 mL; cross-section 2 cm<sup>2</sup>; bed height 2.5 cm) (Cytiva, Sweden). The flow rate was 2 mL/min (linear velocity 60 cm/h; residence time 2.5 min). Prior to loading, the desalted TB002 solution was adjusted to pH 8.3–8.5 and conductivity verified. In Trials 1 and 2, columns were equilibrated with Buffer A (50 mM Tris, 7 M urea, pH 8.5) and the elution was performed with Buffer B (50 mM Tris, 7 M urea, 1 M NaCl, pH 8.5). A load

volume of 15 mL was applied per run. Trial 1 employed a linear gradient from 0–100% B (0 M – 1 M NaCl) over 10 CV (25 min), yielding a FT and an eluate fraction. Trial 2 used a restricted gradient (0–30% B over 10 CV) followed by a direct strip step with 100% B, producing a FT, an eluate and a strip fraction. In Trial 3, samples were first desalted on a PD-10 column to reduce urea concentration and exchanged into 50 mM Tris, 0.5 M urea, pH 8.5. The column was equilibrated with Buffer A (50 mM Tris, 0.5 M urea, pH 8.5), eluted with Buffer B (50 mM Tris, 0.5 M urea, 1 M NaCl, pH 8.5), and finally stripped with Buffer C (50 mM Tris, 7 M urea, 1 M NaCl, pH 8.5). After a 10 CV wash with Buffer A, the flow-through was collected. Elution 1 was performed with a gradient from 0–20% B over 10 CV (25 min). Elution 2 was carried out with a gradient from 20–100% B over 10 CV (25 min), followed by a strip with 100% Buffer C. All post-AEX fractions were concentrated with Vivaspin 20 PES concentrators (Sartorius, Germany) and desalted on PD-10 columns (Cytiva, Sweden) into 50 mM tris buffer supplemented with 0.05% Tween-20. Fractions were analysed by SDS-PAGE and Western blot. In the first two trials, protein recovery occurred largely in the flow-through, while eluates contained low-molecular-weight species and aggregates. The third trial, performed after reducing urea to 0.5 M, yielded cleaner elution peaks with enrichment of monomeric TB002, although purity was still insufficient. For this reason, AEX was not integrated into the final downstream process, which proceeded with SEC for polishing and formulation.

**Table 6.** Summary of anion-exchange chromatography (AEX) trials performed on TB002 using Q-Sepharose Fast Flow resin. Three gradient strategies were tested under different urea concentrations.

| <i>Trial</i> | <i>Pre-treatment</i>                   | <i>Buffer A</i>                    | <i>Buffer B</i>                              | <i>Buffer C</i>                            | <i>Gradient/Steps</i>                            | <i>Load</i> | <i>Outcome</i>                                                         |
|--------------|----------------------------------------|------------------------------------|----------------------------------------------|--------------------------------------------|--------------------------------------------------|-------------|------------------------------------------------------------------------|
| <b>1</b>     | None                                   | 50 mM Tris, 7M urea<br>– pH:8.5    | 50 mM Tris, 7 M urea, 1 M NaCl<br>– pH 8.5   | ---                                        | 0–100% B, 10 CV                                  | 15 mL       | Protein found in FT. Eluate containing low-MW species and aggregates.  |
| <b>2</b>     | None                                   | 50 mM Tris, 7M urea<br>– pH:8.5    | 50 mM Tris, 7 M urea, 1 M NaCl<br>– pH 8.5   | ---                                        | 0–30% B, 10 CV; strip w/ 100% B                  | 15 mL       | As for the Trial 1, TB002 mostly found in FT.                          |
| <b>3</b>     | PD-10 columns (desalted in 0.5 M urea) | 50 mM Tris, 0.5 M urea<br>– pH 8.5 | 50 mM Tris, 0.5 M urea, 1 M NaCl<br>– pH 8.5 | 50 mM Tris, 7 M urea, 1 M NaCl<br>– pH 8.5 | 0–20% B, 10 CV; 20–100% B, 10CV; strip w/ 100% C | 15 mL       | Cleaner Elution peaks. Enrichment of TB002. Still insufficient purity. |

### **3.2.9 Sterilization and Storage**

Aliquots of the formulated bulk were subjected to sterile filtration tests using 0.22 µm syringe filters of different materials (CA, Nylon, PES, PTFE). PES 0.22 µm syringe filter (Sartorius, Germany) enabled only partial recovery, with substantial protein adsorption; the other materials led to more severe losses. As an alternative, UV exposure (2 h, RT) was applied to



bulk aliquots, which did not induce visible degradation or aggregation by SDS-PAGE/Western blot. Final bulks were aliquoted and stored at 4 °C and –20 °C for stability tracking.

### **3.2.10 SDS-PAGE Analysis of TB002**

Protein purity and molecular weight estimation were assessed by SDS-PAGE. Prior to gel loading, protein concentrations were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, USA), using bovine serum albumin (BSA) standards (Bio-Rad, USA) for calibration. This ensured that equivalent protein amounts were loaded in each well for comparative analysis across process intermediates. Samples from different purification steps were mixed with Laemmli sample buffer (Bio-Rad, USA) supplemented with  $\beta$ -mercaptoethanol and denatured by boiling at 95 °C for 5 min. Equal volumes (10  $\mu$ L, corresponding to 20–25  $\mu$ g of protein depending on sample concentration) were loaded onto 4-20% polyacrylamide gels and run at 120 V for approximately 60 min in Tris-Glycine SDS running buffer (Bio-Rad, USA) in electrophoresis tank (Bio-Rad, USA). Following separation, gels were stained with Coomassie Brilliant Blue R-250 and destained in methanol–acetic acid solution until background was clear. TB002 appeared as a distinct band at ~56 kDa.

### **3.2.11 Western Blot Analysis of TB002**

Western blotting was employed to confirm TB002 identity and monitor His-tag removal during processing. Proteins resolved by SDS-PAGE (4-20% polyacrylamide gels) were transferred to Trans-Blot Turbo Midi 0.2  $\mu$ m Nitrocellulose Transfer Packs (Bio-Rad, USA) using a Trans-Blot Turbo Transfer System (Bio-Rad, USA). Membranes were blocked in 5% (w/v) non-fat dry milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 h at room temperature. After blocking, the membranes were incubated overnight at 4 °C with either:

- anti-6xHis monoclonal antibody (1:1000; Abcam, UK), to detect His-tagged TB002.
- polyclonal anti-TB002 antibody (1:1000; in-house, SPL, Italy) to confirm identity of cleaved protein.

Following washes in TBS-T, membranes were incubated for 1 h with HRP-conjugated secondary antibodies: anti-mouse IgG (H+L)-HRP 1:100000 (Cytiva, Sweden) for anti-His detection. Signals were visualized by enhanced chemiluminescence with Clarity western ECL substrate (Bio-Rad, USA) and captured using a ChemiDoc imaging system (Bio-Rad, USA). Western blotting revealed a consistent ~56 kDa band for TB002 intermediates, with a molecular shift of ~6 kDa observed post-thrombin digestion, consistent with His-tag removal.

### **3.2.12 Dot Blot Analysis**

Dot blot assays were employed for rapid screening of TB002 expression and purification intermediates. PVDF membranes (Bio-Rad, USA) were activated in methanol for 30 s and equilibrated in transfer buffer. 2–5 µL of protein samples were spotted directly onto membranes and allowed to air-dry. Membranes were blocked with 5% non-fat dry milk in TBST for 1 h at room temperature, then incubated overnight at 4 °C with a mouse anti-6xHis monoclonal antibody 1:1000 (Abcam, UK). Following washing, membranes were incubated for 1 hour with HRP-conjugated anti-mouse secondary IgG antibody, 1:10000 dilution (Cytiva, Sweden). Signal detection was performed using enhanced chemiluminescence (Clarity wester ECL, Bio-Rad, USA) and images were acquired with a ChemiDoc system (Bio-Rad, USA). This method allowed rapid, semi-quantitative detection of His-tagged TB002 without prior electrophoretic separation, enabling fast screening of plant extracts and purification intermediates. However, this approach did not provide molecular weight information and was therefore used only as a complementary assay during early process development.

### **3.2.13 UHPLC-SEC Analytic Analysis**

The oligomeric state and aggregation profile of TB002 were evaluated by ultra-performance liquid chromatography size-exclusion chromatography (UPLC-SEC). Analyses were performed on a Waters Acquity UPLC system (Waters, USA) equipped with a UV detector set at 280 nm. Samples from different purification stages were injected onto an Acquity UPLC Protein BEH SEC column (200 Å pore size, 1.7 µm particle size, 4.6 × 150 mm; Waters, USA). The column was equilibrated and run isocratically with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 at a flow rate of 0.3 mL/min. Chromatograms were analysed for monomeric and aggregate content. TB002 eluted as a tetramer as principal peak at a retention time of 7,8-8 minutes corresponding to a molecular weight of 220 kDa. In certain IMAC eluates, a single main peak was observed but corresponded to aggregated species rather than the monomer. This behaviour was consistent across several purification trials.

## ***3.3 Methods from Chapter 6***

### **3.3.1 Plasmid Construct Validation**

Plasmid constructs carrying the GFP reporter sequence for mRNA expression were validated prior to transformation of *Agrobacterium tumefaciens*. Quality control included PCR amplification (PCRmax Ltd, UK), restriction digestion, and Sanger sequencing (Plasmidsaurus, USA). PCR amplification was performed using construct-specific primers under standard cycling conditions (initial denaturation at 95 °C for 3 min, followed by 30 cycles

of 95 °C for 30 s, annealing at 55–60 °C for 30 s, and extension at 72 °C for 30 sec/kb, with a final extension of 72 °C for 10 min). PCR products were visualized on 1% agarose gels and compared with molecular weight markers to confirm the expected insert size. Restriction digestion was carried out on purified plasmid DNA using Nocl or SacI enzymes (New England Biolabs, UK) targeting unique recognition sites within the construct. Digested products were separated on 1% agarose gels to confirm the expected fragment pattern. Sequencing of validated constructs was performed by Sanger sequencing (Plasmidsaurus service, USA) and was analysed using SnapGene/Benchling software to confirm sequence fidelity of the GFP insert and regulatory elements. Validated plasmids were used for *Agrobacterium* transformation and preserved as reference stocks for subsequent experiments.

### **3.3.2 Gibson Assembly and PCR Amplification of DNA Fragments**

DNA fragments containing overlapping regions for Gibson Assembly were generated by polymerase chain reaction (PCRmax Ltd, UK) using the VeriFi™ polymerase system (PCR Biosystems, UK), which includes a high-fidelity polymerase and GC enhancer. Reactions were performed in 50 µL total volume, consisting of: 10 µL 5× VeriFi Buffer (containing 15 mM MgCl<sub>2</sub> and 5 mM dNTPs), 5 µL 10× VeriMax Enhancer, 2 µL of each primer (10 µM; diluted 1:10 from stock), ~100 ng template DNA, 0.5 µL polymerase enzyme, and nuclease-free water up to final volume. A touchdown PCR protocol was applied: initial denaturation at 95 °C for 3 min, followed by 30–35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C (30 s per kb). A final extension was carried out at 72 °C for 10 min. PCR products were analysed on 1% agarose gels alongside molecular weight markers. Desired bands were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Germany), according to the manufacturer's instructions. DNA concentration and purity were determined using a Nanodrop™ spectrophotometer (DeNovix, USA). Purified fragments were subsequently assembled into the pGreen vector backbone using the Gibson Assembly® Master Mix (New England Biolabs, USA), following the supplier's protocol. The reaction was incubated at 50 °C for 1 hour to allow enzymatic joining of overlapping DNA fragments via exonuclease-mediated strand pairing, polymerase filling, and ligase sealing. Reaction products were transformed into chemically competent *E. coli* cells and electrocompetent *A. tumefaciens* cells for propagation and validation.

### **3.3.3 Transformation of *E. coli* with Assembled Constructs**

Chemically competent *E. coli* Top10 cells (Thermo Fisher Scientific, USA) were used for transformation of assembled plasmids. For each reaction, 50  $\mu$ L of competent cells were mixed with 2  $\mu$ L of Gibson assembly product (diluted 1:4) and incubated on ice for 30 min. Cells were heat-shocked at 42 °C for 1 min, followed by immediate incubation on ice for 2 min. Subsequently, 950  $\mu$ L of LB medium were added and the suspension incubated at 37 °C with shaking (250 rpm) for 2 h to allow recovery. Following recovery, cells were plated on LB agar supplemented with kanamycin (50  $\mu$ g/mL) and incubated overnight at 37 °C. Transformed colonies appeared the following morning and were selected for downstream plasmid preparation and sequence validation.

### **3.3.4 Transformation of *Agrobacterium tumefaciens* via Electroporation**

Transformation of *A. tumefaciens* strain LBA4404 (GoldBio, USA) was performed by electroporation using a Bio-Rad Pulse Controller (Bio-Rad, USA). Since pGreen-based constructs require the pSoup helper plasmid for replication, co-transformation was carried out with pSoup, which confers resistance to tetracycline (5  $\mu$ g/mL). For each transformation, 25  $\mu$ L of competent *A. tumefaciens* cells were mixed with 2  $\mu$ L of plasmid DNA (100–200 ng) and transferred into a pre-chilled 0.2 cm electroporation cuvette (Bio-Rad, USA). Electroporation was performed under the following conditions: 2.5 kV, 25  $\mu$ F, 400  $\Omega$ , corresponding to a field strength of 12.5 kV/cm and a time constant of ~7-8 ms. Immediately following the pulse, 200  $\mu$ L of SOC medium (GoldBio, USA) were added to the cuvette, and the suspension was transferred into a sterile 15 mL Falcon tube containing an additional 800  $\mu$ L of sterile SOC medium. Recovery was carried out at 29 °C with agitation at 230 rpm for 4 h. During the first 2 h, tubes were incubated horizontally to enhance oxygen transfer, then returned to vertical orientation. After recovery, cells were plated on LB agar supplemented with rifampicin (25  $\mu$ g/mL), streptomycin (100  $\mu$ g/mL), and kanamycin (50  $\mu$ g/mL). Plates were incubated at 29 °C for 48-72 hours to allow colony development. In some cases, cells were concentrated by centrifugation at 6,000 x g for 10 min at 30 °C prior to plating to increase colony yield.

### **3.3.5 Overnight Cultures and Resuspension for Agroinfiltration**

*A. tumefaciens* clones carrying the pGreenII construct were initially grown in preinoculum cultures of 10 mL LB medium supplemented with kanamycin (50  $\mu$ g/mL), rifampicin (25  $\mu$ g/mL), and streptomycin (100  $\mu$ g/mL). Cultures were incubated overnight at 29 °C with

shaking at 230 rpm. The following morning, the optical density at 600 nm ( $OD_{600}$ ) typically reached 1.5–1.8. Subsequently, 5 mL of pre-inoculum were transferred into 2 L Erlenmeyer flasks containing 250 mL of LB medium with the same antibiotic supplementation. Cultures were incubated overnight under identical conditions at 29 °C and 230 rpm, reaching  $OD_{600}$  = 2.8–3.5 after ~16 h. Cells were harvested by centrifugation at  $6,000 \times g$  for 15 min at 25 °C. Pellets were resuspended in 50 mL of agroinfiltration buffer (10 mM MES, 10 mM  $MgCl_2$ , adjusted to pH 5.7). The pooled bacterial suspension reached an  $OD_{600}$  of ~15.8. Using the dilution equation ( $C_1V_1 = C_2V_2$ ), the required volume was calculated to adjust the suspension to a final  $OD_{600}$  of 0.4 in 8 L total volume for vacuum infiltration.

### 3.3.6 Agroinfiltration via Vacuum Infiltration

Vacuum agroinfiltration was performed using an 8 L plastic vacuum desiccator (Cole-Parmer, UK) connected to a Microsart® E-Jet vacuum pump (Sartorius, Germany). *Nicotiana benthamiana* plants were inverted and their aerial tissues immersed in the *Agrobacterium tumefaciens* suspension prepared at a final optical density of  $OD_{600}$  = 0.4 in infiltration buffer (10 mM MES, 10 mM  $MgCl_2$ , pH 5.7). A negative pressure of -600 mbar was applied for 3 min to facilitate infiltration of bacterial cells into the leaf intercellular spaces. The pressure was then rapidly released to atmospheric level, ensuring uniform penetration of the suspension throughout the lamina. After infiltration, plants were gently rinsed with deionized water to remove excess bacterial suspension from the leaf surface and returned to the greenhouse under the controlled conditions described in Section 3.1.2. Wild-type plants and plants infiltrated with a pGreenII-GFP construct were maintained in parallel as negative and positive controls, respectively.

### 3.3.7 Expression Monitoring

Expression of GFP in *N. benthamiana* plants infiltrated with *A. tumefaciens* (pGreenII-GFP) was monitored daily from 1 to 7 days post-infiltration (dpi) using a UV Light Torch (395 nm) (Darkbeam, UK). Leaves were illuminated in a darkened environment, and visual inspection was used to detect GFP fluorescence as a bright green signal against the natural leaf background. Peak fluorescence was consistently observed at 4–5 dpi, followed by a progressive decline thereafter. Wild-type plants (negative control) were analysed in parallel under identical conditions to confirm signal specificity and infiltration success.

### **3.3.8 RNA Extraction: Principles and Contaminant Removal**

Total RNA was extracted from infiltrated *N. benthamiana* leaves at peak GFP expression (5 dpi). Young, non-lignified leaves were selected to facilitate extraction and improve RNA quality. Harvested leaves were immediately stored at  $-80^{\circ}\text{C}$  until processing. For each extraction, frozen leaves were weighed and homogenized in a mechanical blender (Nutribullet, USA) with ice-cold extraction buffer (10 mM sodium phosphate, 2 mM EDTA, 150 mM NaCl, pH 7.4) at a ratio of 1 g tissue : 2 mL buffer. Homogenization was carried out for 1 min at  $4^{\circ}\text{C}$ . Maintaining low temperature was essential to inactivate endogenous ribonucleases. The homogenate was clarified by centrifugation at  $15,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ , and the supernatant was carefully recovered. The clarified extract was filtered through a  $0.65\ \mu\text{m}$  cellulose acetate membrane (Millipore, Germany) to reduce particulate load prior to downstream application. Extracts were processed immediately or stored on ice for no longer than 2 h before purification to minimize degradation.

### **3.3.9 Anion Exchange Chromatography (CaptoQ ImpRes)**

Clarified RNA extracts were subjected to anion exchange chromatography (AEX) using a pre-packed HiTrap CaptoQ ImpRes column (5 mL; Cytiva, Sweden) operated at  $4^{\circ}\text{C}$ . Samples were pre-filtered ( $0.65\ \mu\text{m}$ ) (Millipore, Germany) prior to loading. Column equilibration was performed with 10 column volumes (CV) of 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl. Samples volumes of 5–10 mL were applied at  $\sim 1.0\ \text{mL min}^{-1}$  ( $\leq 20\% \text{ CV min}^{-1}$ ), followed by a wash with 5 CV of equilibration buffer to remove unbound components. RNA was eluted using a stepwise NaCl gradient in the same Tris buffer, with salt concentrations ranging from 0.15 M to 1.0 M NaCl. Fractions of 1 mL were collected throughout the gradient. Collected fractions were analysed by UV absorbance ( $A_{260}/A_{280}$ ) and by 1% agarose gel electrophoresis to assess RNA integrity and distribution across the gradient. Peak fractions enriched in RNA were pooled and either concentrated by ethanol precipitation or processed directly in downstream assays. Despite successful binding and elution of nucleic acids, protein and phenolic contaminants were frequently co-purified, and RNA integrity varied depending on leaf batch and extraction conditions.

### **3.3.10 Oligo (dT) Affinity Chromatography**

mRNA was enriched from clarified plant extracts by oligo(dT) affinity chromatography on a pre-packed oligo(dT) (Thermo Fisher Scientific, USA) column operated on an ÄKTA Go system (Cytiva, Sweden) at  $4^{\circ}\text{C}$ . All solutions were prepared with nuclease-free reagents

and handled on ice. The column was equilibrated with binding buffer (Tris-HCl 50 mM, NaCl 0.5–1.0 M, pH 7.5) for 10 column volumes (CV). Clarified RNA extract was pre-filtered (0.65 µm) (Millipore, Germany) immediately prior to loading. The sample was applied at ~0.5–1.0 mL min<sup>-1</sup> (≤ 20% CV min<sup>-1</sup>). The column was washed with 10 CV of binding buffer to remove non-polyadenylated RNA and proteinaceous contaminants, followed by an additional 5 CV wash with low-salt Tris-HCl (50 mM, NaCl 0.1–0.2 M, pH 7.5). Poly(A)-RNA was eluted with nuclease-free water. 1 mL fractions were collected on ice in RNase-free tubes. Eluate pools were ethanol-precipitated and concentrated if required and immediately chilled. mRNA integrity and recovery were assessed by  $A_{260}/A_{280}$  and 1% agarose gel electrophoresis. The column was regenerated according to the manufacturer's instructions and re-equilibrated in binding buffer. Binding in high-salt Tris/NaCl favoured selective capture of polyadenylated species, while the plant-matrix carry-over was mitigated by the two-step wash with salt.

### **3.3.11 Oligo-Specific Magnetic Beads**

Magnetic beads functionalized with GFP-specific oligonucleotides were prepared starting from Sera-Mag carboxylate-modified beads (Cytiva, Sweden). A 5' amino-C6 modified GFP probe was coupled to the beads using EDC-mediated amide bond formation in imidazole buffer. Beads were washed extensively and blocked with sodium bicarbonate prior to use, and stored in PBS at 4 °C. For each assay, clarified plant extracts (1–2 mL) were incubated with 50 µL of functionalized beads under gentle rotation at room temperature to allow hybridization of target mRNA. Beads were collected on a magnetic stand and washed three times with PBS to remove unbound material. Elution was performed with nuclease-free water at 80 °C, followed by immediate cooling on ice. Control experiments with in vitro transcribed (IVT) GFP mRNA confirmed probe functionality and specific capture. However, plant-derived samples yielded low recovery. Elution fractions were concentrated by ethanol precipitation when possible and analysed by 1% agarose gel electrophoresis and spectrophotometry ( $A_{260}/A_{280}$ ). Comparative assays with wild-type and agroinfiltrated samples showed slightly higher signals in infiltrated leaves, although overall recovery remained below preparative thresholds.

### **3.3.12 Precipitation with Ammonium Sulphate**

Clarified extracts (15 mL) from *N. benthamiana* leaves were subjected to sequential ammonium sulphate precipitation at 20–80% saturation. For each step, the appropriate mass of ammonium sulphate was added directly to the extract (e.g., ~1.6 g for 20% saturation; 0.8–

1.0 g for subsequent increments) and dissolved by gentle agitation at room temperature. Samples were incubated for 1 h at 25 °C and centrifuged at 5,000 rpm for 15 min at 4 °C. The supernatant from each precipitation step was transferred to a fresh tube containing the salt required for the next saturation level, until 80% was reached. Pellets were resuspended in 100 µL extraction buffer (10 mM phosphate, 2 mM EDTA, 150 mM NaCl, pH 7.4) for analysis. Precipitation profiles were assessed by 2% agarose gel electrophoresis and SDS-PAGE (Bis-Tris 4–12%; Bio-Rad, USA) to evaluate nucleic acid enrichment and protein co-precipitation across the different saturation levels.

### **3.3.13 Size Exclusion Chromatography (SEC)**

Size exclusion chromatography was performed on an ÄKTA Pure system (Cytiva, Sweden) using a Superose 6 Increase 10/300 GL column (Cytiva, Sweden) operated at 4 °C. The column was equilibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, and run at a flow rate of 0.5 mL/min. Clarified RNA extracts (0.5–1.0 mL) were loaded onto the column, and fractions were collected across the elution profile. RNA content was monitored by UV absorbance at 260 nm and at 280 nm, and selected fractions were analysed by 1% agarose gel electrophoresis to confirm RNA presence and size distribution. SEC allowed separation of RNA from low-molecular-weight contaminants but did not achieve sufficient purity.

### **3.3.14 RNeasy Plant Mini Kit**

Total RNA was extracted from *N. benthamiana* leaves using the RNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's instructions with minor adaptations. Approximately 100 mg of frozen leaf tissue was ground to a fine powder in a mortar and immediately resuspended in RLT buffer supplemented with β-mercaptoethanol (10 µL per 1 mL buffer). The lysate was homogenized by pipetting and centrifuged at 15,000 × g for 5 min at 4 °C to remove debris. The clarified supernatant was transferred to a QIAshredder spin column (Qiagen, Germany) and centrifuged for 2 min at 15,000 × g. The flow-through was mixed with an equal volume of 70% ethanol and loaded onto a RNeasy spin column (Qiagen, Germany). Columns were washed sequentially with RW1 and RPE buffers (Qiagen, Germany), and RNA was eluted in 30–50 µL nuclease-free water. RNA yield and purity  $A_{260}/A_{280}$  were evaluated by spectrophotometry via Nanodrop (DeNovix, USA) and by 1% agarose gel electrophoresis.



### **3.3.15 RNA Quantification and Analytical Assessment**

RNA concentration and purity were determined by spectrophotometry via Nanodrop (Denovix, USA). Absorbance was recorded at 260 nm, with  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  ratios used as purity indicators. Integrity and size distribution were evaluated by agarose gel electrophoresis. Aliquots (typically 200–500 ng) were mixed with loading dye (Invitrogen, USA) and run on 1% agarose in 1×TAE buffer at constant voltage. Gels were stained with a nucleic-acid dye (Invitrogen, USA) and imaged immediately after the run. Crude extracts, flow-throughs, wash and elution fractions from each purification strategy were analysed in parallel with wild-type (non-infiltrated) controls to assess enrichment and matrix carryover.

## **3.4 Methods from Chapter 7**

### **3.4.1 Lysis and Extraction of CCMV**

The CCMV coat protein (CP) sequence was obtained as a synthetic DNA fragment (GenScript, USA) and used for cloning into both microbial and plant expression vectors. To perform the adequate QC prior plant expression, the pGreenII backbone was linearized by PCR using primers with homology arms specific for the CCMV insert. Reactions (50  $\mu$ L) contained 10  $\mu$ L VeriFi buffer (PCR Biosystems, UK), 5  $\mu$ L VeriMax enhancer (PCR Biosystems, UK), 2  $\mu$ L forward primer, 2  $\mu$ L reverse primer (both diluted 1:10), 0.1  $\mu$ L template DNA (pGreenII), 0.5  $\mu$ L VeriFi polymerase (PCR Biosystems, UK), and nuclease-free water to volume. PCR products were resolved on 1% agarose gels, and bands around ~6 kb were excised and purified (QIAquick Gel Extraction Kit, Qiagen, Germany). Gibson Assembly reactions were performed with 10  $\mu$ L Gibson Master Mix (New England Biolabs, USA) combined with purified pGreenII backbone and CCMV insert at molar ratios optimized to achieve a 3:1 insert-to-vector ratio. Mixtures were incubated at 50 °C for 1 h. Gibson products were then transformed into chemically competent *E. coli* DH5 $\alpha$  (New England Biolabs, USA). After heat shock (30 min on ice, 30 s at 42 °C, 5 min on ice), cells were recovered in 950  $\mu$ L SOC medium at 37 °C, 250 rpm for 2 h, and later plated on LB agar containing kanamycin (50  $\mu$ g/mL). Colonies were obtained with an estimated transformation efficiency of  $\sim 9 \times 10^6$  CFU/ $\mu$ g DNA. Colonies were expanded in LB-kanamycin liquid culture, purified by miniprep (GeneJet Miniprep Kit, Thermo Fisher Scientific, UK), and confirmed by restriction digestion with enzyme Bsp68I (NruI) (Thermo Fisher Scientific, UK) and sequencing (Plasmidsaurus, USA).

For bacterial expression, the CCMV CP sequence was also cloned into the pSB1C3 vector (chloramphenicol resistance) by Gibson Assembly following PCR linearization of the plasmid backbone. Transformation into *E. coli* BL21(DE3) (New England Biolabs, USA) cells was performed using the same heat shock procedure. Colonies were screened by miniprep, restriction digestion, and sequencing. Glycerol stocks of confirmed clones were prepared and stored at  $-80^{\circ}\text{C}$ .

#### **3.4.2 Expression of CCMV Coat Protein in *E. coli* BL21(DE3)**

*E. coli* BL21(DE3) cells (New England Biolabs, USA) carrying pSB1C3-CCMV (T7 promoter; chloramphenicol-resistant) were grown in LB supplemented with chloramphenicol (25  $\mu\text{g/mL}$ ). Overnight pre-cultures (10 mL,  $37^{\circ}\text{C}$ , 230 rpm) were used to inoculate 250 mL LB with chloramphenicol (25  $\mu\text{g/mL}$ ) in 1 L baffled flasks. Cultures were grown at  $37^{\circ}\text{C}$ , 230 rpm to  $\text{OD}_{600} \approx 0.4\text{--}0.6$ , then induced with IPTG 0.5 mM and shifted to  $29^{\circ}\text{C}$  for  $\sim 16\text{--}20$  h shaking at 230–250 rpm. A non-transformed BL21(DE3) culture was run in parallel as negative control. Cells were harvested by centrifugation ( $6,000 \times g$ , 10 min,  $25^{\circ}\text{C}$ ), the supernatant discarded, and pellets stored at  $-20^{\circ}\text{C}$  or processed immediately for lysis. For expression checks, aliquots were resuspended in Laemmli buffer (1 $\times$ ), boiled ( $95\text{--}97^{\circ}\text{C}$ , 7–5 min), and analysed by SDS-PAGE (Bis-Tris 4–12%; Thermo Fisher Scientific, USA) to verify the  $\sim 22$  kDa band corresponding to CCMV CP.

#### **3.4.3 Cell Lysis and Clarification**

Cell pellets of *E. coli* BL21(DE3) expressing CCMV coat protein (CP) were lysed by sonication in either a neutral or mildly acidic buffer. The two extraction buffers used were:

- Neutral buffer (pH 7.4): 50 mM Tris-HCl, 150 mM NaCl, 2 mM DTT, 10 mM  $\text{MgCl}_2$ , 0.1% Triton X-100; supplemented with lysozyme and DNase.
- Acidic buffer (pH 5.0): 50 mM sodium acetate, 150 mM NaCl, 2 mM DTT, 10 mM  $\text{MgCl}_2$ , 0.1% Triton X-100; supplemented with lysozyme and DNase.

Pellets were resuspended (typically 30 mL per culture) and sonicated (Soniprep 150; MSE, UK) on ice using 10 s ON - 10 s OFF cycles. An initial 10-cycle program was used; optimization experiments established 20 cycles as preferable, yielding higher soluble CP recovery. Lysates were clarified by centrifugation at 8,500 rpm for 30 min at  $4^{\circ}\text{C}$ . Supernatants (soluble fraction) and pellets were retained for analysis. Under both pH conditions, a band at  $\sim 22$  kDa consistent with CCMV CP was detected in the soluble fraction

by SDS-PAGE, with yield/purity varying by buffer and pH. Clarified lysates were used for downstream optimization trials.

#### **3.4.4 Precipitation with Ammonium Sulphate**

Clarified *E. coli* lysates containing CCMV CP were subjected to salting-out with ammonium sulphate under pH 5.0 and pH 7.4 conditions. Pellets were resuspended in extraction buffer, clarified (8,500 rpm, 30 min, 4 °C), and the supernatant was subdivided into 5 mL aliquots. Solid ammonium sulphate was added to reach 20%, 30%, 40%, 50%, and 60% saturation (approx. 1.0 g; 1.5 g; 2.0 g; 2.5 g; 3.0 g per 5 mL, respectively), mixed to dissolve, incubated briefly, and centrifuged (15,000 rpm, 15 min, 4 °C). Pellets were resuspended in 50 µL PBS for analysis. Identical precipitations (30–50% salt concentration) were run in parallel on negative control lysates (cells without CCMV). Precipitation profiles were assessed by SDS-PAGE. Resuspended pellets were mixed with Laemmli buffer (1×), boiled (~97 °C, 5 min), and loaded on Bis-Tris 4–12% gels (Thermo Fisher Scientific, USA); selected supernatants and starting material were included for comparison. A distinct ~22 kDa band attributable to CCMV CP was enriched in pellets at 20–40% saturation, with reduced recovery at ≥60%. These data established the precipitation threshold used to condition samples for subsequent HIC trials.

#### **3.4.5 Hydrophobic Interaction Chromatography (HIC)**

Hydrophobic interaction chromatography was performed on clarified *E. coli* lysates expressing CCMV CP. Three chromatographic supports were tested: Butyl Sepharose FF (5 mL; Cytiva, Sweden), Benzyl Ultra (1 mL; Thermo Fisher Scientific, USA) and Phenyl Sepharose FF (5 mL; Cytiva, Sweden). All runs were conducted on an ÄKTA GO system (Cytiva, Sweden). HIC columns were equilibrated with 10 column volumes (CV) of high-salt buffer consisting of either 50 mM Tris-HCl, pH 7.5, 1.5 M ammonium sulphate or 50 mM sodium acetate, pH 5.0, 1.5 M ammonium sulphate, depending on the pH condition tested. Lysates were adjusted to the same buffer composition prior to loading. Samples (5–10 mL) were applied at a flow rate of 1 mL/min, corresponding to ≤20% of the column volume per minute. After sample application, columns were washed with 5 CV of equilibration buffer. Initial elution was performed using a linear gradient decreasing the ammonium sulphate concentration in over 20 CV. Fractions were collected and concentrated by precipitation with four volumes of cold acetone at –20 °C, followed by SDS-PAGE analysis to assess retention and elution profiles. The initial screening showed that Butyl Sepharose provided weak

retention, with most protein appearing in the flow-through fractions, while Benzyl Ultra exhibited strong retention with diffuse and poorly resolved elution. Based on these results, Phenyl Sepharose was selected for in-depth optimization. Under high-salt conditions (1.5 M ammonium sulphate), elution was performed using step gradients: 5 CV at 45% B, 5 CV at 75% B, and 5 CV at 100% B, where Buffer B represented the same equilibration buffer without ammonium sulphate. Under this protocol, CCMV CP reproducibly eluted primarily in the 75% step, corresponding to approximately 0.38 M ammonium sulphate.

The optimized method was then transferred and scaled up on a CIMmultus OH monolithic HIC column (1 mL; Sartorius, Germany). Lysates conditioned with 1.5 M ammonium sulphate were loaded at 2 mL/min. The column was equilibrated with 5 CV of binding buffer and elution employed stepwise gradients of 40%, 75%, and 100% elution buffer, each for 5 CV at 5 mL/min. Fractions were collected and maintained on ice until analysis. The elution behaviour of CCMV CP was comparable to that observed on Phenyl Sepharose, but the monolithic resin allowed processing of substantially larger sample volumes, offering a significant advantage for preparative scale.

#### **3.4.6 Affinity Chromatography (IMAC)**

Immobilized metal affinity chromatography (IMAC) was tested to purify His-tagged CCMV coat protein (CP) expressed in *E. coli*. Clarified lysates were applied to a HisTrap HP column (5 mL; Cytiva, Sweden) connected to an ÄKTA GO system (Cytiva, Sweden). The column was equilibrated with 10 column volumes (CV) of binding buffer consisting of 50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 7.5. Lysates were adjusted to the same buffer composition before loading. Samples (5–10 mL) were applied at a flow rate of 5 mL/min. After sample application, the column was washed with 10 CV of binding buffer to remove unbound proteins. Elution was performed stepwise using increasing imidazole concentrations in the same buffer system: 45 mM (5 CV), 100 mM (5 CV), 200 mM (5 CV). Fractions of 1 mL were collected throughout the elution. The column was stripped with 5 CV of 0.5 M imidazole and re-equilibrated with 5 CV of binding buffer before subsequent runs.

Collected fractions were analysed by SDS-PAGE (Bis-Tris 4–12%, Thermo Fisher Scientific, USA) and Western blot (anti-His antibody) to confirm the presence of CCMV CP. The elution of CCMV CP was consistently observed around 200 mM imidazole, confirming effective binding of the His-tagged protein to the resin.

#### **3.4.7 Size Exclusion Chromatography (SEC)**

Size exclusion chromatography was employed to assess the oligomeric state of CCMV coat protein (CP) and to further purify assembled particles. Runs were performed on an ÄKTA GO system (Cytiva, Sweden) using a Superose 6 Increase 10/300 GL column (Cytiva, Sweden) with a bed volume of 24 mL. The column was equilibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, and operated at a flow rate of 0.5 mL/min, corresponding to a linear velocity of approximately 15 cm/h. Lysates or partially purified fractions (0.5–1 mL injection volume) were loaded. Elution was performed isocratically with equilibration buffer, and fractions of 2 mL were collected across the elution profile. Fractions were examined by SDS-PAGE to confirm the presence of CP. SEC allowed separation of monomeric CP from higher-order aggregates and larger particles.

#### **3.4.8 Expression of eGFP mRNA and CCMV CP in *Nicotiana benthamiana***

The pGreenII-049 backbone (AddGene, USA) was linearised by PCR using primers designed with homology arms specific for either the GFP mRNA insert or the CCMV coat protein (CP) coding sequence (GenScript, USA). PCR reactions (50 µL) contained 10 µL VeriFi buffer (PCR Biosystems, UK), 5 µL VeriMax enhancer (PCR Biosystems, UK), 2 µL forward primer (1:10 diluted), 2 µL reverse primer (1:10 diluted), 0.5 µL VeriFi polymerase (PCR Biosystems, UK), and 0.3–1 µL (200 ng) DNA template, with nuclease-free water up to volume. Amplified products were resolved on 1% agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen, Germany). DNA concentrations were determined by Nanodrop spectrophotometry (DeNovix, UK). Gibson Assembly was carried out with 10 µL Gibson Master Mix (New England Biolabs, USA), maintaining vector : insert molar ratios of 1:3. Reactions were incubated at 50 °C for 1 h. Assembly products were transformed into chemically competent *E. coli* DH5α (New England Biolabs, USA) following standard heat-shock (30 min on ice, 30 s at 42 °C, 5 min on ice) and recovered in SOC medium (950 µL, 37 °C, 250 rpm, 2 h). Transformants were plated on LB agar with kanamycin (50 µg/mL) and incubated overnight at 37 °C. Colonies were screened by plasmid miniprep (GeneJet Miniprep Kit, Thermo Fisher Scientific, UK), restriction digestion with Bsp68I (Thermo Fisher Scientific, UK), and Sanger sequencing (Plasmidsaurus, USA). Confirmed clones (pGreenII-mRNA and pGreenII-CCMV) were archived as glycerol stocks at – 80 °C.

#### **3.4.9 Transformation of *Agrobacterium tumefaciens***

Electrocompetent *A. tumefaciens* strain LBA4404 was thawed on ice and mixed with 2–3  $\mu\text{L}$  of plasmid DNA (pGreenII-mRNA or pGreenII-CCMV, 20–40 ng/ $\mu\text{L}$ ). The suspension was transferred into pre-chilled 2 mm electroporation cuvettes and pulsed using a Gene Pulser (Bio-Rad, USA) under optimised conditions (2.5 kV, 25  $\mu\text{F}$ , 400  $\Omega$ ). Immediately after electroporation, sterile SOC medium was added to recover the cells, which were incubated at 28 °C for 4 h with agitation at 200 rpm. Transformants were plated on LB agar containing kanamycin (50  $\mu\text{g/mL}$ ), streptomycin (100  $\mu\text{g/mL}$ ), and rifampicin (25  $\mu\text{g/mL}$ ). Colonies were obtained after 2–3 days and verified by plasmid miniprep (GeneJet Miniprep Kit, Thermo Fisher Scientific, UK) and Sanger sequencing (Plasmidsaurus, USA).

#### **3.4.10 Agroinfiltration of *Nicotiana benthamiana***

Pre-cultures of *A. tumefaciens* harbouring pGreenII-mRNA or pGreenII-CCMV were initiated in 10 mL LB containing kanamycin (50  $\mu\text{g/mL}$ ), streptomycin (100  $\mu\text{g/mL}$ ), and rifampicin (25  $\mu\text{g/mL}$ ) and incubated overnight at 28 °C with shaking at 230 rpm. Pre-cultures were used to inoculate 200 mL LB with the same antibiotic composition in 2 L baffled flasks. Cultures were grown at 28 °C, 230 rpm to  $\text{OD}_{600} \sim 5\text{--}7$ , harvested by centrifugation (6,000  $\times g$ , 15 min, 25 °C), and resuspended in infiltration buffer (10 mM MES, 10 mM  $\text{MgCl}_2$ , pH 5.7). Bacterial suspensions were adjusted to  $\text{OD}_{600}$  0.2 and mixed 1:1 (v/v) to combine mRNA and CCMV constructs. For vacuum infiltration, 6 weeks old *N. benthamiana* plants were inverted into the infiltration solution inside the desiccator (Cole-Parmer, UK). A vacuum of -600 mbar was applied for 4 min, followed by rapid restoration to atmospheric pressure. Plants were returned to greenhouse conditions (25 °C, 16 h light/8 h dark) and leaves were harvested at 5 days post infiltration (dpi), then stored at – 80 °C.

#### **3.4.11 Extraction of eGFP mRNA from Infiltrated Leaves**

50–100 mg of frozen infiltrated leaves were ground in cold mortar and processed using the RNeasy Plant Mini Kit (Qiagen, Germany) according to manufacturer instructions. Samples were eluted in 40  $\mu\text{L}$  nuclease-free water and RNA concentration assessed by Nanodrop spectrophotometry (DeNovix, UK). RNA integrity was evaluated by electrophoresis on 2% agarose gels against an in vitro transcribed (IVT) eGFP positive control.

#### **3.4.12 Extraction of CCMV CP from Infiltrated Leaves**

20 g of frozen agroinfiltrated leaves were homogenised in a chilled blender (Nutribullet, USA) with extraction buffer at a ratio of 1:2 (w/v). Two buffers were used to reflect the pH-dependent properties of CCMV CP:

- Neutral buffer (pH 7.4): 50 mM Tris-HCl, 150 mM NaCl, 2 mM DTT, 10 mM MgCl<sub>2</sub>, 1% Triton X-100.
- Acidic buffer (pH 5.0): 50 mM sodium acetate, 150 mM NaCl, 2 mM DTT, 10 mM MgCl<sub>2</sub>, 1% Triton X-100.

Homogenates were clarified by double centrifugation at 15,000 × g for 30 min at 4 °C. The clarified solution was filtered through 0.65 µm membranes (Millipore, Germany). Clarified extracts were analysed by SDS-PAGE (Bis-Tris 4–12%; Thermo Fisher Scientific, USA) alongside wild-type controls to verify expression of CCMV CP.

## 4. UPS – Upstream Process Optimization

### 4.1 *Introduction to Upstream Processing in Plant Molecular Farming*

Plant Molecular Farming represents a versatile and increasingly sophisticated platform for the production of high-value recombinant proteins in whole plant systems. Compared with conventional microbial and mammalian cell-based expression systems, PMF offers several intrinsic advantages, including reduced risk of contamination with human pathogens, cost-effective scalability, and the ability to perform complex post-translational modifications critical for the functionality of many biopharmaceuticals (133, 134).

Upstream processing in PMF encompasses the entire sequence of activities from host plant selection to plant cultivation, transgene delivery, and the conditions for optimal gene expression, preceding product extraction and purification. Among these, the cultivation environment plays a critical role, as it directly influences plant growth, health, and recombinant protein yield (135). Controlled Environment Agriculture (CEA), particularly when implemented in greenhouse systems, allows precise regulation of parameters such as light spectrum and intensity, photoperiod, humidity, temperature, CO<sub>2</sub> concentration, and nutrient availability (136). Tight control over these factors is essential to ensure uniform biomass accumulation, consistent morphology, and reproducible levels of transgene expression – all features that are indispensable for translation of PMF into industrial applications. Greenhouse-base systems also provide a protective barrier against biotic threats (e.g., insect pests, microbial pathogens) and abiotic stressors (e.g., fluctuating temperature, drought, drastic climatic conditions) (137). This containment reduces experimental variability and facilitates process reproducibility, both of which are fundamental in the context of the Good Manufacturing Practice (GMP) requirements for a PMF platform. Within greenhouse facilities, hydroponic cultivation is often preferred due to its precision in delivering water and nutrients, as well as its compatibility with automation (138). Among hydroponic substrates, rockwool has become particularly relevant in PMF as it combines high water retention with efficient aeration, thereby promoting vigorous root development. Being inert and sterile, rockwool also minimizes risks of microbial contamination while allowing nutrient delivery (139). Different hydroponic system designs have been successfully adapted to PMF. Nutrient Film Technique (NFT) provides a continuous, shallow stream of nutrient-rich water flowing over the roots, ensuring efficient uptake and oxygenation (140). Alternatively, Deep Water Culture (DWC) systems immerse roots in aerated nutrient solutions, supporting rapid vegetative growth (141). Floating root systems, in which plants are placed on buoyant rafts above nutrient reservoirs, have proven attractive for



large-scale applications due to their simplicity, scalability, and cost-effectiveness. More recently, vertical farming has emerged as a complementary strategy for PMF (142). By integrating multiple cultivation layers within enclosed and fully climate-controlled units, vertical farming maximizes spatial efficiency while enabling uninterrupted year-round production (143). Advanced LED lighting systems within these facilities allow fine-tuning of light quality, intensity, and photoperiod, which are known to directly influence photosynthetic efficiency, metabolite accumulation, and ultimately recombinant protein expression levels. The environmental homogeneity provided by such systems reduces physiological variability across plants and enhances reproducibility in downstream processing. The conditioning of plants prior to transgene delivery is also a critical, though often underappreciated, component of upstream processing. Parameters such as nutrient formulation, temperature acclimation, and humidity at the time of infiltration can significantly affect plant receptiveness and transgene expression (37). Pre-infiltration adjustments – including management of oxygen levels and pH – have been reported to enhance *Agrobacterium* uptake while reducing plant stress responses (144). Overall, the design and optimization of cultivation technologies ranging from greenhouse-based hydroponics to vertical and aeroponic farming have a direct impact on yield, product consistency, and economic viability. As PMF technologies continue to mature, the integration of real-time sensor networks, automated climate control, and precision agriculture tools is expected to transform upstream cultivation from an empirical practice into a standardized and scalable process science (145). This transition will be essential for positioning PMF as a competitive manufacturing platform in the biopharmaceutical industry.

#### **4.2 Overview of the Host Plant: *Nicotiana benthamiana***

The choice of host plant is a central decision in upstream processing, as it determines not only the expression efficiency but also the scalability, the product quality, and the regulatory acceptability of the PMF platform. Among the species explored, *Nicotiana benthamiana* has become the model of choice for transient expression systems due to a unique combination of biological and agronomic traits (146). *Nicotiana benthamiana* has established itself as the host of choice for transient expression systems in PMF. A member of the Solanaceae family, this species exhibits several unique traits that make it particularly amenable to genetic manipulation and recombinant protein production (147). Originally discovered in Australia, *N. benthamiana* has become a global research tool due to its natural susceptibility to a wide range of viruses and its compatibility with *Agrobacterium*-mediated transformation, both of which have accelerated its adoption in plant biotechnology (148).



**Figure 5.** *Nicotiana benthamiana*, the reference host for transient expression in PMF, widely adopted due to its short growth cycle, high biomass, and compatibility with *Agrobacterium*-mediated transformation. Drawn from living plants; image adapted from Chase et al. (2022) (253)

One of the most valuable characteristics of *N. benthamiana* is its weak RNA interference (RNAi) system (149), which allows unusually high levels of transgene expression by preventing rapid post-transcriptional silencing. This feature, combined with its relatively simple genome, broad leaf surface area and high biomass yield, makes this plant particularly well suited for transient expression platforms. Its short growth cycle of only five to six weeks and its status as a non-food, non-feed species further increase its attractiveness for pharmaceutical applications, both from a practical and regulatory standpoint (150). Agroinfiltration with *Agrobacterium tumefaciens* carrying expression constructs has become the standard method to exploit these traits. By introducing bacteria into the intercellular space of the leaves, high levels of recombinant protein can be achieved within a few days, providing a speed advantage that is unmatched by other plant or cell-based systems. This rapid expression capability is crucial not only for construct screening but also for urgent biomanufacturing needs, such as vaccine production during emergency outbreaks (65). Although *N. benthamiana* has become the reference host for transient expression, other plant species have been explored. *Nicotiana tabacum* produces high biomass and has a long history in molecular farming but is less amenable to transient expression due to stronger RNA silencing (151). Cereals such as *Oryza sativa* and *Zea mays* offer seed-based storage and edible vaccine possibilities, but they are limited by longer generation times, reduced infiltration efficiency, and complex regulatory pathways (152). Legumes such as *Glycine max* provide protein-rich seeds that may stabilize

recombinant proteins, yet they lack optimized agroinfiltration protocols and infrastructure (153). The broad adoption of *N. benthamiana* by both academia and industry reflects its superior compatibility with transient transformation, rapid growth, and regulatory suitability compared with these alternatives. Nevertheless, *N. benthamiana* presents several biological and technical limitations. The plant expresses more than 1,200 active proteases, many of which are upregulated during infiltration stress and that can significantly degrade recombinant proteins leading to yield loss (154). Various mitigation strategies have therefore been explored, ranging from co-expression of protease inhibitors and the development of low-protease genome-edited lines to directing recombinant proteins to subcellular compartments less exposed to proteolysis. In addition, agroinfiltration often triggers post-infiltration necrosis and the accumulation of reactive oxygen species (ROS), both of which compromise protein stability (155). Supplementation with antioxidants such as lipoic acid, acetosyringone, or ascorbate has shown to alleviate some of these effects (156), and advanced lines with reduced ROS responses are currently under development. These challenges have also spurred innovation in molecular tools. Techniques such as CRISPR/Cas9 genome editing and Virus-Induced Gene Silencing (VIGS) are increasingly applied in *N. benthamiana* to study host–pathogen interactions, reconfigure glycosylation pathways, and boost recombinant protein yield (67). The widespread reliance on *N. benthamiana* by commercial PMF platforms, including Medicago and Kentucky Bioprocessing, underscores its pivotal role in bridging laboratory discovery with industrial-scale production (41, 157).

In conclusion, *N. benthamiana* represents a highly versatile and experimentally tractable host that has become a cornerstone of upstream plant-based biomanufacturing. Its unparalleled suitability for transient expression, combined with continuous improvements through molecular breeding and cultivation practices, ensures its central role in the future development of PMF pipelines.

**Table 7.** Comparative overview of plant species used in molecular farming, highlighting their advantages, limitations, and suitability for pharmaceutical applications.

| Host plant                   | Advantages                                                                                                                                                                                                                | Limitations                                                                                                                                                                          |
|------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Nicotiana benthamiana</i> | <ul style="list-style-type: none"> <li>- Weak RNA silencing</li> <li>- Highly amenable to agroinfiltration</li> <li>- Rapid growth (5–6 weeks)</li> <li>- Large leaf surface area</li> <li>- Non-food/non-feed</li> </ul> | <ul style="list-style-type: none"> <li>• High protease activity</li> <li>• Prone to necrosis post-infiltration</li> </ul>                                                            |
| <i>Nicotiana tabacum</i>     | <ul style="list-style-type: none"> <li>- High biomass yield</li> <li>- Extensive agronomic knowledge</li> <li>- History in molecular farming</li> </ul>                                                                   | <ul style="list-style-type: none"> <li>• Stronger RNA silencing that reduces transient expression efficiency</li> <li>• Slower response compared to <i>N. benthamiana</i></li> </ul> |
| <i>Oryza sativa (rice)</i>   | <ul style="list-style-type: none"> <li>- Edible seeds for oral delivery</li> <li>- Stable transgenic expression</li> <li>- Long-term storage stability</li> </ul>                                                         | <ul style="list-style-type: none"> <li>• Long generation time</li> <li>• Limited transient expression</li> <li>• Complex regulatory pathway for edible vaccines</li> </ul>           |
| <i>Zea mays (maize)</i>      | <ul style="list-style-type: none"> <li>- Potential for large-scale cultivation</li> <li>- Seed-based protein accumulation</li> </ul>                                                                                      | <ul style="list-style-type: none"> <li>• Long generation cycle</li> <li>• Limited transient expression</li> <li>• Challenging downstream recovery</li> </ul>                         |
| <i>Glycine max (soybean)</i> | <ul style="list-style-type: none"> <li>- Protein-rich seeds that may stabilize recombinant proteins</li> <li>- Potential for oral delivery</li> </ul>                                                                     | <ul style="list-style-type: none"> <li>• Long generation cycle</li> <li>• Limited infiltration protocols</li> <li>• Less developed infrastructure</li> </ul>                         |

*N. benthamiana* thus provides the biological foundation for the upstream strategies explored in this work. However, the effectiveness of plant molecular farming does not depend solely on the host species, but also on the way plants are cultivated and the method by which the transgene is delivered. The upstream process for plant-based recombinant protein production can therefore be broadly divided into two core components: the cultivation system and the agroinfiltration strategy. In this chapter, these elements are examined and compared through two complementary approaches: a laboratory-scale model developed inside the project developed at UCL, designed to provide experimental flexibility and proof-of-concept validation, and an industrial-scale platform implemented inside the pharmaceutical company Special Product's Line (SPL), aimed at process robustness and regulatory alignment. The description of these two models will be followed by an analysis of key optimization parameters and a critical assessment of both platforms, highlighting the opportunities and limitations of scaling PMF upstream processes from research to manufacturing environments.

### 4.3 Laboratory-Scale Upstream Processing: UCL Prototype System

The global demand for recombinant biopharmaceuticals continues to rise, often outpacing the production capacity of conventional microbial and mammalian systems (158). This trend has reinforced the need for alternative expression platforms that combine scalability with cost-effectiveness. As previously stated, PMF platforms have emerged as a promising approach, and cultivation in controlled greenhouse environments represents a safe and manageable option. Such systems reduce the risk of cross-contamination with other crops, minimize exposure to outdoor contaminants, and do not require sterile conditions since the host plants are not susceptible to human pathogens (147). To address the needs at a research level, a hydroponic greenhouse for laboratory-scale experiments was designed and assembled within the Department of Biochemical Engineering at UCL. The system was designed to support early-stage investigations of novel plant-based biopharmaceuticals, with an emphasis on sustainability, ease of operation, and accessibility to non-specialized personnel. Although constructed with modest resources, the unit incorporates features consistent with pharmaceutical-grade cultivation. The overall goal was to provide a lab-scale hydroponic system that could reproduce, in simplified form, the principles of industrial-scale cultivation while remaining practical for academic research. The greenhouse was dimensioned to accommodate the growth of up to fifteen *Nicotiana benthamiana* plants.

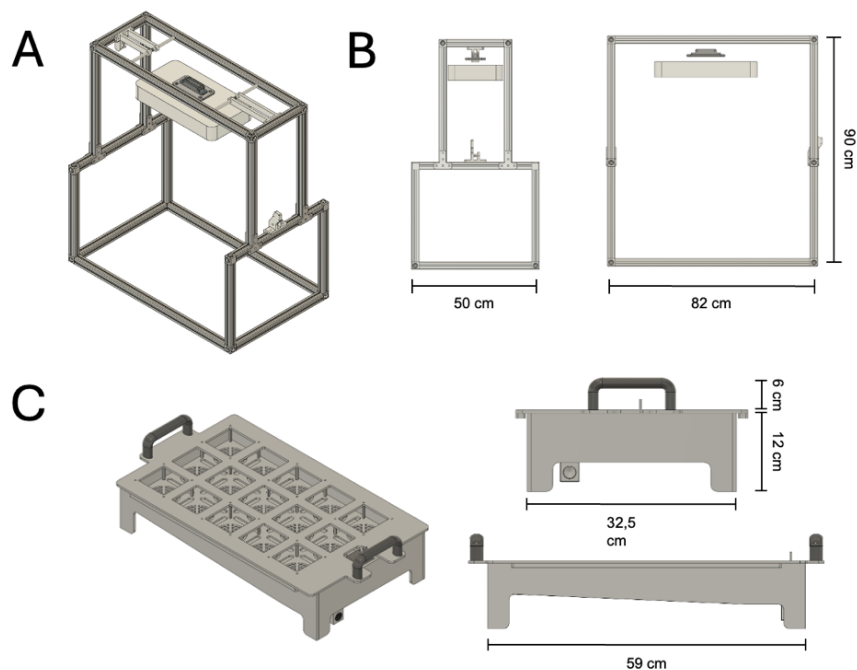
Soil-based systems represent the traditional approach, with plants deriving nutrients and minerals directly from the soil. In contrast, soilless cultivation – or hydroponics – delivers mineral nutrients directly to the roots in aqueous solution, enabling precise control of plant nutrition and growth dynamics. By providing essential nutrients in ionic form, hydroponics eliminates the variability associated with soil composition and allows tailored adjustment of nutrient supply throughout the plant growth cycle. Critical parameters for hydroponic cultivation include composition of the nutrient solution, pH, and electrical conductivity (EC). The pH strongly influences nutrient solubility and availability, with optimal values for *N. benthamiana* growth ranging from 5.8 to 6.5 (37). Electrical conductivity serves as an indicator of the overall ionic strength of the nutrient solution and therefore of nutrient availability. Excessively high EC values can impair nutrient uptake due to osmotic stress, whereas too low values may result in nutrient deficiency (159). Regular monitoring and adjustment – typically maintaining EC between 1.5 and 2.5 dS/m – are therefore essential to sustaining optimal plant development and ensuring reproducibility in recombinant protein expression. An important feature was the use of rockwool as the cultivation substrate. Rockwool offers high water retention and excellent

root aeration, which together support vigorous plant growth. Being inert and sterile, it minimizes the risk of microbial contamination and allows precise delivery of nutrients through the hydroponic solution (160). Its widespread use in commercial greenhouse cultivation also increases the comparability of lab-scale findings with industrial practice, making it a strategic choice for a system designed to emulate large-scale platforms. In addition to nutrient management and substrate selection, environmental parameters within the greenhouse were also considered critical variables for upstream bioprocessing. Light spectrum and intensity, temperature, humidity, and CO<sub>2</sub> concentration directly affect photosynthesis, biomass accumulation, and ultimately recombinant protein yield (161). Although the lab-scale system did not replicate the full automation of industrial controlled-environment agriculture, these factors were monitored and adjusted within practical ranges to sustain consistent plant growth. Such regulation was particularly important for ensuring that agroinfiltration and downstream experiments were conducted on plants with comparable physiological status. Overall, the UCL hydroponic greenhouse provided a cost-effective and flexible platform to explore upstream variables in PMF. Its design – combining pharmaceutical-grade control with academic accessibility – enabled reproducible cultivation of *N. benthamiana*. The controlled environment established in this system formed the foundation for the transient expression experiments described in Chapter 6 and Chapter 7.

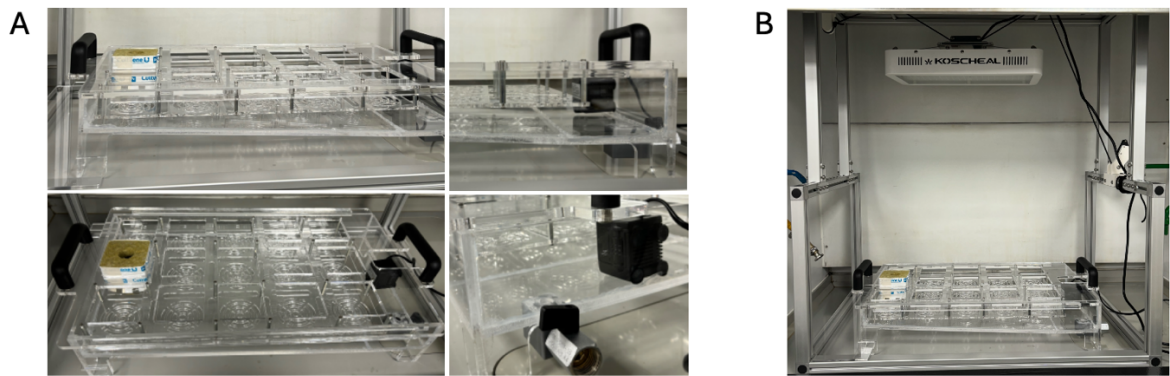
#### 4.3.1 Lab-Scale Cultivation Conditions

The lab-scale cultivation platform developed was designed to integrate essential hydroponic advantages (e.g. tight control of nutrition, reduced contamination risk, and resource efficiency) within a small-scale pharmaceutical-grade setting. Commercial hydroponic platforms such as the Radix system from Sananbio demonstrate the potential for automation and modularity. However, they are designed for large-scale production, demanding substantial investment and space, while smaller market solutions are often oriented toward hobby cultivation and lack adequate environmental control. This gap underscores the need for laboratory-scale systems that are affordable, reproducible, and compatible with GMP principles. In response, this custom hydroponic greenhouse was developed to support early-stage studies on plant-based biopharmaceuticals (Figure 6, Figure 7). The unit accommodates up to fifteen *Nicotiana benthamiana* plants and relies on a circulating nutrient solution enriched with oxygen to sustain optimal growth and root health. Illumination is provided by LED panels programmed to a photoperiod optimized for *N. benthamiana* growth (16 hours of light, 8 hours of dark), while a

submersible pump ensures continuous recirculation and aeration of the solution (162). The system is housed within a filter hood, which enables constant air renewal and provides an additional safeguard against external contaminants. Compact and modular, this design requires only a temperature- and humidity-controlled room, making it adaptable to small laboratory spaces. By combining low-cost construction with essential process-control features, the system mirrors key principles of industrial cultivation while remaining tailored to exploratory upstream research.



**Figure 6.** 3D model of the laboratory-scale greenhouse facility designed for Plant Molecular Farming. (A) Aluminium frame supporting the LED lighting unit; (B) side vision of the aluminium frame supporting the lighting unit; (C) cultivation tray to accommodate the hydroponic substrate and the nutrient solution.

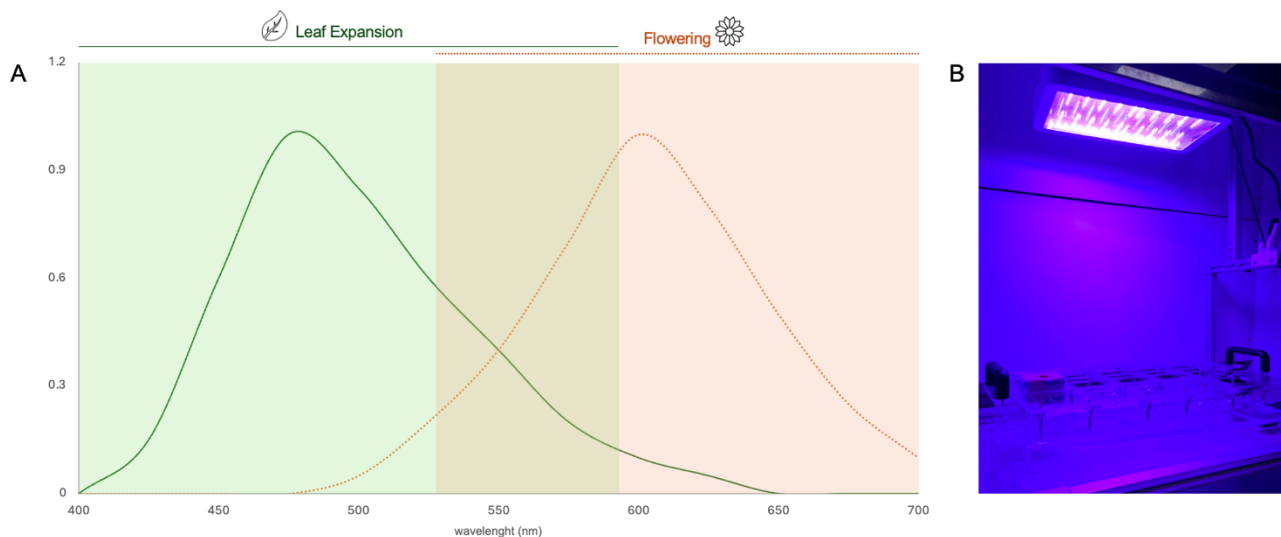


**Figure 7.** Laboratory-scale greenhouse system assembled, showing the hydroponic cultivation unit (A) and the whole infrastructure (B).

The hydroponic greenhouse assembled consisted of a modular cultivation unit equipped with a tray for the nutrient solution and a supporting grid to hold the rockwool cubes used as substrate. Rockwool was selected as the preferred medium because of its high porosity, which ensures effective water and oxygen retention while supporting root development (139). Prior to use, the substrate was conditioned by soaking overnight in an acidic solution at pH ~3.0 to neutralize its intrinsic alkalinity, followed by rinsing with water and drying. The tray was designed with a sloping bottom to facilitate emptying and cleaning, while a submersible pump placed in the nutrient tank ensured continuous recirculation of the solution. For ease of handling, the supporting grid was fitted with side handles.

An external support frame sustained the LED lighting unit, positioned above the cultivation tray. The lamp height was adjustable to maintain a constant distance from the plants during growth. Light quality and intensity are recognized as critical determinants of plant development and recombinant protein production in *N. benthamiana*, as they directly influence photosynthesis, photomorphogenesis, and secondary metabolism. Among available technologies, light-emitting diodes (LEDs) have become the standard in controlled-environment agriculture, offering precise spectral control with high energy efficiency, long operational life, and minimal heat generation (163). By contrast, fluorescent lamps represent a lower-cost option but are associated with shorter lifespan and higher energy consumption, making them less suitable for continuous operation in PMF. Plant growth is most effective within the Photosynthetically Active Radiation (PAR) range of 400–700 nm, with peak chlorophyll absorption in the blue (~450 nm) and red (~660 nm) regions. Blue light (400–500 nm), mediated by photoreceptors such as cryptochromes and phototropins, promotes leaf expansion, chloroplast development, and stomatal regulation, thereby enhancing vegetative growth. Red light (~660 nm), perceived through phytochrome activation, supports photosynthetic efficiency, biomass accumulation, and flowering induction (164-166). In the context of PMF, lighting conditions can influence metabolite accumulation, chlorophyll content, and oxidative stress responses, all of which ultimately impact recombinant protein yield and stability (167). Based on these principles, the LED setup for the greenhouse developed was optimized to prioritize emissions at ~450 nm. A photosynthetic photon flux density (PPFD) of 100–150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  under an 16 h light / 8 h dark photoperiod was adopted for vegetative growth (Figure 8). The lamp was positioned at a fixed distance of 25 cm from the plant canopy to ensure uniform light distribution.





**Figure 8.** Light conditions in the Lab-scale greenhouse. (A) Light spectral distribution that shows the ideal PAR range of the LED system for the leaves expansion; (B) lab-greenhouse lighting setup.

The cultivation module was designed with optimized dimensions to ensure uniform light distribution and homogeneous plant development. Based on the tray measurements, a custom grid was engineered to accommodate fifteen *Nicotiana benthamiana* plants, with spacing of 3 cm between plants to allow sufficient room for leaf expansion and canopy formation. Each grid cell was fitted with a groove to securely hold the rockwool cubes, ensuring stable contact with the nutrient solution. The grid was also dimensioned to leave adequate space in the tank below, allowing roots to extend freely and to be properly oxygenated during growth. An additional compartment was incorporated into the tank, positioned outside the main light beam. This section housed the submersible pump and the electrodes for continuous monitoring of pH and conductivity and provided direct access for the addition of nutrients during plant growth. This layout facilitated system maintenance while minimizing disruption of the plants. The small aquarium air pump maintained oxygenation and fluid movement within the hydroponic medium, also ensuring adequate root aeration. Once assembled, the cultivation unit was positioned inside a filter hood, providing both physical protection and controlled airflow. To prevent interference from external light sources, the hood was shielded with a dark panel that blocked ambient light without reflecting the internal LED illumination. This precaution served two purposes: protecting the plants from light overexposure and protecting operators from the high-intensity LED light. From germination onwards, plant substrates were kept in contact with a nutrient solution formulated by mixing three commercial components, obtained from Growth Technology. The pH of the solution was maintained at 5.7 through continuous monitoring with an in-tank probe, while electrical conductivity was regularly tracked as an indirect measure of ionic nutrient uptake. The nutrient solution was replaced every 7–10 days to prevent nutrient

imbalance and microbial growth. Daily supplementation with an oxygen solution was performed to avoid algae formation and maintain solution clarity. The Table 8 below shows the quantity calculated and optimized for the 3 components of the nutrient solution. The nutrient solution composition and concentration were calculated and optimized starting from the specifics given by the provider.

**Table 8.** Nutrient solution composition across six cultivation weeks in the lab-scale cultivation system.

| Growth conditions          | 1 <sup>st</sup> week | 2 <sup>nd</sup> week | 3 <sup>rd</sup> week | 4 <sup>th</sup> week | 5 <sup>th</sup> week | 6 <sup>th</sup> week* |
|----------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|-----------------------|
| <i>Ionic solution</i>      | 3 mL/lt              | 4 mL/lt              | 5 mL/lt              | 9 mL/lt              | 13 mL/lt             | 13 mL/lt              |
| <i>Silicon solution</i>    | 0.1 mL/lt            | 0.2 mL/lt            | 0.4 mL/lt            | 0.5 mL/lt            | 0.7 mL/lt            | 0.7 mL/lt             |
| <i>Oxygen solution</i>     | 0.2 mL/lt            | 0.2 mL/lt            | 0.2 mL/lt            | 0.2 mL/lt            | 0.2 mL/lt            | 0.2 mL/lt             |
| <i>Conductivity (dS/m)</i> | 0.8                  | 1.2                  | 1.5                  | 2.5                  | 3.6                  | 3.6                   |
| <i>pH</i>                  | 5.7 ± 0.2            | 5.7 ± 0.2            | 5.7 ± 0.2            | 5.7 ± 0.2            | 5.7 ± 0.2            | 5.7 ± 0.2             |

\*The 6<sup>th</sup> week represents the week after the agroinfection, where the solution doesn't need to be changed because the plants don't have to go further with the growing cycle.

To evaluate the practicality of this hydroponic greenhouse system, a cost analysis of the consumables was performed. Calculations were based on a weekly nutrient solution replacement strategy, reflecting the standard operating procedure adopted in this study. For a complete growth cycle, the total consumption was estimated at 315.6 mL of Ionic Solution, 17.4 mL of Silicon Solution, and 8 mL of Oxygen Solution. Solid supports included 30 rockwool plugs for seed germination and 15 rockwool cubes for plant growth. The resulting cost per batch, corresponding to one full cultivation cycle of 15 *Nicotiana benthamiana* plants, was approximately £20.9, as shown in Table 9. This low cost per run highlights the economic accessibility of the system for routine laboratory use.

**Table 9.** Operational costs per cultivation cycle in the laboratory-scale cultivation system. Breakdown of consumables expenses.

| Consumable                         | Quantity | Unit Cost   | Subtotal      |
|------------------------------------|----------|-------------|---------------|
| <i>Ionic solution (Hydro Grow)</i> | 315.6 mL | 13.9 £/lt   | 4.38 £        |
| <i>Silicon solution</i>            | 17.4 mL  | 12.7 £/lt   | 0.22 £        |
| <i>Oxygen solution</i>             | 8 mL     | 12 £/lt     | 0.1 £         |
| <i>Rockwool plugs</i>              | 30 units | 0.50 £/unit | 15 £          |
| <i>Rockwool cubes</i>              | 15 units | 0.08 £/unit | 1.2 £         |
| <b>Total Cost</b>                  |          |             | <b>20.9 £</b> |

At the end of five weeks, when plants reached the optimal stage for downstream processing, rockwool cubes were removed and leaves harvested for extraction and purification of the target product. Residual nutrient solution was discharged via the siphon located at the base of the tray, which allowed safe emptying into a sink. The tray and grid were subsequently cleaned with detergent and bleach, ensuring adequate hygienic conditions and rapid preparation for the next cultivation cycle.

The modular platform designed thus supports a variety of applications:

- Laboratory-scale studies on transgenic plant growth for biopharmaceutical production.
- Optimization of *N. benthamiana* growth parameters in a controlled system.
- Refinement of agroinfiltration protocols and subsequent expression studies.

Furthermore, the system can be adapted to other plant species by modifying the grid dimensions and spacing, underscoring its flexibility as a tool for upstream PMF research.

In summary, the hydroponic greenhouse developed at UCL provided a reproducible and accessible platform for the controlled cultivation of *N. benthamiana*. Its main advantages lie in the low cost of consumables, modularity, and the ability to precisely regulate parameters such as light spectrum, photoperiod, nutrient delivery, and aeration. These features make it a powerful tool for exploratory and proof-of-concept studies, while at the same time reflecting key principles of larger-scale industrial platforms. Nevertheless, the system also presents inherent limitations, including its restricted throughput and reliance on manual monitoring compared with fully automated industrial setups. For these reasons, experimental validation of the platform was considered essential, not only to confirm its capacity to sustain healthy and homogeneous plant growth but also to demonstrate its suitability as a model for upstream process optimization. The following section therefore focuses on the validation of the system,

assessing its performance as a reliable foundation for downstream applications in plant molecular farming.

#### *4.3.2 Optimization and Validation of the Lab-Scale Greenhouse*

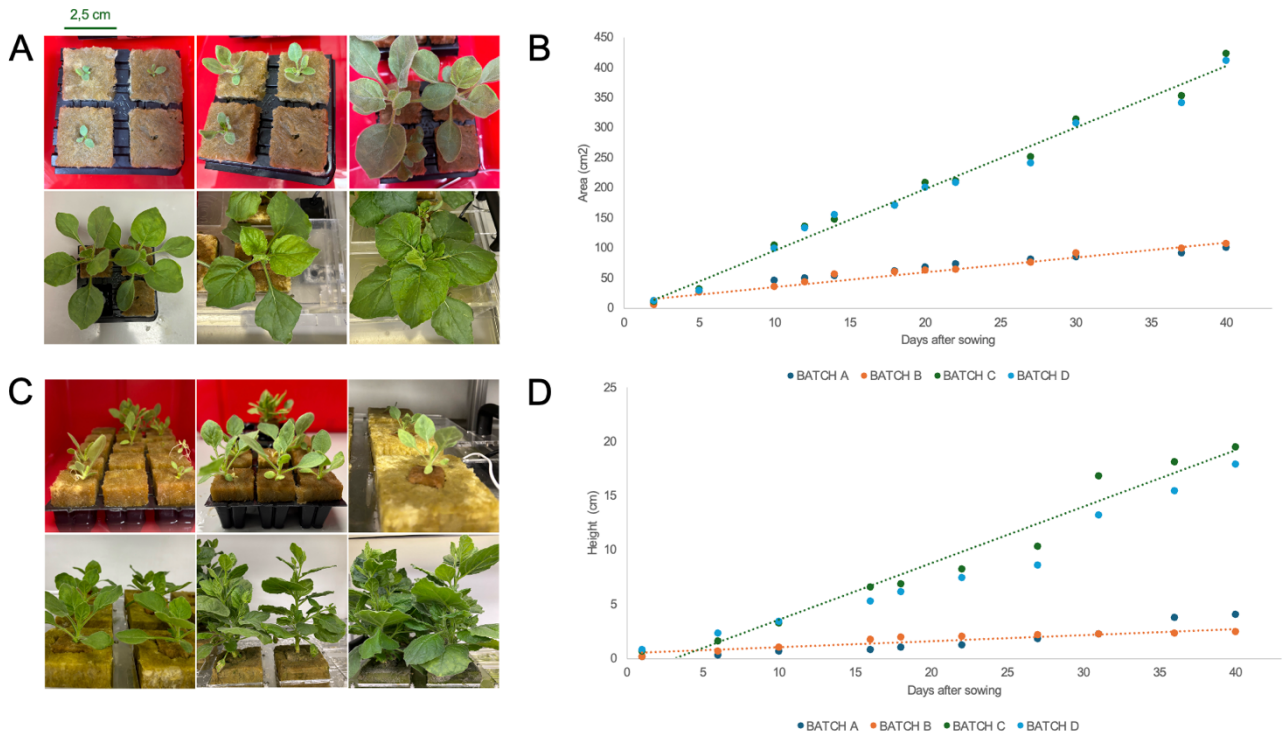
To validate the efficiency of the hydroponic greenhouse, plant performance and environmental stability were monitored across four independent cultivation batches. A set of six criteria was defined to assess the reproducibility and suitability of the system for plant molecular farming:

- I. Seed germination: time from sowing to cotyledon appearance.
- II. Cotyledon emergence: percentage of seeds germinated.
- III. Vegetative growth 1: measurement of leaf area, monitored every 2-3 days using digital imaging (ImageJ).
- IV. Vegetative growth 2: measurement of plant height, monitored every 2-3 days using manual measurements.
- V. Light distribution: quantification of light intensity at the plant canopy level using a LuxMeter.
- VI. Environmental stability: recording of temperature and relative humidity.

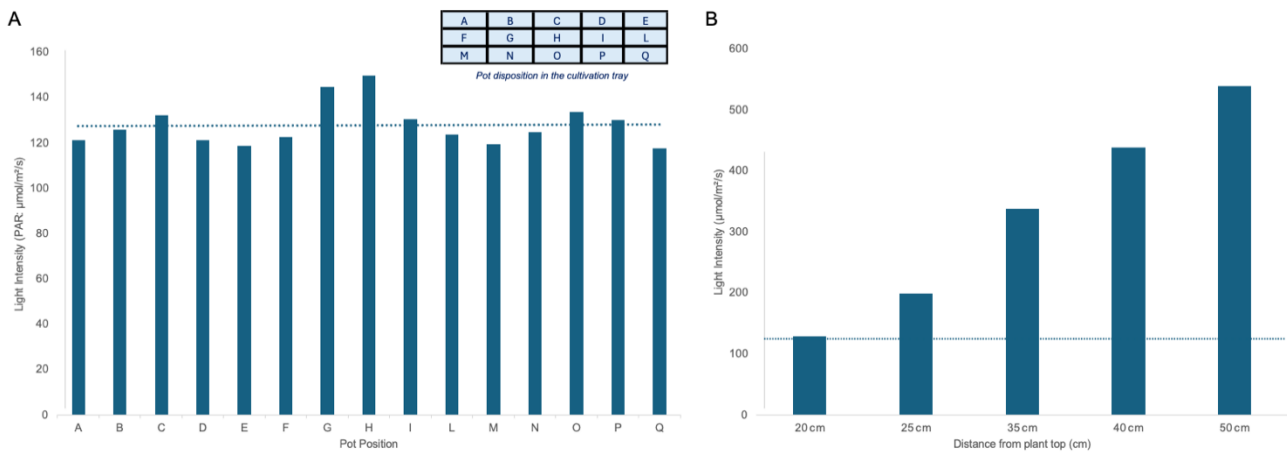
In addition to the data collected from these measurements, visual health parameters were continuously checked, through the observation of leaf coloration, root aeration and time to flowering. Below are the results obtained in the validation process.

**Table 10.** Initial validation parameters of the greenhouse system: (I) time from sowing to cotyledon appearance; (II) percentage of seeds germinated.

| Batch | Days to Germination | Seeds Germinated (%) |
|-------|---------------------|----------------------|
| A     | 5                   | 61,5                 |
| B     | 6                   | 60                   |
| C     | 5                   | 62,1                 |
| D     | 5                   | 62                   |



**Figure 9.** Vegetative growth validation of the greenhouse system (measures III and IV). (A) Top-view photographs of plants showing leaf area expansion; (B) quantification of leaf area increase over time; (C) side-view photographs showing progressive increase in plant height; (D) quantification of plant height increase over time.



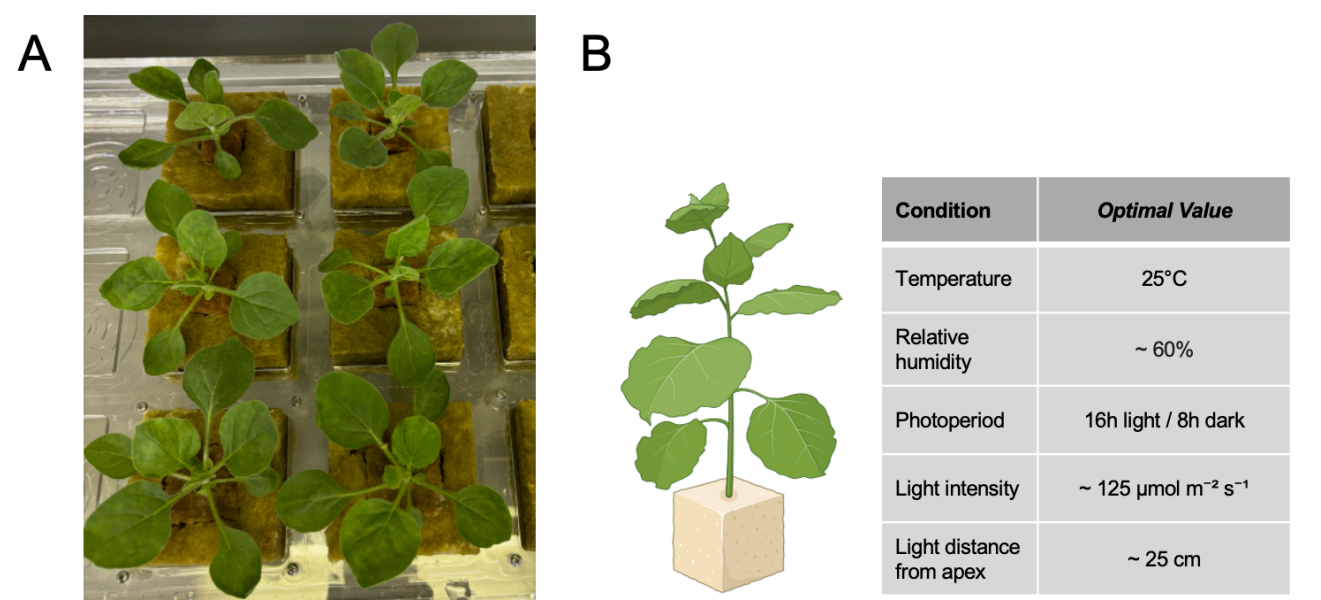
**Figure 10.** Light distribution in the greenhouse system measured using a LuxMeter (measure V). (A) Light intensity across different pot positions; (B) light intensity at varying distances from the plant apex.



**Figure 11.** Environmental stability monitoring in the greenhouse system (measure VI). (A) Temperature profile recorded during cultivation; (B) relative humidity profile recorded during cultivation. Both measures were recorded with and without the presence of an humidifier.

Germination efficiency proved consistent across batches (Table 10), with cotyledons emerging within five/six days under controlled conditions of 24–26 °C and ~60% relative humidity. These environmental parameters ensure the breakdown of the protective outer layer and promote the development of cotyledons under stable and uniform conditions (168). The germination pattern confirmed that the system was capable of supporting early developmental stages reliably, with only minor variability. Within one week, root systems extended beyond the rockwool plugs, indicating readiness for transfer to larger cubes in the cultivation tray. Vegetative growth, assessed through height and leaf area measurements, revealed differences between the first two batches (A and B) and subsequent runs (C and D) (Figure 9). In the initial cycles, fluctuations in temperature and humidity led to irregular growth, despite otherwise controlled conditions. Investigation revealed that the continuous airflow provided by the hood, while effective in reducing contamination risk, caused localized cooling and drying of the environment. To address this, a humidifier was integrated into the system. Following this modification, environmental parameters stabilized, and plant performance improved markedly, with typical values of 20–25 cm in height and ~400 cm<sup>2</sup> of total leaf area achieved at maturity. Light measurements confirmed that the LED lamp provided adequate intensity (PAR ~125  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at the optimal canopy distance of 25 cm (Figure 10). Distribution across the cultivation grid was generally uniform, though plants in central positions received marginally higher irradiance, a known feature of point-source LED setups. Temperature and humidity were continuously monitored with probes placed both inside the nutrient tank and in

the external structure of the greenhouse. Optimal values for *N. benthamiana* (25 °C, 60% RH) (169) were consistently achieved only after the integration of the humidifier, which eliminated irregular fluctuations observed in the earlier batches (Figure 11). From that point onwards, environmental control was stable and reproducible.



**Figure 12.** Validation of the laboratory-scale greenhouse system. (A) Photograph of *Nicotiana benthamiana* plants cultivated under optimized and controlled conditions; (B) schematic representation of the optimal growth parameters identified for *N. benthamiana*.

Together, these results demonstrate that the UCL hydroponic greenhouse system provides a validated environment for consistent plant cultivation. Key parameters of growth and physiology were monitored, adjusted, and optimized, ensuring robust performance across multiple runs. In addition to reproducibility, the system confirmed its strengths in terms of cost-effectiveness and sustainability. The estimated cost per batch, including nutrient solution and substrates, was approximately £20.9, confirming its accessibility compared with conventional culture systems. The breakdown of the entire infrastructure costs (illustrated in Table 11) confirms that, although an initial investment is required, the overall expenses remain modest and compatible with routine implementation in research laboratories. This affordability allows the system to be adopted as a practical upstream platform and to serve as a benchmark when compared with commonly used cultivation approaches such as microbial or mammalian cell systems. The system was validated as a scalable and sustainable upstream platform, adaptable to GMP standards and suitable for early-stage studies in plant molecular farming. Its ability to provide reproducible growth under controlled conditions makes it a reliable foundation for the downstream experiments described in the following chapters.

**Table 11.** Infrastructure costs for the construction of the entire laboratory-scale greenhouse system. Breakdown of capital expenditures for aluminium frame, LED lighting, hydroponic system, and auxiliary equipment.

| Components Description                                                             | Supplier            | Quantity | Unit Cost | Sub Total      |
|------------------------------------------------------------------------------------|---------------------|----------|-----------|----------------|
| RS PRO Silver Aluminium, Anodized Profile Strut, 30 x 30 mm, 6mm Groove, 2m Length | RS Components       | 4        | £35.75    | £143.00        |
| RS PRO Silver Aluminium, Anodized Profile Strut, 30 x 30 mm, 6mm Groove, 1m Length | RS Components       | 1        | £18.17    | £18.17         |
| RS PRO Cube Connector, 3 Way, Strut Profile 30 mm, Groove Size 6mm                 | RS Components       | 4        | £9.75     | £39.00         |
| RS PRO Cube Connector, 2 Way, Strut Profile 30 mm, Groove Size 6mm                 | RS Components       | 4        | £8.20     | £32.80         |
| RS PRO M6 T-Slot Nut, Connecting Component, Strut Profile 30 mm, Groove Size 6mm   | RS Components       | 3        | £8.58     | £25.74         |
| RS PRO Black Plastic Handle 50 mm Height, 25mm Width, 137mm Length                 | RS Components       | 2        | £12.85    | £25.70         |
| HIROSUGI Standoff, M3 x M3 Thread, 35mm Body, POM, Female/Female                   | RS Components       | 3        | £7.46     | £22.38         |
| Acrylic 6mm Thick Sheet 1000mm x 1000mm                                            | Direct Plastics     | 1        | £89.65    | £89.65         |
| Acrylic 5mm Thick Sheet 1000mm x 500mm                                             | Direct Plastics     | 1        | £39.22    | £39.22         |
| Acrylic 4mm Thick Sheet 1000mm x 500mm                                             | Direct Plastics     | 1        | £31.38    | £31.38         |
| 12mm Diameter Aluminium Bar 500mm long                                             | Metals 4u           | 1        | £9.77     | £9.77          |
| 8mm Thick Aluminium Plate 200mm x 100mm                                            | Aluminium Warehouse | 1        | £11.10    | £11.10         |
| Resin 3D Printed Parts                                                             | N/A                 | 1        | £20.00    | £20.00         |
| Filament 3D Printed Parts                                                          | N/A                 | 1        | £15.00    | £15.00         |
| Assorted Screws                                                                    | N/A                 | 1        | £20.00    | £20.00         |
| 2000W LED Plant Grow Light                                                         | Koscheal            | 1        | £119.77   | £119.77        |
| 450L/H Submersible Water Pump                                                      | Decdeal             | 1        | £12.99    | £12.99         |
| Blackout Window film 900x2000mm                                                    | VSDUO               | 1        | £19.99    | £19.99         |
| <b>Total</b>                                                                       |                     |          |           | <b>£695.66</b> |

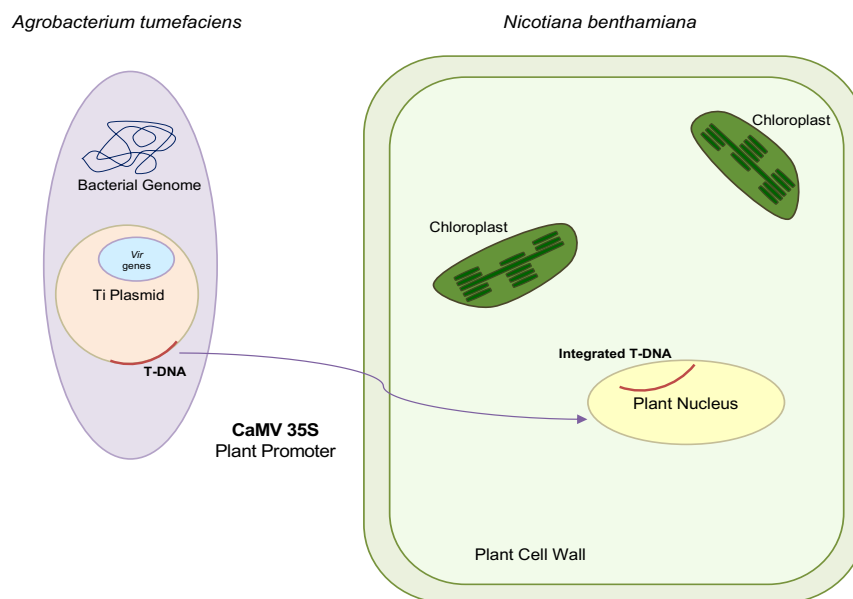


#### 4.3.3 *Agrobacterium tumefaciens* – Transformation and Agroinfection on Lab-scale

Agroinfection, or *Agrobacterium*-mediated transformation, represents the cornerstone of transient gene expression in PMF. This technique enables the rapid and flexible production of recombinant proteins, drastically shortening development timelines compared with stable transgenic systems (170). In practice, transient expression can be exploited to test new genetic constructs, perform rapid sampling of recombinant proteins for functional analysis, investigate subcellular localization and protein–protein interactions, or even support large-scale production when optimized for yield (171). The process harnesses the natural ability of *Agrobacterium tumefaciens*, a soil-borne Gram-negative bacterium, to transfer a defined segment of DNA – known as transfer DNA T-DNA – from its tumour-inducing (Ti) plasmid into the nuclear genome of host plant cells (172). In its natural context, this mechanism underlies crown gall disease in dicot plants. Phenolic compounds such as acetosyringone, released by wounded tissues, induce the bacterial vir (virulence) genes, which orchestrate the excision, processing, and delivery of the T-DNA into the plant cell nucleus (173). This unique feature has been repurposed for the delivery of synthetic expression cassettes in plants, enabling high-yield production of recombinant proteins. The Ti plasmid contains the T-DNA region, delimited by imperfect 25-bp repeats known as right and left borders (RB and LB), and a set of ~35 vir genes clustered in the vir region. Together, these elements provide the machinery for T-DNA transfer. Importantly, the oncogenic genes normally encoded within the T-DNA can be removed without impairing transfer efficiency. This “disarmed” configuration preserves the transformation capability of the bacterium while eliminating tumour formation (174, 175). This principle has been adapted into the binary vector system, where *Agrobacterium* is engineered to carry a plasmid containing the gene of interest flanked by T-DNA borders and driven by plant-active promoters (Figure 13). Widely used vectors include pBIN19, pCAMBIA, and pEAQ-HT, which share several essential features: left and right T-DNA borders, multiple cloning sites, replication origins compatible with both *E. coli* and *A. tumefaciens*, selectable markers for plants and bacteria, reporter genes, and regulatory elements for expression tuning (176). This modularity allows flexibility in construct design and broad applicability across host strains and plant species, establishing the binary vector system as a versatile standard for PMF. A critical step preceding agroinfection is the introduction of the binary vector into the *Agrobacterium* host strain. This transformation represents a decisive bottleneck, since the successful delivery of the construct into bacterial cells directly determines the efficiency of subsequent plant infection. Among the available methods, electroporation was selected over

chemical or freeze–thaw approaches because of its higher transformation efficiency and reduced cellular stress (177). The electroporation principle relies on the transient permeabilization of bacterial membranes through high-voltage pulses, allowing uptake of plasmid DNA, including large or complex constructs. In practice, achieving consistent results required careful optimization of the protocol. Critical parameters included the intensity (kV/cm) and the duration of the electric pulse (time constant), which are influenced by the resistance setting ( $\Omega$ ) of the electroporator and by the ionic strength of the suspension medium. To maximize efficiency, in-house electrocompetent cells were prepared through repeated washes to remove salts, in order for plasmid DNA to be introduced in a low-conductivity solution. However, the process was not without challenges. Early attempts were complicated by the absence of the helper plasmid pSoup, an element required for the maintenance of certain binary vectors, including pGreen (178). This omission initially prevented successful recovery of transformed colonies and delayed the workflow considerably. Identifying and addressing this issue was essential to establish a reliable protocol. Once corrected, the electroporation conditions could be systematically refined, and validated constructs were preserved as glycerol stocks for reproducibility in subsequent experiments. Confirmation of transformation efficiency combined quantitative and qualitative assessments. Colony-forming units (CFUs) were normalized to DNA input to calculate transformation efficiency, which typically ranged between  $5 \times 10^5$  and  $5 \times 10^6$  CFU/ $\mu$ g DNA. Colony validation was performed by antibiotic selection, plasmid recovery, restriction analysis, and sequencing, ensuring construct integrity prior to infiltration.

Following transformation, *Agrobacterium tumefaciens* cultures were cultured under selective conditions, centrifuged and resuspended in the infiltration buffer optimized for gene delivery. This buffer typically included magnesium chloride – MES buffer (pH ~5.6). The optical density of the bacterial suspension was adjusted between OD<sub>600</sub> 0.3–0.4, depending on plant age and physiological state, to balance efficient gene delivery with minimal phytotoxicity. Optimizing this bacterium–plant interface proved critical, as the integrity of the bacterial strain, the plant developmental stage, and the response of wounded tissues could all influence outcomes, with stress frequently manifesting as leaf necrosis. Despite initial challenges including fluctuations in environmental parameters and the need to fine-tune optical density and vacuum pressure the final protocol provided reproducible expression outcomes suitable for downstream applications.



**Figure 13.** Schematic representation of *Agrobacterium tumefaciens*–mediated gene transfer into plant cells. *A. tumefaciens* is used to insert the T-DNA carrying the foreign gene into the chromosome of the plant cell.

The preliminary results validated the agroinfiltration workflow as a robust method for transient expression in *N. benthamiana*. Pre-cultures of *A. tumefaciens* LBA4404 grown overnight at 29°C to high density (OD<sub>600</sub> 1.5–1.8) were scaled up in baffled flasks and harvested in late log phase (OD<sub>600</sub> 3.0–3.5). After centrifugation at 6,000 × *g* for 15 minutes at 25 °C, pellets were resuspended in infiltration buffer (10 mM MES, 10 mM MgCl<sub>2</sub>; pH 5.7) and diluted to a final OD<sub>600</sub> of ~0.4, a condition determined to maximize transient expression while minimizing tissue damage. Agroinfiltration was performed in *Nicotiana benthamiana* plants at the end of the fifth week of growth, when vegetative tissues were fully expanded and physiologically receptive to transformation. Vacuum infiltration was chosen as the delivery method, in which plants were inverted and submerged in the bacterial suspension inside a desiccator connected to a vacuum pump. To stabilize plant handling during the procedure, a custom 3D-printed support was developed to immobilize pots inside the desiccator, ensuring uniform exposure and reproducibility. Vacuum (-600 mbar) was applied for 3 minutes per plant, followed by rapid release, which facilitated infiltration through stomatal openings. Post-infiltration, plants were rinsed with sterile water and returned to controlled growth conditions. Under optimized parameters, recombinant expression was consistently achieved within 5–7 days. Once the expression was finalized, infiltrated leaves were harvested and immediately stored at -80 °C until further processing.

While the laboratory-scale platform at UCL demonstrated the feasibility of establishing a controlled and cost-effective upstream process for *N. benthamiana*, its throughput and degree

of automation remain inherently limited. To fully explore the scalability and industrial relevance of plant molecular farming, it is therefore essential to examine how upstream cultivation and agroinfiltration are implemented at commercial scale. The following section describes the industrial platform developed at Special Product's Line (SPL), highlighting process optimization strategies, GMP-oriented design, and the critical differences compared with the lab-scale model.

#### **4.4 Industrial-Scale Upstream Processing: The SPL Case Study**

In recent decades, controlled environment agriculture has become a cornerstone technology for industrial-scale PMF (179), enabling the reliable production of recombinant proteins in species such as *Nicotiana benthamiana*. Although open-field cultivation remains attractive from a cost perspective and allows plants to grow under their most natural conditions, it poses considerable risks. Exposure to pests, parasites, anthropogenic pollutants, and fluctuating environmental conditions compromises batch-to-batch reproducibility and raises significant biosafety concerns, especially under GMP requirements for plant-derived pharmaceuticals. CEA systems – including hydroponic greenhouses, vertical farming facilities, and fully enclosed phytotrons – overcome these challenges by providing precise control of growth conditions. Such platforms significantly reduce biological variability, stabilize transgene expression, and improve overall process reproducibility (107). In GMP-oriented facilities, the cultivation environment must be tightly regulated to meet standards of reproducibility, biosafety, and environmental containment. Among CEA strategies, vertical farming has gained particular relevance, enhancing productivity in limited spaces. By cultivating plants in stacked layers under artificial lighting and hydroponic irrigation, vertical farms maximize yield per unit area while minimizing water consumption and pesticide use. Advanced systems incorporate automation to regulate lighting spectra, humidity, nutrient delivery, and air circulation. Tools such as pH probes, EC sensors, and programmable controllers ensure environmental stability and reproducibility (180, 181). For biopharmaceutical companies, these platforms provide the scalability and responsiveness needed to meet increasing global demand for flexible and rapid biologics manufacturing. Despite these advantages, the implementation of large-scale vertical or greenhouse facilities entails considerable technical and economic challenges. High upfront investment, calibration of environmental systems, and the need for robust process validation represent major barriers. Even greenhouse systems, often positioned as a cost-effective compromise, require extensive optimization. Key hurdles include balancing photoperiod

cycles, fine-tuning nutrient delivery, calibrating HVAC systems, and ensuring continuous online monitoring of pH and electrical conductivity. Furthermore, maintaining agroinfiltration readiness across batches requires strict control of morphological traits such as leaf expansion, internode length, and tissue hydration status (182, 183).

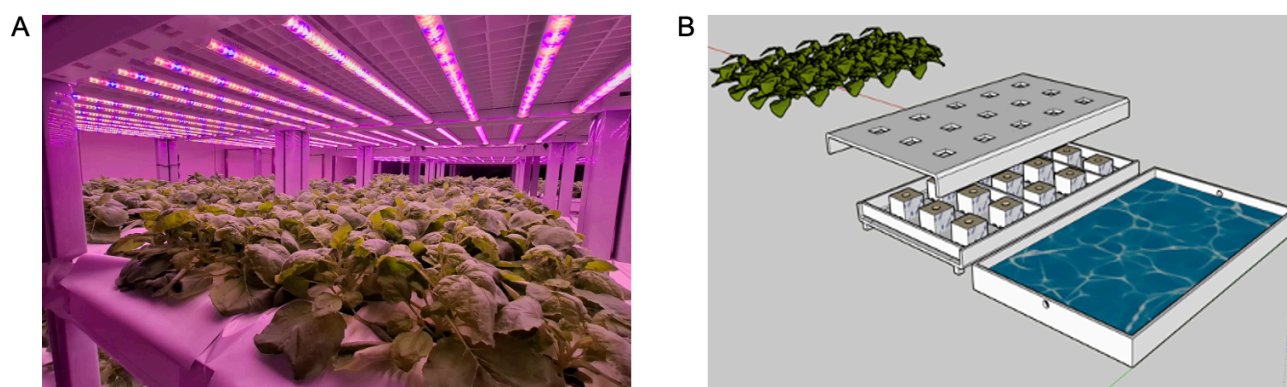
This case study focuses on the upstream system developed by SPL, a biopharmaceutical company that has implemented a vertically integrated hydroponic platform for *Nicotiana benthamiana*. The system was designed to combine biotechnological precision with industrial robustness integrating custom-fabricated hardware, real-time environmental monitoring, and modular tray configurations tailored to transient expression workflows. Its primary objective is to deliver a cultivation system that is scalable, reproducible, and compliant with pharmaceutical quality standards. Over time, the platform has undergone several iterations, reflecting continuous adaptation to the requirements of agroinfiltration processes, downstream compatibility, and biosafety. These refinements have aimed not only at enhancing expression consistency but also at aligning the infrastructure with GMP-oriented standards for containment and product safety. The following section describes the cultivation infrastructure in detail, including the rationale for hardware selection, environmental control strategies, and the key lessons learned through successive rounds of process optimization.

#### 4.4.1 Industrial Scale Plant Cultivation System

The cultivation strategy adopted at SPL relies on a vertically integrated hydroponic greenhouse, purpose-built to maximize production rates in *Nicotiana benthamiana*, being able to accommodate up to three sequential cultivations of 1440 plants each one. At the core of the platform are modular trays designed and fabricated to support high plant densities, enabling large-scale production while maintaining consistency across batches. Unlike the laboratory-scale system developed at UCL, which was primarily intended for proof-of-concept studies, the SPL setup was engineered combining industrial robustness with GMP-oriented precision. Emphasis was placed on cultivation techniques, since upstream performance strongly influences all subsequent process steps and overall facility design. To this end, the greenhouse was conceived as a fully enclosed environment, physically isolated from external conditions to ensure biosafety and stability of growth parameters. The platform integrates principles of Industry 4.0, with automation, real-time monitoring, and adaptive control strategies designed to enhance robustness, minimize operator variability, and facilitate GMP compliance. A detailed definition of batch handling further strengthened process

reproducibility. All production plants are derived from a single working seed bank, seeded on the same day, and cultivated under identical environmental conditions. Transplanting is synchronized, and harvesting is carried out simultaneously across the cohort. This standardized schedule defines a 40-day growth cycle: 14 days of seedling development, 21 days of vegetative growth, and a final 5-day incubation following agroinfiltration. Together, these measures reduce heterogeneity and ensure batch-to-batch consistency, a prerequisite for reliable downstream processing and regulatory acceptance. To achieve this, plant propagation begins from a Master Seed Bank (MSB), periodically renewed and genetically validated to guarantee stability and traceability. Both wild-type and genetically modified lines are employed in PMF, and in both cases extensive testing of seed identity and genetic stability is required (37). From the MSB, production seeds are germinated in rockwool plugs pre-treated with acidified ultrapure water (pH 5.5). Rockwool was selected as the growth substrate due to its sterility, inert nature, high porosity, and ability to retain both moisture and oxygen, making it highly suitable for hydroponic systems (139). Germination (0–14 DAS) occurs under strictly controlled conditions in climate chambers, with regulated temperature, humidity, and hydration using low-salinity nutrient solutions. At day 14, once cotyledons are fully developed, seedlings are transferred to vegetative growth modules. Vegetative development (15–35 DAS) takes place in vertical hydroponic racks (Sananbio design) integrating Nutrient Film Technique (NFT) and Deep-Water Culture (DWC). The modular and multilevel architecture minimizes floor-space requirements while maximizing productivity, with each rack accommodating several trays fabricated by Ferriplastic to ensure planting uniformity at a density of 27.77 plants/m<sup>2</sup>. A customized water distribution system delivers uniform irrigation enriched with nutrients, maintaining consistent growth across the facility. Environmental control is achieved through LED lighting systems delivering 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of PAR and positioned 500 mm above the canopy. The LED technology was selected because it provides a wide range of advantages compared with standard fluorescent lighting. While fluorescent lamps offer a broad spectrum and lower installation costs, they lack sufficient photic energy, are less energy-efficient, and generate more heat, leading to inefficiencies in plant growth and facility operation. In contrast, LEDs offer spectral tunability, long lifespan, and minimal heat emission, making them the preferred solution for controlled environment agriculture (184). A distributed lighting configuration ensures uniform illumination across trays, reducing shading effects and ensuring that all plants develop under equivalent light conditions. Environmental stability is further reinforced by HVAC and PLC systems, which maintain temperatures at  $23 \pm 2^\circ\text{C}$  and regulate photoperiod cycles of 16 h light / 8 h dark. Nutrient management is tailored to the

developmental stage of the plants and refreshed weekly. The nutrient solution is based on formulations provided by Growth Technology Ltd., using Ionic Hydro Grow, Silicon supplements, and liquid Oxygen. Inline sensors continuously monitor pH (maintained at 5.7–6.0) and EC (reaching between 2.0 and 2.5 mS/cm in the later growth stages). These parameters are regulated in real time via automated dosing systems, ensuring that optimal conditions are sustained throughout the cycle. This closed-loop regulation minimizes operator intervention and variability, improving yield uniformity and addressing one of the primary challenges in scaling PMF from laboratory to industrial settings (185). To complement production modules, additional experimental hydroponic units were also constructed, being designed to test cultivation parameters under identical HVAC and lighting conditions. Each experimental unit contained six independent circuits (two trays each), allowing parallel studies on parameters such as nutrient formulation, lighting regimes, and transient GFP expression (see Section 4.4.3). Finally, the cultivation system was designed with the possibility of integrating robotics for fully automated production. Automating processes from seeding to harvesting would eliminate the need for human entry into the facility, reducing biosafety risks while increasing usable space. Without the need for walkways or operator access, plants could be stacked at greater heights, maximizing the cultivation area. This approach aligns with the broader Industry 4.0 framework, where robotics, sensor networks, and adaptive control converge to enable highly reproducible, scalable, and safe plant-based biomanufacturing environments.



**Figure 14.** Cultivation modules for *Nicotiana benthamiana*. (A) Photograph of plants grown under controlled conditions with LED lighting. (B) Schematic representation of the hydroponic growth module, illustrating the structural components and water reservoir.





**Figure 15.** Vertical hydroponic platform for *Nicotiana benthamiana* plants growth. The setup integrates Sananbio growth modules with controlled environmental parameters.

#### 4.4.2 Agroinfiltration at Industrial Scale

Agroinfiltration is a pillar technique in transient plant expression systems, enabling the delivery of recombinant genes into plant tissues via *Agrobacterium tumefaciens*. On an industrial scale, the method must be optimized for efficiency, reproducibility, and scalability (65). The procedure involves the preparation of *Agrobacterium* cultures harbouring binary vectors, adjusting cell density to achieve optimal optical density ( $OD_{600}$ ), and delivering the bacterial suspension to plant leaves via vacuum infiltration. Within the SPL industrial platform, this required fine-tuning of inoculum preparation, infiltration conditions, and post-infiltration handling to ensure high transformation efficiency while minimizing plant stress. As in laboratory workflows, the agroinfiltration process begins with the introduction of binary vectors into *Agrobacterium tumefaciens*. Plasmids are introduced into electrocompetent *Agrobacterium* cells using electroporation, a method favoured over the heat-shock one for its higher transformation efficiency and reliability, especially in industrial workflows (177). Colonies are selected under antibiotic pressure and expanded in liquid culture. To optimize bacterial growth, different media formulations were compared, with performance assessed in terms of growth rate, final density, and suitability for downstream infiltration. Based on these trials, an enriched medium designated LB++ was selected as the most effective, consisting of LB supplemented with  $FeSO_4$ ,  $MnSO_4$ ,  $ZnSO_4$ , and  $MgSO_4$ , adjusted to pH 7.0. This formulation supported robust and reproducible biomass accumulation, which was essential for consistent infiltration outcomes.



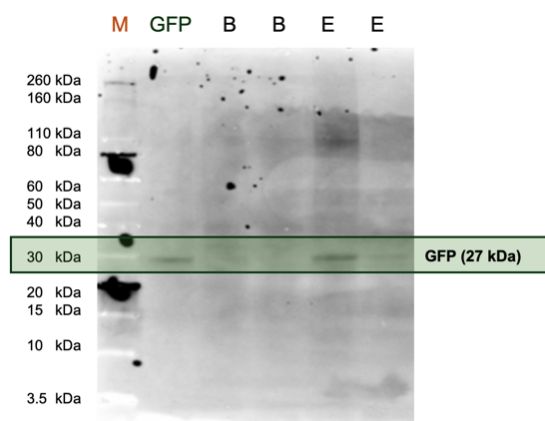
Cultures were then scaled up in successive volumes until reaching OD<sub>600</sub> values of 1.0–1.2. After harvesting by centrifugation and washing in infiltration buffer (10 mM MES, 10 mM MgCl<sub>2</sub>, pH 5.7), suspensions were diluted to a final OD<sub>600</sub> of 0.4. This range was empirically determined to achieve optimal transformation efficiency. Equal volumes of different strains could be combined at this stage, depending on the constructs of interest. The infiltration step itself was performed using a custom-designed, PLC-controlled vacuum system. Plants at 35 days of age were inverted into a 56 L infiltration tank, with trays processed in pairs. Vacuum pressure of -700 mbar (relative vacuum) was applied for 4 minutes, followed by rapid restoration of atmospheric pressure. This transient pressure differential evacuated intercellular air from the mesophyll via stomata and facilitated penetration of the bacterial suspension into the leaf tissue. As a result, *Agrobacterium* cells carrying high-copy-number vectors were uniformly introduced into the leaf parenchyma, initiating transient expression. Following infiltration, plants were rinsed, returned upright, and incubated for 5 days under controlled conditions (22 ± 2 °C, 16 h light/8 h dark photoperiod). During this period, the bacterial T-DNA was delivered to the plant cell nucleus, enabling expression of the protein of interest. To maintain biosafety, infiltrated plants were segregated from non-infiltrated plants in a dedicated, spatially isolated room. At the end of the incubation phase, leaves were harvested, portioned into 2 kg bags, and stored at -80 °C pending downstream processing. The entire process was refined over multiple experimental cycles, which identified bacterial OD, infiltration time, vacuum pressure, and incubation period as the most critical variables. The ideal agroinfiltration process conditions (vacuum, time) were identified through experiments using Green Fluorescence Protein (GFP) and are furtherly described in Section 4.4.3. Through this systematic optimization, SPL established a robust and scalable agroinfiltration procedure suitable for industrial PMF applications.

#### 4.4.3 Optimization of the Agroinfiltration parameters

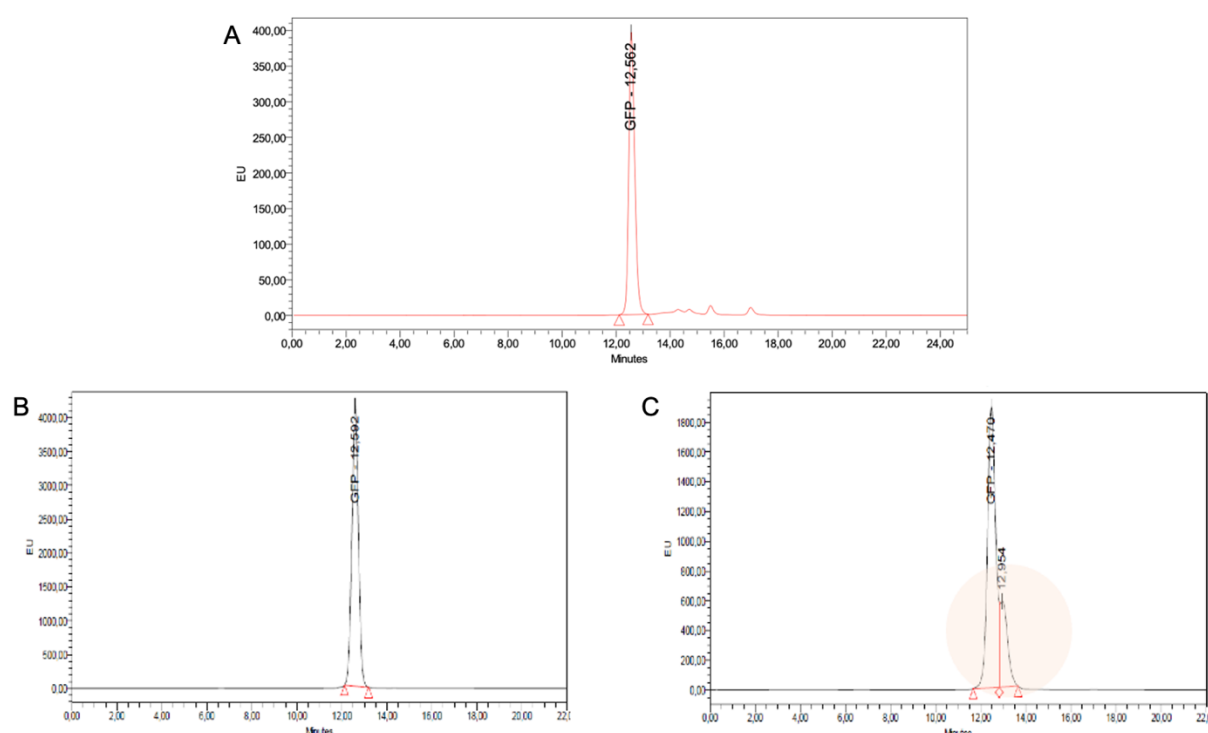
To ensure a high and reproducible yields of recombinant proteins expressed in *Nicotiana benthamiana*, a series of optimization studies were conducted at SPL to refine the vacuum-based agroinfiltration protocol. These studies focused on identifying the physical parameters most critical to infiltration efficiency and protein expression, namely vacuum pressure, duration of vacuum application, and post-infiltration incubation period. Green Fluorescent Protein (GFP) was used as a reporter system to allow rapid and quantitative assessment of protocol performance. Over the course of three experimental campaigns, a total of 24 infiltration conditions were tested. The studies systematically varied infection time, incubation

duration, and vacuum pressure, combining fixed and variable setups to simulate industrial deviations and to prove the robustness of the system. Performance was assessed through total soluble protein quantification, GFP fluorescence intensity via TECAN, GFP quantification via SEC-UHPLC and visual tissue integrity. To ensure comparability, all experiments were performed using plants at equivalent developmental stages and standardized bacterial inoculum (strain *A. tumefaciens* LBA4404, OD<sub>600</sub> adjusted). This section provides a consolidated view of the optimization trials performed using the GFP system and highlights the key outcomes that influenced SPL's industrial protocol for vacuum agroinfiltration. The first two experimental campaigns investigated the influence of infection duration (length of vacuum application) and post-infiltration incubation time before harvesting. Six different conditions were tested in each study, with up to ten biological samples per condition. Fluorescence intensity measurements, supported by SEC-UHPLC and TECAN analysis, showed that GFP accumulation increased progressively with longer incubation times, reaching a maximum at 5 days post-infiltration. However, protein degradation also rose significantly after this point with extended incubation (7 dpi). After six days, SDS-PAGE revealed partial breakdown of GFP, while HPLC profiles displayed additional low-molecular-weight peaks consistent with proteolysis. These results indicated that although longer incubation enhanced yield, protein stability declined due to heightened proteolytic activity, particularly under conditions of elevated humidity and suboptimal light. From the perspective of vacuum application, infection times of 4 minutes proved optimal. Durations shorter (2 minutes) produced incomplete infiltration, visible as uneven GFP distribution under UV light, whereas longer infiltration time would have increased tissue collapses and necrosis. Biomass recovery post-infiltration also correlated negatively with excessive vacuum exposure, confirming that prolonged treatments impaired tissue viability. The third campaign evaluated the effect of vacuum pressure and its stability during infiltration. Three pressures were tested (550, 625, and 700 mbar), each combined with standard agroinfection time of 4 minutes and  $\pm 10\%$  variation in vacuum duration. The best performance was consistently observed at 700 mbar for 4 minutes, which yielded the highest GFP intensity with minimal tissue disruption. At 550 and 625 mbar, results became more variable between replicates, affecting up to 40% of the leaf surface. Quantitative protein analysis of homogenized tissue confirmed a 2.5-fold reduction in soluble protein yield at longer vacuum time, underscoring the detrimental effects of mechanical stress caused by over-infiltration. Overall, these optimization trials demonstrated that protein yield and stability in *N. benthamiana* are highly sensitive to even small deviations in infiltration timing and pressure control. By systematically refining these

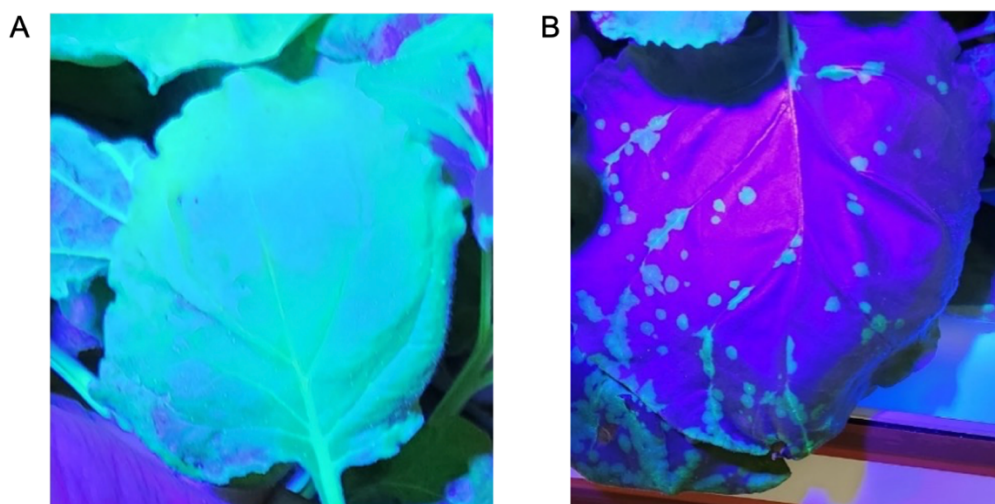
parameters, SPL established a robust and reproducible vacuum agroinfiltration protocol at industrial scale, ensuring scalability, process consistency, and product quality for plant-derived biopharmaceuticals.



**Figure 16.** SDS–PAGE analysis of crude plant extracts. Lane M: molecular weight standard. Lane GFP: standard GFP commercially purchased. Lane B: negative control. Lane E: agroinfiltrated leaves expressing GFP. A clear band at the expected molecular weight of ~27 kDa is visible only in agroinfiltrated samples.



**Figure 17.** SEC-UHPLC chromatograms of GFP detection. (A) Chromatogram of sample obtained under optimized agroinfiltration conditions. (B) Chromatogram of a commercially purchased GFP standard. (C) Chromatogram of GFP extracted at 7 dpi, showing evident degradation (highlighted in orange).



**Figure 18.** Visualization of GFP expression in *Nicotiana benthamiana* leaves under UV light. (A) Leaf from a plant agroinfiltrated under optimized parameters, showing uniform GFP distribution. (B) Leaf from a plant agroinfiltrated under non-optimized parameters.

#### 4.5 Comparative Analysis: Lab-Scale vs Industrial Systems

The upstream processing strategies previously described represent two distinct but complementary approaches to plant molecular farming. The lab-scale platform relied on compact hydroponic growth chambers and manual agroinfiltration, offering experimental flexibility ideally suited for early-stage development. Such lab-scale systems are cost-effective, adaptable, and valuable for parameter testing, rapid prototyping, and proof-of-concept studies. Their advantages include reduced infrastructure requirements, ease of modification, and minimal space demand. However, they are inherently limited by batch-to-batch variability, restricted scalability, and labour-intensive workflows. In contrast, SPL's industrial platform was designed for throughput, robustness, and reproducibility. The combination of vertical hydroponic farming with PLC-monitored environmental controls enabled high-biomass cultivation under GMP-oriented conditions. This setup provided the stability needed for pharmaceutical production but came at the cost of high capital investment, greater operational complexity, and the need for stringent process standardization to prevent contamination and necrosis. Upstream decisions strongly influence downstream outcomes in PMF. Parameters such as plant density, infiltration uniformity, and stress-induced necrosis directly impact the concentration, purity, and structural stability of the recombinant product. Inconsistent infiltration, for instance, can lead to heterogeneous expression levels, complicating purification and increasing product loss (186). Environmental conditions – including relative humidity and light spectrum – not only affect biomass yield but can also alter glycosylation patterns and oxidative stability of expressed proteins, thereby influencing

product homogeneity and therapeutic efficacy (187, 188). Therefore, optimizing upstream conditions is indispensable to securing downstream quality.

This chapter has explored the upstream strategies used in both setups, emphasizing how scale affects priorities and constraints. While each presents unique strengths, the choice ultimately depends on the desired output scale, regulatory framework, and economic resources. While academic platforms provide agility and cost-effectiveness for method development, industrial infrastructures are indispensable for consistent, large-scale manufacturing. A hybrid perspective that integrates lessons from both could yield systems that combine the flexibility of lab-scale platforms with the robustness of industrial production. Looking forward, scalable plant-based production platforms will benefit from synergizing biotechnology and automation. Emerging technologies such as sensor-guided fertigation, automated canopy imaging for growth monitoring, and AI-driven environmental feedback loops promise to reduce operator variability and enhance reproducibility (189). Advances in transient expression vectors – such as viral replicons and inducible promoters – are further improving yields without compromising system stability (190). At the same time, host engineering strategies, including CRISPR/Cas9 knockouts of protease genes or the introduction of molecular chaperones, are helping overcome persistent bottlenecks in protein stability (191). As regulatory frameworks evolve, these innovations are expected to expand the role of plant molecular farming in areas such as pandemic preparedness, where responsiveness and scalability are crucial.

## 5. THE TB002: A Recombinant Subunit Vaccine Produced in Plants

This chapter describes the downstream process development and optimization activities conducted at Special Product's Line (SPL), a company specialized in plant molecular farming and biopharmaceutical production. The work focused on the "TB002" recombinant protein, a vaccine candidate against tuberculosis produced in *Nicotiana benthamiana*. All experiments were carried out under controlled conditions, with the aim of establishing a scalable and robust purification process suitable for preclinical applications.

### 5.1 Introduction and Scientific Background

Tuberculosis (TB) is still one of the leading causes of death by infectious disease worldwide and continues to be a major global health concern (192). The only currently licensed vaccine, Bacillus Calmette-Guérin (BCG), provides limited protection, particularly failing to protect adults against pulmonary TB, which represents the most transmissible and life-threatening form of the disease (193, 194). These limitations have driven a renewed focus on novel vaccine strategies capable of inducing long-lasting immunity and preventing infection or reactivation of latent TB. Among these, recombinant subunit vaccines have emerged as a safe and rational alternative. Unlike whole-pathogen or inactivated vaccines, subunit vaccines are composed of selected antigenic fragments such as isolated proteins or peptides, capable of inducing the immune response without the risks associated with live or attenuated organisms. These vaccines are advantageous for their defined composition, consistent quality, and lower risk of adverse reactions (195). In addition, their modular design allows flexible design and production across different heterologous expression systems (196). As previously stated, PMF has emerged as a competitive platform for the production of recombinant subunit vaccines (197).

In the context of this project, a recombinant protein candidate called "TB002" was designed as a subunit vaccine against *Mycobacterium tuberculosis*. Its production was carried out in *Nicotiana benthamiana* plants using a transient expression system. This approach allowed rapid and contained production under controlled conditions in a vertical farming system, as previously described in Chapter 4. Plant-based platforms like the one developed at SPL represent a promising route for cost-effective vaccine manufacturing (198).

## 5.2 Experimental Design

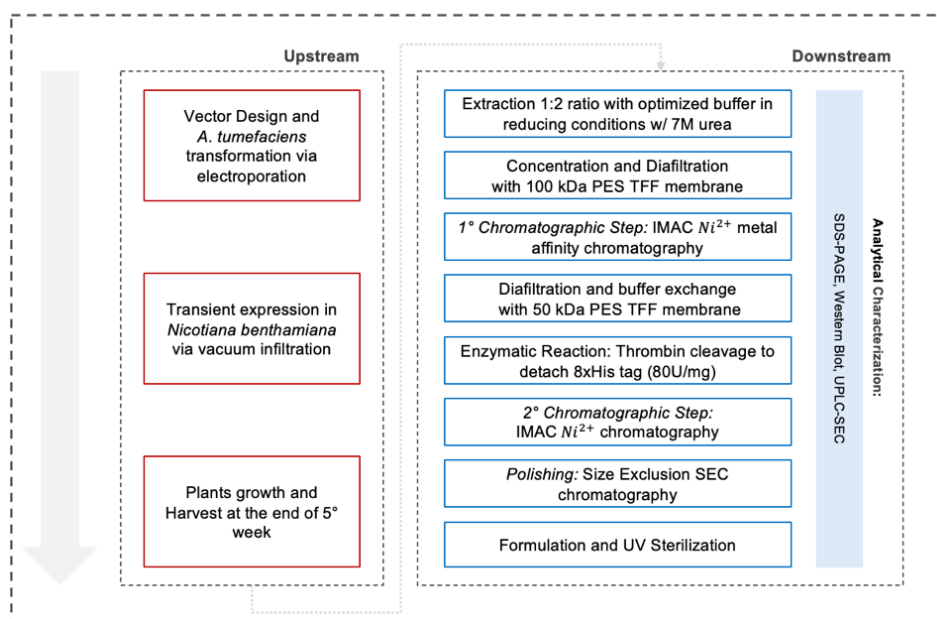
The TB002 subunit vaccine candidate was designed as a recombinant protein targeting *Mycobacterium tuberculosis*. The gene sequence was codon-optimized for plant expression and cloned into a binary expression vector under the control of a constitutive promoter. The construct included a C-terminal 8xHis-tag to facilitate downstream purification via affinity chromatography. The resulting plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 using electroporation. Following confirmation of transformation and sequence validation, glycerol stocks of the transformed *A. tumefaciens* were prepared and stored at  $-80^{\circ}\text{C}$ . Pre-infiltration cultures were grown in LB++ medium supplemented with appropriate antibiotics, and cells were harvested at mid-log phase. Prior to infiltration, cells were resuspended in infiltration buffer containing 10 mM MES, 10 mM  $\text{MgCl}_2$ , and 100  $\mu\text{M}$  acetosyringone, adjusted to an optical density of 0.35–0.4 at 600 nm to ensure optimal infection efficiency. Transient expression of TB002 was carried out in *Nicotiana benthamiana* plants cultivated under controlled conditions in a vertical farming system. Plants aged 5 weeks were selected for vacuum-assisted agroinfiltration. The infiltration process was performed under sterile conditions using a custom-designed vacuum chamber operating at  $-700$  mbar, ensuring homogeneous infiltration of the leaf tissue. Following agroinfiltration, plants were incubated for five days under optimized growth conditions ( $23^{\circ}\text{C}$ , 16-hour light/8-hour dark photoperiod, 60% humidity), allowing accumulation of the recombinant protein within the intercellular space and cytoplasm. At harvest, only the infiltrated leaves were collected, excluding stems. Samples were frozen and stored at  $-80^{\circ}\text{C}$  until extraction. Biomass was quantified immediately after harvest, and plant material was processed within two months of storage. This experimental setup was designed to evaluate both expression yield and process compatibility with downstream purification protocols, described in detail in the following sections. The aim was to obtain a purified, stable protein suitable for analytical characterization and future immunogenicity studies.

## 5.3 Downstream Process development and optimization

Downstream processing (DSP) encompasses all purification and formulation steps required to recover biologically active recombinant proteins after their production in host organisms. In the context of PMF platforms, DSP poses significant technical and economic challenges. Plant tissues contain a wide array of endogenous compounds – including phenolics, proteases, and polysaccharides – that can interfere with protein stability and purification

efficiency, thereby complicating the isolation of high-purity biologics and representing significant hurdles to achieving industrial-grade product quality and consistency (199). In industrial settings, DSP operations are considered a major bottleneck in PMF workflows, often representing up to 80% of the total production cost (200). This is primarily due to the complexity and variability of plant biomass, the typically low protein concentration per gram of tissue, and the necessity for multi-step purification strategies to achieve regulatory-grade quality. To meet current Good Manufacturing Practice (GMP) requirements, the downstream workflow must ensure high product purity (>95%), removal of host-related impurities, process consistency, and long-term stability – all while maintaining scalability and cost-effectiveness (201). A typical plant-based DSP workflow includes: (I) protein extraction and clarification, (II) primary capture via affinity or ion-exchange chromatography, (III) polishing through size exclusion or orthogonal techniques, and (IV) formulation and sterile filtration. Each step requires careful optimization based on resin performance, compatibility with buffer systems, process yield, and compliance with GMP guidelines.

In this chapter, the downstream process development for the TB002 recombinant protein is presented (Figure 19). All activities were conducted at SPL under controlled process development conditions, with the aim of establishing a robust, scalable purification pipeline for plant-derived subunit vaccines. The following sections describe the major steps of the DSP strategy, including extraction and buffer selection, IMAC purification, 8xHis-tag removal, polishing strategies, and final formulation.



**Figure 19.** Workflow of TB002 production in *Nicotiana benthamiana*, from vector design and transient expression to downstream purification and analytical characterization.

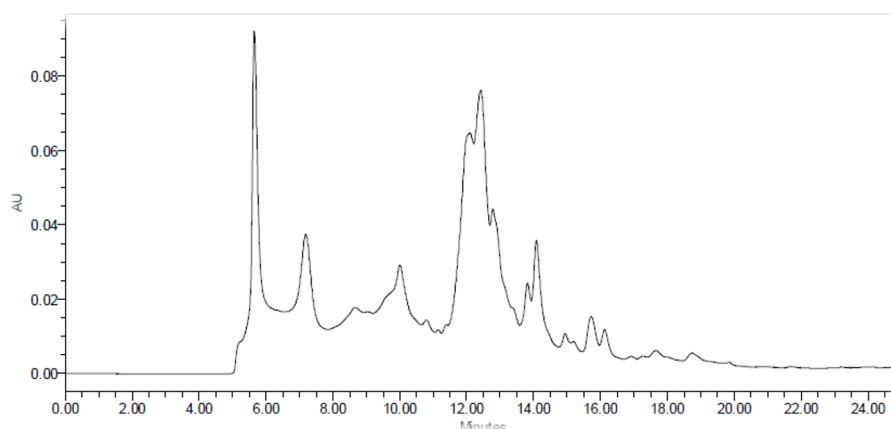


### 5.3.1 Protein Extraction: Buffer Screening and Clarification

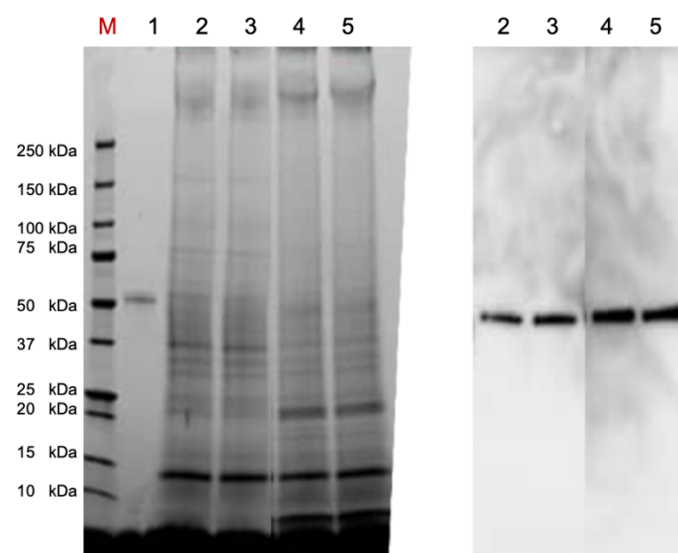
Protein extraction from *Nicotiana benthamiana* leaves was conducted using various buffer compositions, with a strong focus on minimizing aggregation and maximizing solubility of the TB002 protein. Early experiments using phosphate-based buffers supplemented with low concentrations of urea (50 mM – 2 M) yielded low process recovery and significant aggregation. SDS-PAGE analysis confirmed the presence of insoluble high molecular weight smears, indicating suboptimal solubilization of the target protein. To mitigate extract viscosity and reduce phenolic interference, 1% (w/v) polyvinylpolypyrrolidone (PVPP) was added to the extraction buffer, which improved the clarification the extract. Following homogenization, a two-step centrifugation protocol was applied: an initial spin at 14,000 rpm for 30 minutes at 4°C, followed by a second centrifugation of the supernatant at 14,000 rpm. This yielded a refined supernatant with reduced particulates and improved processability. To further improve solubilization, the urea concentration in the phosphate buffer was gradually increased. First, urea was introduced at 2 M concentration in phosphate buffer. However, SDS-PAGE (Figure 21) still revealed partly solubilization of TB002 and visible high molecular weight smears. Increasing the urea concentration to 7 M significantly improved protein solubility and resulted in a distinct ~56 kDa band corresponding to TB002. Despite similar overall protein yields between 2 M and 7 M urea, the latter significantly consistently minimized aggregation and clarified the extract enabling a more efficient downstream purification, becoming the standard extraction condition for subsequent purification steps. The transition to a 7 M urea-based buffer became a critical step in the process, as it allowed reliable extraction of the target protein in a form suitable for downstream processing. All further purification steps were based on this extraction condition. The final optimized extraction buffer included: 20 mM sodium phosphate, 5 mM TCEP, 7 M urea, 1 mM PMSF, 0.5 M NaCl, and 10 mM imidazole at pH 7.4. Frozen leaves were mechanically homogenized in a 1:2 ratio (w:v) with the extraction buffer and clarified through the double centrifugation (Figure 20). The pH of the extract was initially acidic (~6.01) and was adjusted to pH 7.4 with 1 M HCl prior to loading onto the purification column. The addition of NaCl (0.5 M) in the extraction buffer was instrumental in improving ionic strength and minimizing nonspecific interactions with the Ni<sup>2+</sup> matrix. Inclusion of 10 mM imidazole in the crude extract and in the IMAC binding buffer reduced nonspecific interactions with the Ni<sup>2+</sup> matrix.

Following the extraction phase, an initial diafiltration step was introduced to reduce the concentration of chaotropic agents and prepare the sample for affinity chromatography. This

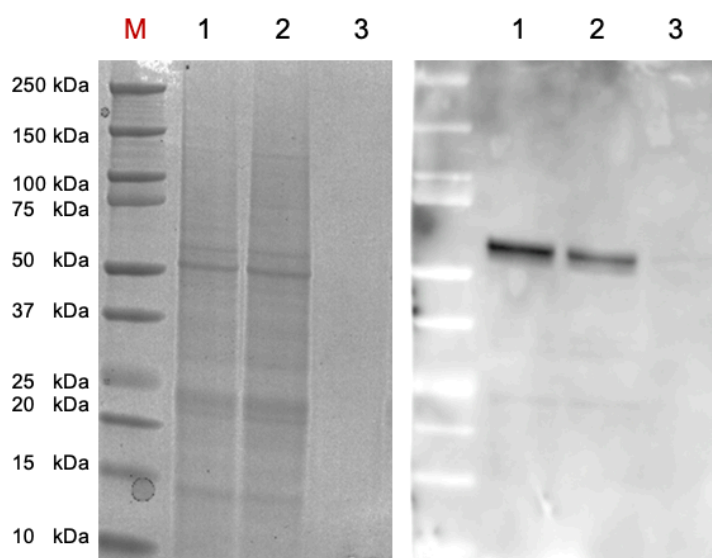
step aimed at clarifying the sample while concentrating the total protein volume, thereby minimizing buffer interference during IMAC binding. Several trials were performed using Vivaflow 200 tangential flow filtration units equipped with polyethersulfone (PES) membranes. A 100 kDa cut-off was selected to retain the target protein (~56 kDa) while eliminating lower molecular weight contaminants and urea in excess. The process was carried out at 4°C to preserve protein stability. Diafiltration typically involved a 5-fold buffer exchange reducing sample volume from ~400 mL to ~50 mL and achieving effective concentration. One of the challenges encountered was protein loss due to membrane adsorption. This issue was mitigated by pre-conditioning the membrane with the extraction buffer and optimizing the flow rate to reduce retention time. SDS-PAGE analysis of the diafiltered extract confirmed retention of TB002 and reduction in background smearing (Figure 22). The processed sample was then directly loaded onto the Ni-NTA column for IMAC purification, with significantly improved binding efficiency compared to non-diafiltered samples.



**Figure 20.** UPLC–SEC chromatogram of clarified leaves extract from *Nicotiana benthamiana* leaves.



**Figure 21.** SDS–PAGE (left) and Western blot analysis with anti-His antibody 1:1000 (right) of TB002 extracts under denaturing conditions. Lane M: molecular weight standard; Lane 1: purified TB002 positive control; Lanes 2–3: raw plant extracts solubilized in 7M urea; Lanes 4–5: raw extracts solubilized in 2M urea. A distinct band corresponding to TB002 is visible in urea extracts, confirming solubilization and recovery.



**Figure 22.** SDS–PAGE (left) and Western blot analysis with anti-His antibody 1:1000 (right) of TB002 samples during extraction and diafiltration with a 100 kDa MWCO PES membrane. Lane M: molecular weight standard; Lane 1: crude extract; Lane 2: retentate after 100 kDa concentration; Lane 3: permeate after 100 kDa concentration. Both results confirm the presence of TB002 at the expected molecular weight and only in the retentate fraction.

### 5.3.2 Immobilized Metal Affinity Chromatography (IMAC)

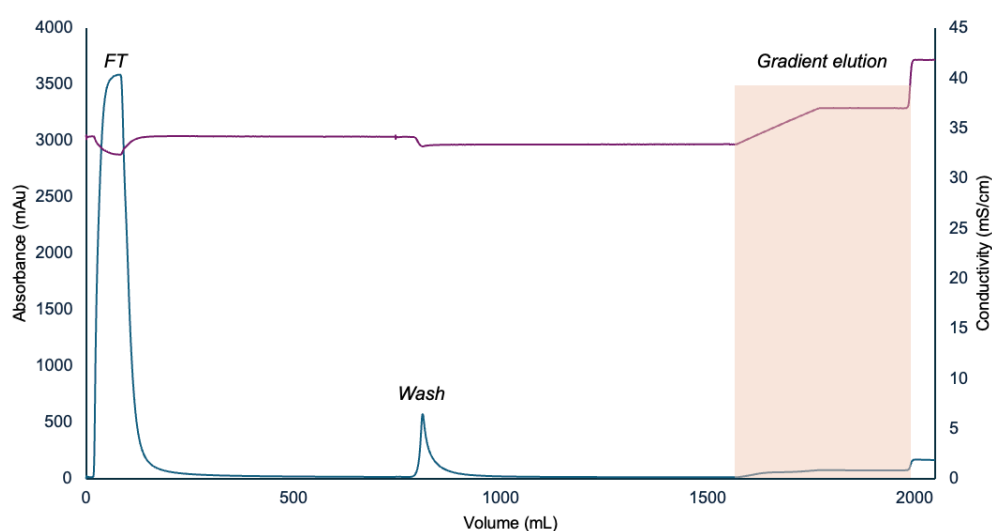
The initial purification step for TB002 was performed by immobilized metal affinity chromatography (IMAC) using nickel-charged agarose matrix. This step was critical to

achieve preliminary enrichment of the His-tagged recombinant protein and was optimized through a series of systematic trials aimed at improving purity, reducing host contaminants, and maximizing yield. Chromatographic runs were carried out on an ÄKTA Pure 150 system using an XK 26/20 column packed with IMAC Sepharose HP resin (Cytiva). Several buffer systems and elution strategies were tested. Particular attention was given to the choice of urea concentration in the binding and wash buffers, which significantly influenced the chromatographic behaviour and the purity of the protein. Two buffer systems were evaluated based on urea concentration – 2 M and 7 M – each containing a base composition of 20 mM sodium phosphate, 5 mM TCEP, 0.5 M NaCl, 1 mM PMSF, and 10 mM imidazole at pH 7.4 (Buffer A). The elution buffer (Buffer B) was formulated similarly, with increasing imidazole concentrations ranging from 40 mM to 500 mM (Figure 23). Initial elution trials employed a three-step gradient: 25% B (125 mM imidazole), 50% B (250 mM), and 100% B (500 mM). Under 2 M urea conditions, TB002 showed broader elution profiles with detectable target protein in both early and late fractions (125 mM to 500 mM imidazole). SDS-PAGE revealed significant co-elution with host contaminants – most notably RuBisCO (~50 kDa) – and protein aggregation. In contrast, under 7 M urea conditions, the elution profile of TB002 shifted noticeably, with the majority of the protein eluting in a sharper, more defined peak, predominantly with 250–300 mM imidazole, showing drastically reduced aggregation and minimal contaminant co-elution. Aggregates were drastically reduced and the clarity of the flow-through and eluates was improved. The presence of contaminant at ~50 kDa was also significantly diminished, likely due to enhanced denaturing conditions that prevented nonspecific binding. Western blot using anti-His antibodies confirmed TB002 presence (~56 kDa) under these conditions (Figure 27).

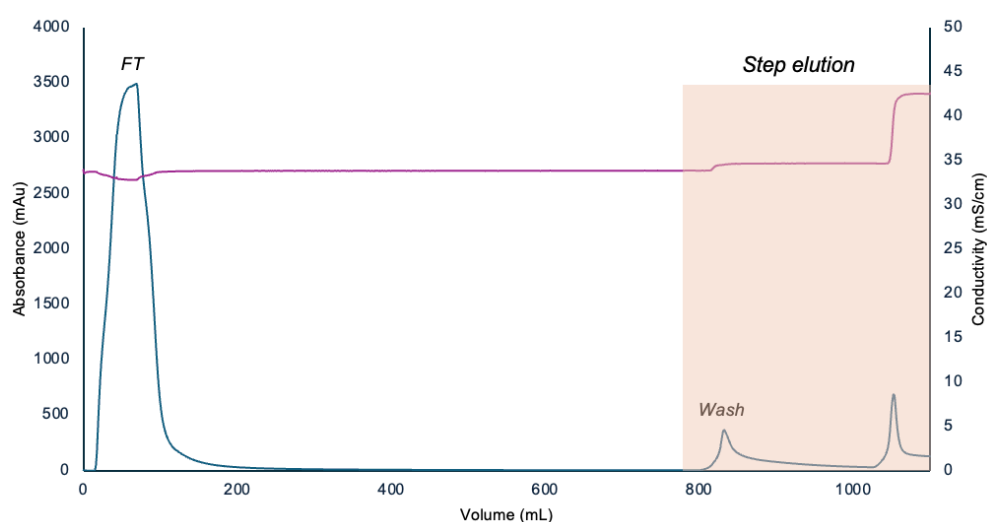
To further refine the separation, intermediate wash steps with 40 mM and 70 mM imidazole were introduced before elution. This extended washing strategy effectively removed residual host proteins while preserving TB002 binding to the resin (Figure 25). The final optimized elution scheme included 5 column volumes (CV) wash with 40 mM imidazole, 10 CV wash with 70 mM imidazole, followed by elution at 300 mM and 500 mM imidazole. Typical loading volumes ranged from 230–250 mL per run, with elution volumes between 50–75 mL per fraction. Flow rates were maintained at 4–5 mL/min with a linear velocity of 57 cm/h and residence time of ~5 minutes. Crude extract pH correction before loading was critical, as precipitation of plant components occurred around pH 6 and was minimized by adjusting to pH 7.4 before loading. Overall, 7 M urea buffer conditions consistently outperformed 2 M in terms of purity, resolution, and process robustness. The use of 7 M urea resulted in better

peak resolution, reduced contamination, and prevented aggregation, as confirmed by analytical tests. This supported the choice of these buffer conditions as the standard protocol for subsequent IMAC purification of TB002. All intermediate and final fractions were analysed by SDS-PAGE and Western Blot using anti-His monoclonal antibody.

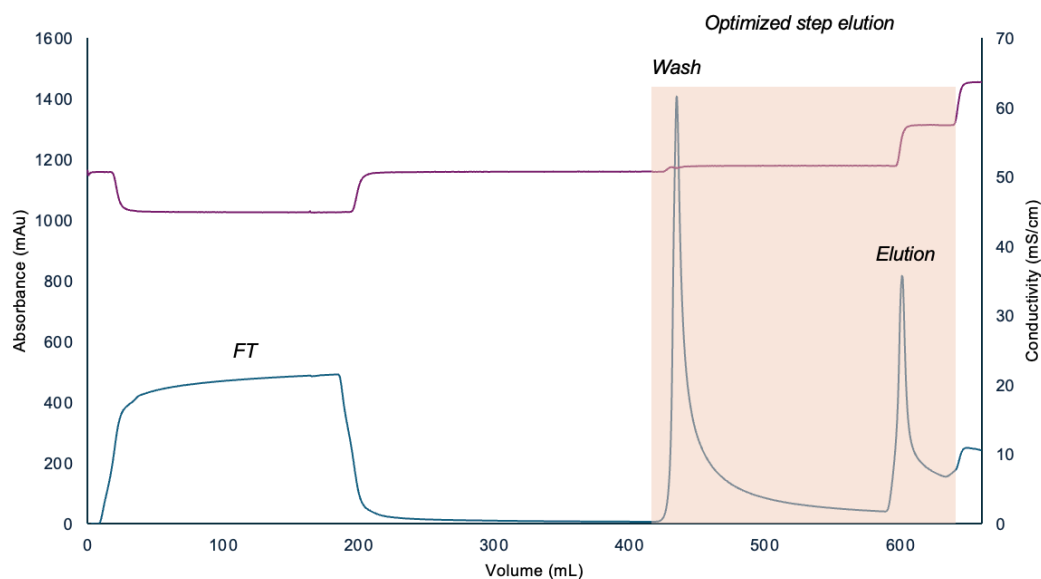
Despite the improved separation, residual impurities were still observed post-IMAC, prompting the need for further purification by diafiltration and polishing steps. A desalting step was carried out immediately after IMAC elution to remove urea and prepare the protein for thrombin-mediated tag removal.



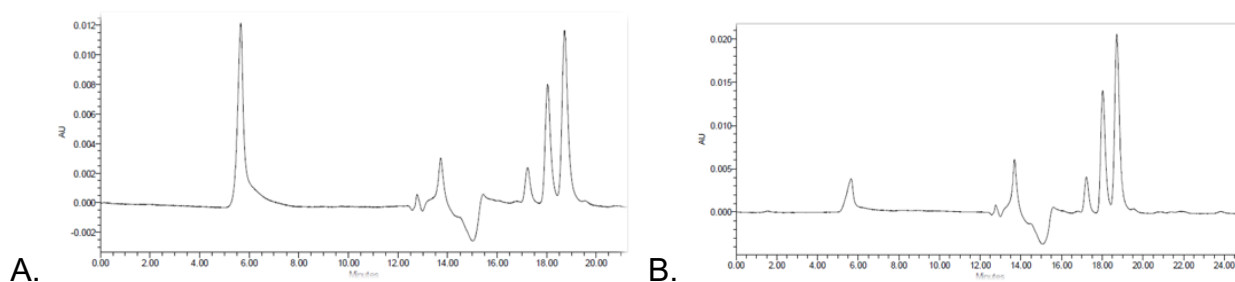
**Figure 23.** IMAC chromatography of the first purification trials of TB002, performed prior optimization. Elution was carried out stepwise and in gradient, resulting in partial recovery and the presence of nonspecific contaminants.



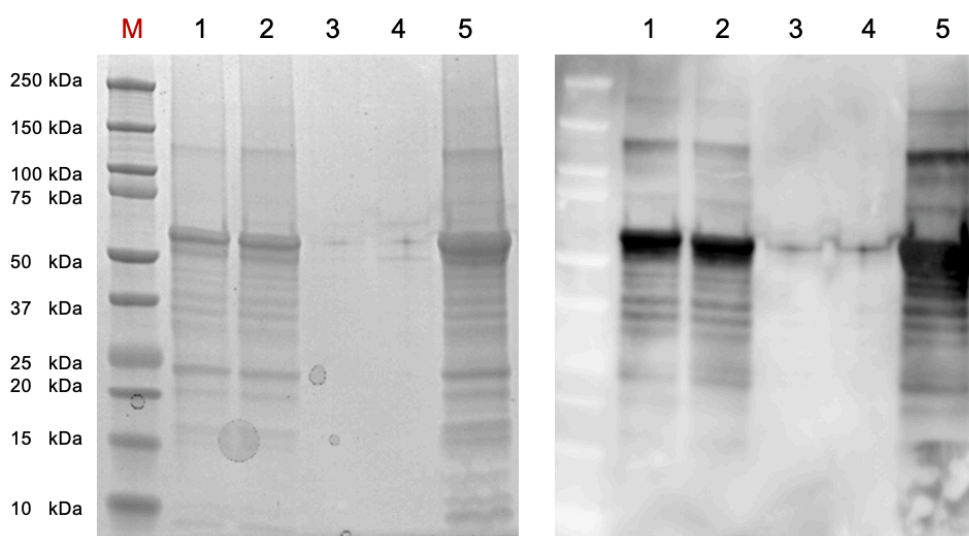
**Figure 24.** IMAC chromatography for the purification of TB002. The use of a step elution with imidazole enabled more selective separation, leading to improved yield and reduced nonspecific protein co-elution.



**Figure 25.** Final optimized IMAC chromatography protocol for the purification of TB002. Through the use of a step elution with imidazole selective separation, improved yield and reduced nonspecific protein co-elution were achieved.



**Figure 26.** UPLC-SEC chromatograms of TB002 after IMAC purification. (A) Eluate fraction obtained with 120 mM imidazole. (B) Strip fraction obtained with 500 mM imidazole. Distinct chromatographic profiles highlight differences in protein content and purity between the two elution steps.



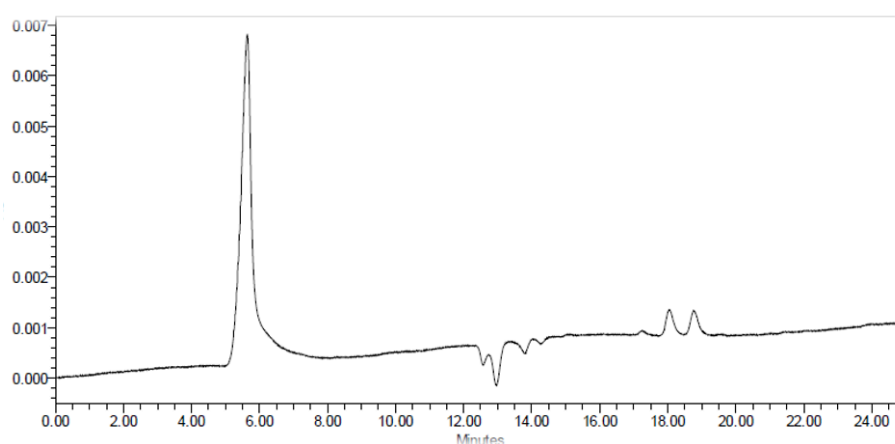
**Figure 27.** SDS-PAGE (left) and Western blot analysis with anti-His antibody 1:1000 (right) of TB002 samples during IMAC purification steps. Lane M: molecular weight standard; Lane 1: crude extract

desalted with 100 kDa; Lane 2: desalted fraction incubated overnight; Lane 3: flow-through from IMAC; Lane 4: wash from IMAC with 75mM imidazole; Lane 5: elution from IMAC with 120 mM imidazole. The Western blot confirms the enrichment of TB002 at the expected molecular weight in the IMAC elution fraction (lane 5).

### *5.3.3 Intermediate Purification and Diafiltration*

Following IMAC purification, TB002-containing eluates underwent intermediate purification steps involving urea dilution, diafiltration, and buffer exchange to prepare the protein for enzymatic tag removal. These steps were essential to eliminate chaotropic agents (urea), protease inhibitors (PMSF), and excess imidazole, all of which could interfere with enzymatic digestion or compromise protein integrity. Initial diafiltration was performed using a buffer composed of 20 mM sodium phosphate, 500 mM NaCl, and 5 mM TCEP at pH 7.4 (Buffer C), which was chosen for its compatibility with subsequent purification stages. Diafiltration was performed using a VivaFlow 200 tangential flow filtration (TFF) systems with a polyethersulfone (PES) membrane. During the optimization of the diafiltration step, both 100 kDa and 50 kDa polyethersulfone (PES) membranes were evaluated using the VivaFlow 200 system to determine the most suitable cut-off for retaining TB002 (~56 kDa). Initial trials with a 100 kDa membrane, using bovine serum albumin (BSA) (66 kDa) as a model protein, confirmed high retention rates (>95%) in the concentrate and minimal loss in the permeate. However, when applied to TB002 samples, partial loss of the target protein was detected in the permeate, likely due to its slightly lower molecular weight. Further analysis by SEC-HPLC and SDS-PAGE confirmed suboptimal retention and increased aggregate formation. The transition to a 50 kDa cut-off membrane significantly improved protein recovery. TB002 was efficiently retained with negligible loss in the filtrate. Based on these observations, the 50 kDa PES membrane was selected as the optimal configuration for both diafiltration and ultrafiltration of TB002 in preparation for thrombin cleavage. The optimized diafiltration protocol was carried out at room temperature using a peristaltic pump with a pressure of approximately 1.5 bar and a flow rate adjusted to maintain optimal transmembrane pressure. The optimized step involved gradual buffer exchange (5-fold) and volume reduction from 150–250 mL to approximately 30–50 mL. This protocol facilitated gradual adaptation of the protein to a more physiological buffer composition. This diafiltration step not only removed urea and imidazole but also cleared PMSF, a protease inhibitor previously included in the extraction buffer that could minimize the thrombin enzyme activity. This was crucial to enable the forthcoming thrombin cleavage. Protein losses were minimal and the clarified retentate was

further concentrated to a final volume of 10–15 mL. Analytical quality control was conducted at this stage using SDS-PAGE, Western blot (anti-His), and UHPLC-SEC to verify the integrity of TB002 and confirm the removal of contaminants. SDS-PAGE and Western blot with anti-His antibody confirmed the presence of intact TB002. UHPLC-SEC analysis showed a monodisperse peak corresponding to the protein (Figure 28). This intermediate purification stage proved essential for ensuring biochemical compatibility with enzymatic tag removal, maintaining protein solubility and structural integrity throughout the workflow.



**Figure 28.** UPLC–SEC chromatogram of TB002 after IMAC purification and diafiltration with 50 kDa MWCO PES membrane. The main peak corresponds to the desalted protein fraction.

#### 5.3.4 Thrombin Cleavage and His-tag Removal

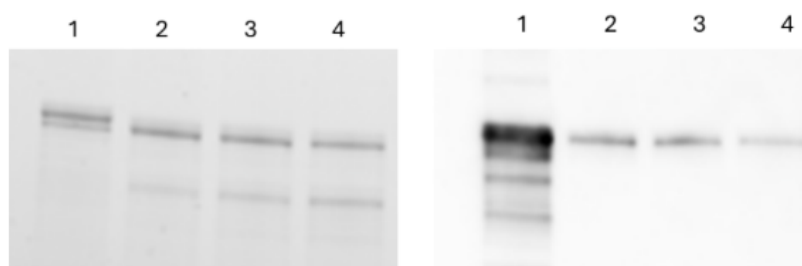
Following concentration and buffer exchange, the TB002 protein was subjected to enzymatic cleavage using thrombin to remove the N-terminal 8xHis-tag. This step was essential to generate a tag-free version of the protein suitable for downstream characterization and potential *in vivo* applications. The expression construct was designed with a specific thrombin cleavage site 8xHis-tag, strategically positioned to avoid the disruption of the immunogenic domain of the protein. Cleavage reactions were performed under controlled conditions, with thrombin doses ranging from 0 to 40 units per mg of protein. A dose-response experiment was conducted to identify the optimal thrombin concentration, testing enzyme levels ranging from 0 to 40 U/mg of TB002. The reactions incubation took place at 22°C for 16 hours in cleavage buffer containing 20 mM Tris, 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, pH 8.0. SDS-PAGE analysis showed a progressive shift in molecular weight corresponding to His-tag removal, with the band shift from ~56 kDa to ~50, consistent with the removal of the His-tag (~6 kDa). Western blot using anti-His antibody confirmed the loss of His-tag, while anti-TB002 antibody validated the presence of the cleaved core protein. Optimal cleavage efficiency (>90%) with



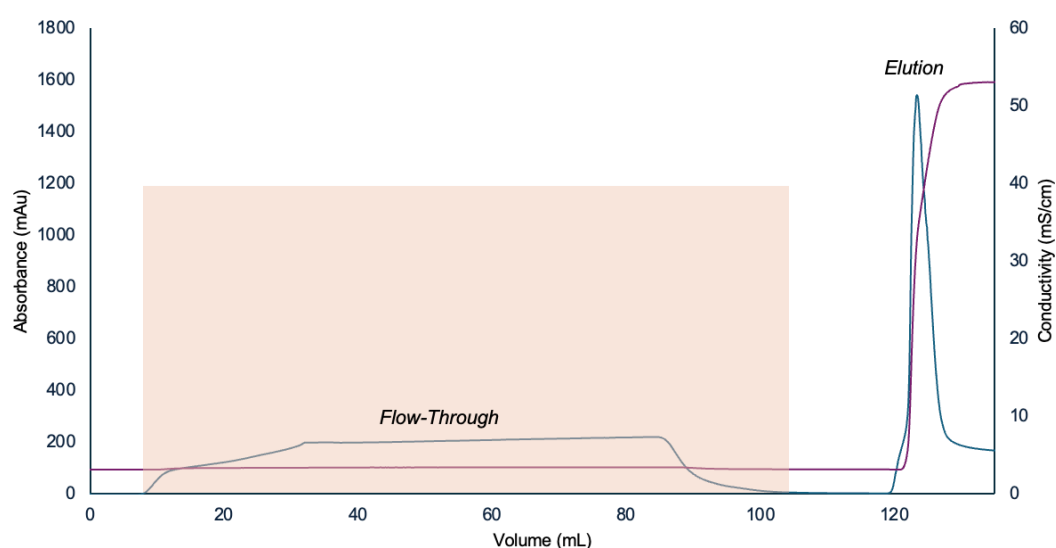
minimal degradation and good protein recovery was achieved at 10–20 U/mg of thrombin. At higher enzyme concentrations ( $\geq 30$  U/mg), nonspecific cleavage and partial degradation of TB002 were observed, evidenced by lower molecular weight bands below 25 kDa and overall protein loss of up to 45% (Figure 29).

In parallel, an on-column cleavage strategy was tested, where thrombin was applied directly to the  $\text{Ni}^{2+}$ -IMAC column after TB002 binding. The goal was to simplify the workflow by performing the enzymatic removal of the His-tag directly on the  $\text{Ni}^{2+}$ -IMAC resin, thereby reducing the need for additional handling and buffer exchanges. A solution containing 240 U of thrombin in 15 mL of cleavage buffer was loaded onto a column with 3.4 mg of bound protein ( $\sim 30$  U/mg), followed by overnight incubation at 26°C under mild agitation. After cleavage, the column was reconnected to the ÄKTA system and eluted using step gradients (40% and 100% Buffer B – same as in Section 5.3.2). Despite the high enzyme concentration and extended incubation time, analytical SDS-PAGE and Western blot showed incomplete cleavage. Anti-His Western blot indicated residual His-tagged protein, likely due to limited enzyme access to the cleavage site when immobilized on resin. Additionally, thrombin-derived degradation products were detected in the eluates, complicating downstream analysis. Additional purification steps were therefore necessary to recover pure, tag-free TB002, diminishing the potential process simplification. Based on these findings, the on-column cleavage strategy was deemed suboptimal. It lacked the reproducibility and cleavage efficiency observed in in-solution protocols.

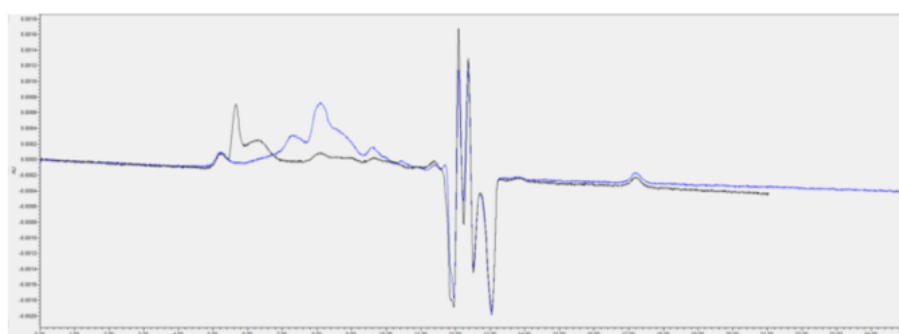
Therefore, in-solution thrombin cleavage remained the preferred and standardized method for TB002 processing. After digestion, a secondary IMAC purification step was performed to remove uncleaved TB002 and residual His-tag-containing fragments. The protein mixture was applied to a  $\text{Ni}^{2+}$ -IMAC column equilibrated with 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, and 2 M urea at pH 7.5. TB002, lacking the His-tag, did not bind to the resin and was collected in the flow-through, while uncleaved or tagged species were retained and later eluted with 500 mM imidazole (Figure 30). This additional IMAC step proved effective in isolating the cleaved protein with high purity and minimal loss, ensuring a homogenous and tag-free TB002 preparation for subsequent polishing and formulation steps.



**Figure 29.** Thrombin concentration optimization for TB002–His tag removal. Stain-free SDS–PAGE (left) and Western blot with anti-His antibody 1:1000 (right) of TB002 incubated with increasing thrombin doses: 0, 20, 40, and 80 U/mg protein (respectively lanes 1–4). The progressive reduction of the anti-His signal at higher enzyme concentrations indicates efficient tag cleavage, with near-complete removal at 80 U/mg and minimal non-specific proteolysis.



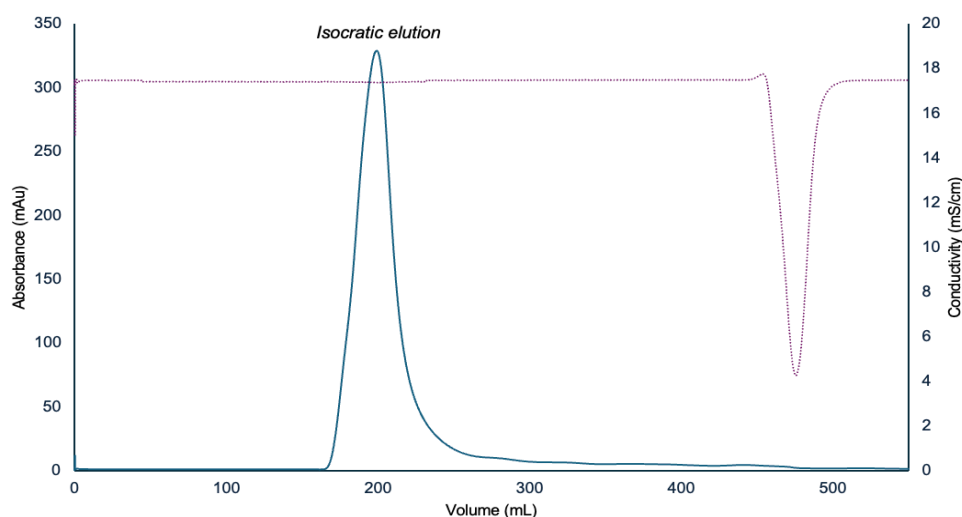
**Figure 30.** IMAC chromatography of TB002 after thrombin digestion. The step was performed to remove the His-tagged forms and residual thrombin, resulting in improved purity of the target protein.



**Figure 31.** HPLC–SEC chromatograms of TB002 before (black trace) and after (blue trace) thrombin cleavage for His-tag removal. The comparison highlights changes in the elution profile upon tag removal, with a shift in the main peak confirming efficient enzymatic processing.

### 5.3.5 Final Polishing and Formulation

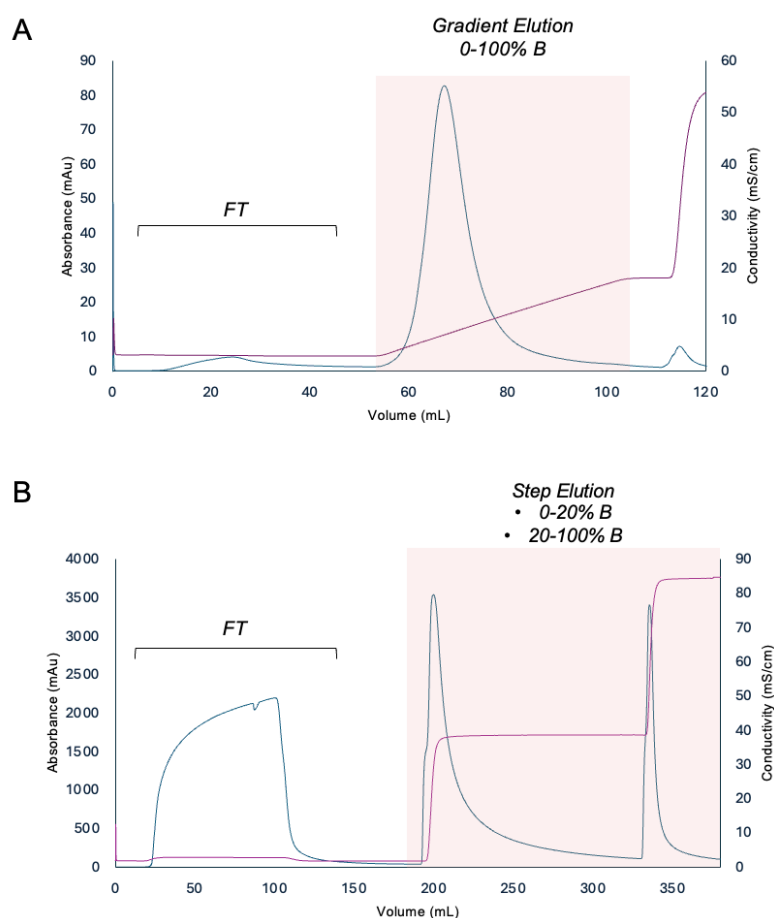
The pooled flow-through from the secondary IMAC step was then subjected to size exclusion chromatography (SEC) using a HiLoad 26/100 Superdex 200 column (530 mL). This step was designed to achieve final product polishing and buffer exchange into the intended formulation conditions. The SEC run was performed using a formulation buffer composed of 50 mM sodium phosphate, 150 mM NaCl, and 1% glycerol at pH 7.4. The chromatographic run revealed a well-resolved main peak corresponding to TB002, typically eluting between 20 and 30 mL (Figure 32). Additional fractions at later retention volumes were collected to assess any tailing material.



**Figure 32.** Size-exclusion chromatography (SEC) used as the final polishing and formulation step for TB002.

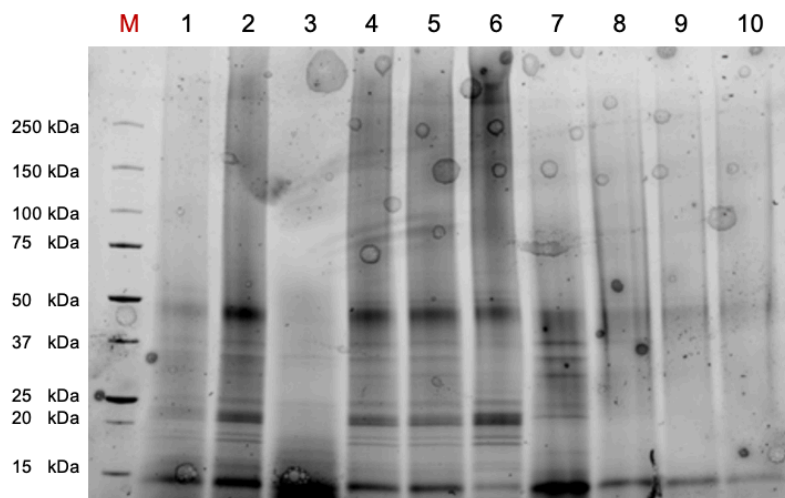
In addition to size-exclusion chromatography (SEC), anion-exchange chromatography (AEX) was explored as a potential alternative polishing step. The rationale behind this investigation was the presence of residual plant-derived contaminants after IMAC, and the need to evaluate methods more compatible with large-scale GMP production. AEX offers several advantages for industrial manufacturing, including resin reusability, low-cost buffers, and effective removal of negatively charged impurities such as host-cell proteins and endotoxins. AEX purification trials were conducted on Q-Sepharose Fast Flow resin (Cytiva) under low-salt loading conditions. The clarified TB002 solution, extracted in the presence of 7 M urea, was concentrated and diafiltered using a 100 kDa PES membrane. Following diafiltration into 50 mM Tris buffer, pH 8.5, the desalted fraction was applied to the column and eluted with increasing NaCl concentrations up to 1 M. SDS-PAGE and Western blot analyses of the flow-

through and eluate fractions showed that TB002 did not bind efficiently to the anion-exchange resin under these conditions (Figure 34). In the first two trials, the majority of the protein was recovered in the flow-through, with eluates enriched only in low-molecular-weight species and aggregates. The third trial, performed after buffer exchange into 0.5 M urea, yielded improved separation, with enrichment of TB002 in the eluted fractions. However, significant amounts of the protein were still detected in both the eluate and the strip, and overall purity remained insufficient (Figure 35). These findings indicate that at the selected pH (8.3–8.5), TB002 carried a net negative charge, which limited its interaction with the positively charged Q matrix. As a result, AEX did not provide a significant purification advantage and was excluded from the final downstream process in favour of a size exclusion chromatography step. By contrast, SEC consistently delivered higher purity, reproducibility across batches, and direct compatibility with formulation conditions. Although AEX was ultimately not integrated into the TB002 process, its evaluation was valuable for benchmarking and confirmed the critical role of high-resolution size-based separation in the final polishing of TB002.

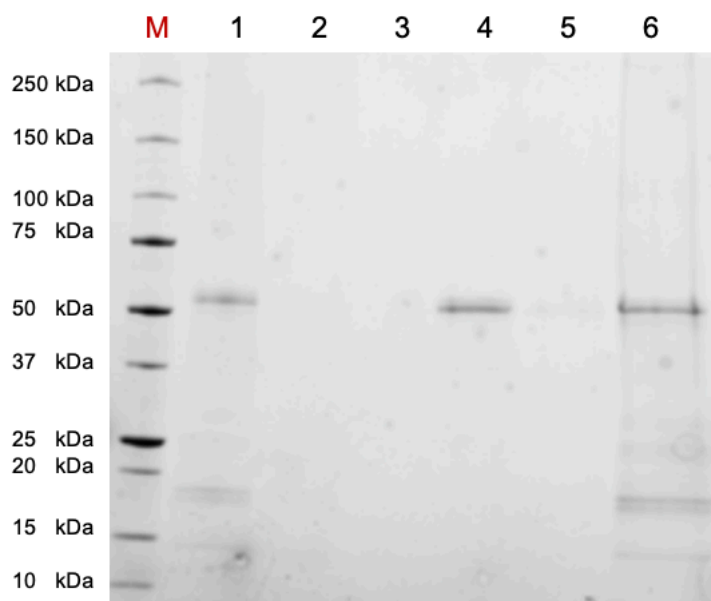


**Figure 33.** Comparison of anion-exchange chromatography (AEX) of TB002 purification trials. Trial 1 (A): linear gradient elution (0–100% Buffer B) resulted in poor separation, with the majority of TB002 recovered in the flow-through and limited resolution of product peaks. Trial 3 (B): stepwise elution (0–

20% Buffer B, followed by 20–100% Buffer B) provided improved peak resolution and partial enrichment of TB002 in the eluted fractions.



**Figure 34.** SDS-PAGE analysis of TB002 fractions collected during anion-exchange chromatography Trial 1. Lane M: molecular weight standard; Lane 1: plant crude extract; Lane 2: retentate 100 kDa; Lane 3: permeate 100 kDa; Lane 4: desalted fraction; Lane 5: desalted fraction incubated o.n. +4°C; Lane 6: FT AEX; Lane 7: Eluate1 AEX; Lane 8: Eluate2 AEX; Lane 9: Strip1 AEX; Lane 10: Strip2 AEX. The gel confirms the limited binding of TB002 to the Q resin under these conditions.



**Figure 35.** SDS-PAGE analysis of TB002 fractions collected during anion-exchange chromatography Trial 3. Lane M: molecular weight marker; Lane 1: Load AEX (desalted IMAC eluate, exchanged into 0.5 M urea); Lane 2: FT AEX; Lane 3: wash AEX; Lane 4: Elution1 AEX w/ 0.3M NaCl; Lane 5: Elution2 AEX w/ 1M NaCl; Lane 6: Strip AEX (100% Buffer C, 7 M urea). Compared to previous trials, elution fractions contained a clearer enrichment of TB002.

The final formulation buffer for TB002 consisted of 50 mM phosphate, 150 mM NaCl, and 1% glycerol, pH 7.4. This buffer composition was selected to ensure isotonicity, minimize aggregation and preserve the solubility of the purified protein. The formulated protein was

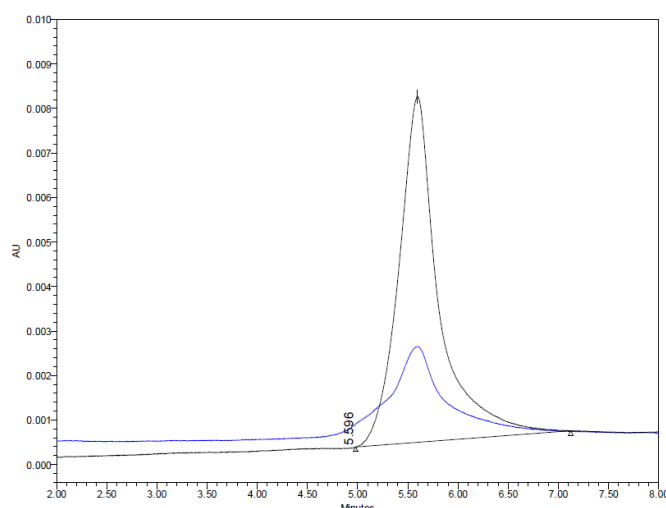
subsequently used for analytical characterization and considered compatible with future in vivo immunogenicity studies. Prior to storage, sterilization was needed. To meet sterility requirements, 0.22 µm syringe filtration was initially considered. A variety of syringe filters with different membrane compositions were evaluated, including cellulose acetate (CA; CHROMACOL 17-SF-02, ADVANTECK 03CP020AS, VWR 28145-477), nylon (CHEBIOS SFNY025022N), and polyethersulfone (PES; Sartorius 16532, VWR 28145-501). SDS-PAGE and Western blot analyses revealed extensive protein loss across all membrane types tested. Most of the TB002 protein was either retained by the membrane or adsorbed to the filter housing (Figure 36). Alternatives such as pre-wetting filters, changing membrane materials (PVDF), and lowering flow rates were also tested, but yielded limited improvement. Of all tested options, the PES Sartorius 16532 filter showed partial protein recovery, although significant losses were still reported. For example, in a representative trial using CA membranes, only 37% of total protein was recovered post-filtration (Table 12). Due to these filtration challenges, UV sterilization was adopted as a non-contact alternative. TB002 samples were irradiated with UV light for 2 hours at room temperature. Post-irradiation analysis using SDS-PAGE and size exclusion chromatography confirmed the absence of degradation, indicating complete sterilization without compromising protein integrity. Final TB002 samples were stored in the formulation buffer and maintained at either 4°C or -80°C. Stability studies revealed that samples stored at -80°C retained structural integrity and solubility for over 3 months, as confirmed by SDS-PAGE.

**Table 12.** Recovery efficiency of TB002 after sterile filtration using different membrane types and brands. Among the tested filters, polyethersulfone (PES) membranes performed better than cellulose acetate (CA) or nylon, with the Sartorius PES filter showing the highest protein recovery (~60%).

| Filter Type            | Brand                 | Recovery (%)             |
|------------------------|-----------------------|--------------------------|
| Cellulose Acetate (CA) | - CHROMACOL 17-SF-02  | • ≤40%                   |
| Cellulose Acetate (CA) | - Advantec 03CP020AS  | • ≤35%                   |
| Cellulose Acetate (CA) | - WVR 28145-477       | • ~37%                   |
| Nylon                  | - CHEBIOS SFNY025022N | • <30%                   |
| Polyethersulfone (PES) | - VWR 28145-501       | • ~45%                   |
| Polyethersulfone (PES) | - Sartorius 16532     | • ~60% (best performing) |



**Figure 36.** SDS–PAGE analysis of TB002 recovery after 0.22  $\mu\text{m}$  sterile filtration. Lane M: molecular weight marker; Lane 1: bulk material; Lane 2: bulk after UV sterilization (2h, RT); Lane 3: bulk after room temperature incubation (RT); Lane 4: cellulose acetate (CA) filter, CHROMACOL; Lane 5: nylon filter, CHEBIOS; Lane 6: cellulose acetate (CA) filter, ADVANTECK; Lane 7: PES filter, Sartorius; Lane 8: cellulose acetate (CA) filter, VWR; Lane 9: PES filter, VWR. The gel confirms that significant protein loss occurs when TB002 is passed through syringe filters, regardless of membrane type.



**Figure 37.** UPLC–SEC chromatograms of TB002 before (black trace) and after (blue trace) sterile filtration through 0.22  $\mu\text{m}$  syringe filter. The post-filtration profile shows a marked reduction in peak intensity, confirming significant protein loss, consistent with SDS–PAGE analysis (Figure 36).

## 5.4 Analytical Characterization

Analytical characterization plays a fundamental role in downstream bioprocess development and validation, particularly in the context of plant molecular farming, where matrix complexity, protein heterogeneity, and host-derived contaminants require rigorous quality assessment. In biopharmaceutical manufacturing, analytical techniques are not only employed to verify product identity and purity, but also to monitor process performance, ensure consistency, inform decision-making during scale-up, and ensure compliance with regulatory guidelines

for critical quality attributes (CQAs). These analytics support both process development and product comparability, which are essential for future GMP implementation and clinical translation. Throughout the downstream workflow of TB002, all process intermediates – from crude extract to final desalted bulk – were systematically analysed using a panel of orthogonal analytical tools.

SDS-PAGE (TGX Stain-Free 4–20%) was used for protein profiling, enabling rapid assessment of extraction efficiency, purification performance, and degradation. Western blotting was employed in parallel to confirm identity and detect tagged versus cleaved protein species. Anti-His monoclonal antibodies allowed specific tracking of His-tagged TB002, while a polyclonal anti-TB002 antibody enabled confirmation of core protein identity post-cleavage. SDS-PAGE results across the process revealed a consistent ~56 kDa band corresponding to the expected TB002 size. In early process steps, additional bands indicated co-eluted plant proteins and partial degradation products, particularly in fractions from IMAC and diafiltration stages. Following thrombin cleavage, a molecular shift of ~6 kDa was observed, consistent with successful His-tag removal. To complement gel-based methods, UPLC-SEC was used to assess the aggregation profile and conformational homogeneity of TB002. The final desalted bulk product consistently displayed a sharp, symmetrical peak with a retention time indicative of a tetramer species, confirming the presence of major aggregates. Aggregation was reduced significantly following diafiltration and polishing, confirming the robustness of the process. Protein quantification was performed using the bicinchoninic acid (BCA) assay. The method was employed throughout the workflow for routine tracking of protein recovery in intermediate fractions. The final purified and formulated TB002 bulk was quantified using a BCA assay calibrated with a BSA standard curve. The concentration was estimated at 200–205 µg/mL, corresponding to a process yield of approximately 65 mg of purified TB002 per batch. These values are consistent with small-scale transient expression yields reported for other recombinant antigens in *N. benthamiana* (202). Collectively, the analytical data confirmed that the downstream process consistently yielded a correctly folded, and tag-free TB002 protein suitable for further immunogenicity evaluation. The analytical platform developed here will serve as a foundation for future GMP-oriented comparability studies.

## 5.5 Discussion and Future Directions

The downstream development of TB002 highlighted several technical challenges commonly associated with plant-based expression systems. These included solubility issues,



aggregation during extraction and purification, degradation during enzymatic tag removal, and significant protein loss during sterilization attempts. Each purification step – ranging from early extraction buffer optimization to final polishing and formulation – was systematically refined to overcome these obstacles and establish a robust process suitable for preclinical application. Solubility and aggregation issues, particularly pronounced during the initial IMAC trials, were effectively mitigated by incorporating 7 M urea into the extraction and binding buffers. This denaturing condition enabled consistent recovery of TB002 and significantly improved process reproducibility. Thrombin-mediated His-tag removal was another critical step, requiring dose-response studies to identify the optimal enzyme-to-protein ratio that maximized cleavage efficiency while minimizing nonspecific degradation. Protein recovery following 0.22 µm filtration proved problematic due to strong adsorption to membrane surfaces. A UV sterilization strategy was therefore implemented as a non-invasive and protein-compatible alternative, offering complete microbial inactivation without compromising structural integrity. The final formulation – with buffer composed of 50 mM sodium phosphate, 150 mM NaCl, and 1% glycerol at pH 7.4 – maintained protein stability and solubility across storage conditions and was deemed compatible with in vivo administration. The resulting TB002 bulk was used for murine immunogenicity studies, demonstrating that the downstream process yielded material suitable for biological evaluation. Across different batches, the process exhibited high reproducibility, with consistent SDS-PAGE profiles and final concentrations of approximately 200 µg/mL. All buffers and materials were selected based on regulatory guidelines and industrial feasibility. Affinity chromatography, diafiltration, and SEC polishing steps were executed using scalable platforms compatible with GMP operations. Importantly, the downstream process was designed with modularity in mind – each unit operation can be linearly scaled by adjusting column volumes, filtration surface area, and flow parameters. This approach enables adaptation to stainless-steel or fixed-bed purification systems, without the reliance on single-use technologies. The process robustness and simplicity make it amenable to both pilot-scale production and future technology transfer. Overall, the downstream platform developed for TB002 constitutes a solid foundation for future scale-up and GMP adaptation. It demonstrates feasibility for plant-based production of recombinant subunit vaccines and may serve as a reference model for similar antigens requiring high purity and structural integrity. The successful implementation of this strategy further reinforces the promise of PMF as a viable alternative to traditional expression platforms in the development of affordable, scalable, and safe human vaccines.

## 6. mRNA Vaccine Platform Produced in Plants

This chapter describes the experimental development of a plant-based platform for the production and purification of messenger RNA (mRNA) in *Nicotiana benthamiana*. The work was designed as a proof-of-concept study to evaluate whether transient expression in plants could be adapted for mRNA construct originally optimised for cell-free transcription systems. The experimental programme encompassed vector design and quality control, plant-based expression, and the evaluation of multiple downstream purification strategies with the objective of defining operational boundaries for the recovery of high-quality plant-derived mRNA. All experiments were conducted using a laboratory greenhouse (Section 4.3) under controlled conditions in the department of Biochemical Engineering at University College London (UCL).

### 6.1 Introduction and Scientific Background

Messenger RNA (mRNA) vaccines have rapidly emerged as a central tool in combating infectious diseases, particularly highlighted by the global response to the COVID-19 pandemic (203, 204). Their key advantages – rapid development cycles, transient expression, and lack of genomic integration – have established mRNA as a highly versatile vaccine platform, with potential applications extending to oncology, personalized medicine, and emerging viral threats (205, 206). Structurally, synthetic mRNAs are designed to mimic eukaryotic transcripts. A typical construct consists of a 5' cap (typically Cap-1), a 5' untranslated region (UTR), an open reading frame (ORF), a 3' UTR, and a poly(A) tail. Each element contributes to mRNA stability, translational efficiency, and immunogenic modulation (207). For example, beta-globin-derived UTRs enhance half-life and translational yield (208), while engineered poly(A) tails and modified nucleotides reduce innate immune activation (209). Manufacturing of mRNA is primarily achieved through cell-free *in vitro* transcription (IVT), catalysed by bacteriophage T7 RNA polymerase and using linearized plasmid DNA template (210). Although efficient and highly productive, the IVT process is prone to generate unwanted double-stranded RNA (dsRNA) contaminants due to template-independent extension and secondary structure formation (211). These by-products can suppress translation and trigger innate immune responses via TLR3, MDA5 and RIG-I recognition (212). To address this challenge, several studies have focused on both process engineering and analytical control. Optimization strategies include the use of optimized promoter variants

(engineered T7 promoter), modified transcription buffers, and advanced enzymatic capping methods (213, 214, 215). Analytical control is equally critical: techniques such as ELISA, reversed-phase UHPLC, and ion-pair chromatography have demonstrated high sensitivity for dsRNA detection, enabling process feedback and optimization (216, 217).

The platform developed by *Rosa et al. (2022)* (218) incorporated many of these advances, integrating process optimization with real-time analytical feedback to achieve yields above 12 g/L with reduced dsRNA burden. However, the reliance on enzymatic and cell-free systems imposes cost and scalability limitations (219). This has prompted exploration into unconventional expression platforms, where plants emerge as an interesting option (220). Plant molecular farming, already validated for complex biologics such as monoclonal antibodies and virus-like particles, offers a cost-effective, inherently safe, and scalable production system (45). *Nicotiana benthamiana*, in particular, is well-suited to transient expression via agroinfiltration, enabling rapid prototyping and flexible manufacturing of biologics, including mRNA constructs (37, 57).

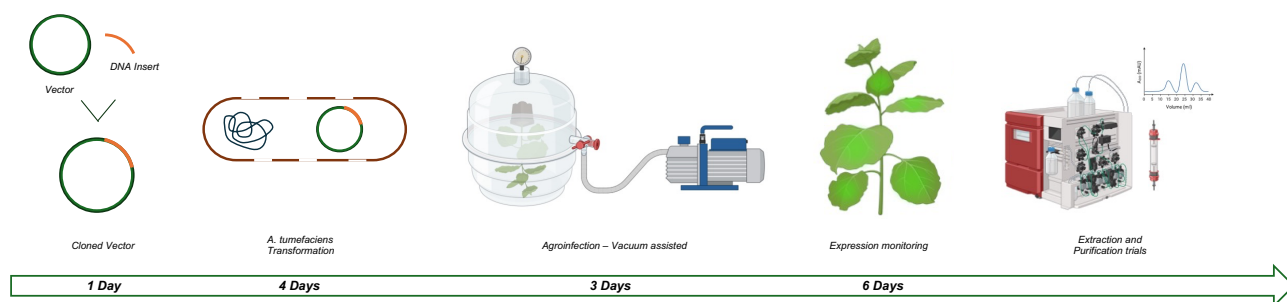
In this context, the present work explores the feasibility of producing a model mRNA vaccine in plants, leveraging the lab-scale greenhouse platform described in Section 4.3. This work builds on the modular synthetic biology framework developed by *Rosa et al. (2022)* (218), using a well-characterized GFP-encoding mRNA construct. The objective was to investigate the potential of *Nicotiana benthamiana* as a host for in planta mRNA delivery and expression. Section 4.3 describes the design of the lab-scale greenhouse platform optimized for controlled infiltration studies, while this chapter focuses on the integration of synthetic mRNA into plant tissues and subsequent evaluation of transcript stability and translational activity. The plant-based approach aims not to replace cell-free IVT but to assess whether living photosynthetic organisms can contribute to the distributed, scalable production of mRNA vaccines. This proof-of-concept project thus addresses both practical and theoretical questions regarding nucleic acid stability, delivery, and bioavailability within a plant cell environment.

## 6.2 Experimental Design

The plant-based expression strategy was designed to test whether an mRNA sequence originally optimized for in vitro transcription (IVT) could be successfully transcribed and expressed in *Nicotiana benthamiana*. The synthetic mRNA sequence used in this project was adapted from the sequence originally optimized for *in vitro* transcription (IVT) by *Rosa et al.*

(2022) (218). The original construct incorporated a 5' untranslated region (UTR) containing a T7 promoter, an eIF4G binding site, and a Kozak consensus sequence, followed by the open reading frame (ORF) encoding enhanced green fluorescent protein (eGFP). The 3' UTR consisted of two tandem  $\beta$ -globin motifs and a segmented poly(A) tail of approximately 120 nucleotides, all of which had been demonstrated to enhance mRNA stability and translational yield in cell-free and mammalian systems (221).

In order to adapt this transcript for plant-based expression, the sequence was cloned into the binary vector pGreenII 0579-1 (222). The pGreenII series of plasmids is specifically designed for plant transformation and offers several advantages. The pGreenII system offers a minimal backbone size, facilitating efficient cloning and transformation, and multiple cloning sites for flexible construct design. There is also proven track record in transient expression studies in *N. benthamiana*. These features make it a widely adopted tool for plant molecular biology, especially for agroinfiltration-based transient expression (223). The CaMV 35S promoter, present in the pGreenII vector, is particularly effective in *Nicotiana benthamiana*, the host plant used in this study, due to its strong interaction with host transcription machinery and its proven performance in transient expression via *Agrobacterium*-mediated delivery (224). The cloning strategy preserved the CaMV polyadenylation signal to ensure transcript stability in planta.



**Figure 38.** Workflow of the mRNA vaccine production platform in *Nicotiana benthamiana*. The process includes vector design and gene construct assembly, plant cultivation and *Agrobacterium*-mediated delivery, followed by mRNA extraction, downstream purification and analytical characterization.

The experimental workflow was structured to ensure reproducibility and efficiency across all trials. The pGreenII-eGFP plasmid was first propagated in *Escherichia coli* DH5 $\alpha$  under kanamycin selection, followed by plasmid purification and quality control checks. To confirm the presence and integrity of the insert, restriction enzyme digestion was performed using Sapl and Nocl enzymes. Furthermore, Sanger sequencing (Plasmidsaurus, USA) was used to verify the fidelity of the eGFP sequence. The pGreenII backbone lacks the origin of replication required for propagation in *Agrobacterium tumefaciens*. To overcome this, all

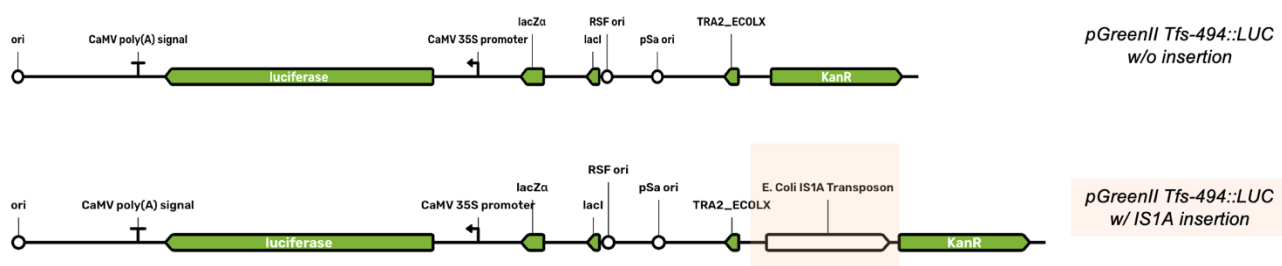
transformations were carried out in combination with the helper plasmid pSoup, which supplies the missing replication functions. The verified plasmid was co-transformed with pSoup into *A. tumefaciens* strain LBA4404 via electroporation, using optimized voltage and recovery conditions. Establishing a robust *Agrobacterium tumefaciens* transformation pipeline required significant troubleshooting and iteration but ultimately enabled reliable delivery of the construct into plant tissues. The transformed *A. tumefaciens* cultures were used at a standardized optical density (OD<sub>600</sub>) for vacuum-assisted agroinfiltration of five-week-old *N. benthamiana* plants grown under controlled hydroponic greenhouse conditions. Vacuum-assisted infiltration was used to deliver transformed *Agrobacterium tumefaciens* into *Nicotiana benthamiana* leaf tissues. Whole plants were immersed in the bacterial suspension during the infiltration process in order to maximise uptake and promote uniform transient expression across the plant canopy (225). Upon release of vacuum pressure, plants were returned to the greenhouse under controlled conditions to allow transient expression. This systematic approach formed the foundation for the subsequent experimental campaigns, which aimed to assess the performance of the adapted construct in planta.

### 6.3 Extraction and Characterization of mRNA produced in plants

#### 6.3.1 pGreenII – Binary Plant Vector

The first set of cloning and transformation experiments employed the binary vector pGreenII 0579-1 (35S::LUC) carrying the eGFP expression cassette under the control of the CaMV 35S promoter. Initial quality control involved diagnostic restriction digestion with *SapI* and *NocI* enzymes, followed by agarose gel electrophoresis to verify cassette size and arrangement. While digestion profiles matched the expected pattern, subsequent sequencing of the backbone revealed the presence of an unexpected insertion. The insertion was located outside the eGFP expression cassette, within a non-coding region of the vector backbone, and was absent from the reference sequence of pGreenII 0579-1 (35S::LUC). Such insertions are typically the result of recombination events during *Escherichia coli* propagation or can be carried over from contaminated upstream cloning stocks. While not directly disrupting the transgene, modifications in backbone architecture can influence plasmid topology, replication in *Agrobacterium tumefaciens*, and, ultimately, transient expression efficiency in planta. To determine whether this was an isolated contamination or a recurring issue, plasmid DNA from multiple *E. coli* colonies was sequenced. The insertion

was present in all tested clones, suggesting that the contamination was already present in the working stock. In an attempt to circumvent the problem, an alternative backbone, pGreenII Tfs-494::LUC, was sourced and tested. This vector retains the same minimal T-DNA region and pSa origin-dependent replication system as pGreenII 0579-1, ensuring compatibility with the pSoup helper plasmid, while differing in multiple cloning site configuration and selectable marker features. However, sequencing of the pGreenII Tfs-494 stock revealed the same insertion (Figure 39), indicating that the issue was not restricted to a single batch or vector type. This finding confirmed that a complete backbone replacement with a verified sequence was necessary. The persistence of the insertion in both pGreenII 0579-1 and pGreenII Tfs-494 prompted an attempt to reconstruct a clean backbone using Gibson Assembly. The kanamycin-resistance region from a verified plasmid free of insertions was amplified and used to replace the corresponding defective fragment in the original construct. The rationale was that this substitution would maintain all functional elements of the vector while eliminating the extraneous sequence. However, post-assembly sequencing revealed that the insertion was still present. This outcome confirmed that the contaminating sequence was more pervasive than initially assumed and was likely embedded in the stocks of both pGreenII variants available for this work. As a result, no fully clean pGreenII backbone could be obtained during the initial experimental timeframe. Consequently, all the transformation and infiltration experiment here described were carried out using sequence-verified plasmids containing the insertion, with the understanding that this structural anomaly represented an intrinsic limitation of the present study. This process, though time-consuming, ensured that all downstream experiments were conducted with vectors of verified sequence.

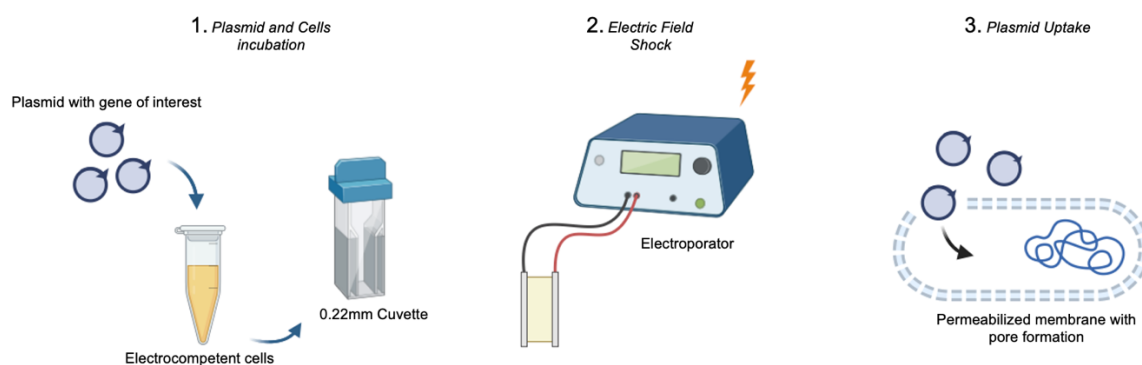


**Figure 39.** Schematic representation of the pGreenII Tfs-494::LUC vector. Construct without insertion (top), containing the luciferase reporter under the control of the CaMV 35S promoter and poly(A) signal, together with the kanamycin resistance marker. Construct with insertion of an E. coli IS1A transposon element (bottom) upstream of the kanamycin resistance cassette.

### 6.3.2 *Agrobacterium tumefaciens* Transformation

The binary vector pGreenII requires co-transformation with the pSoup helper plasmid to replicate in *Agrobacterium tumefaciens*. For this work, we selected the disarmed strain of *A. tumefaciens* LBA4404, a derivative of the octopine-type C58 lineage, widely used in plant biotechnology for its high transformation efficiency and reduced virulence in non-target hosts (226). LBA4404 carries intrinsic resistance to rifampicin and streptomycin, allowing straightforward selection in co-transformation experiments when combined with kanamycin resistance from the pGreenII backbone. The compatibility between pGreenII, pSoup, and LBA4404 was essential to maintain both plasmids stably during propagation and plant infiltration (227).

Electroporation was chosen over other transformation methods (such as freeze–thaw or triparental mating) due to its reproducibility, shorter turnaround time, and lower requirement for specialized reagents (174, 177) (Figure 40). To generate electrocompetent LBA4404 cells, fresh cultures were grown in LB medium at 28 °C to mid-log phase ( $OD_{600} \sim 0.5\text{--}0.6$ ), then harvested by centrifugation and subjected to multiple washes in ice-cold 10% glycerol to remove ionic contaminants. All steps were carried out at 4 °C to preserve cell viability. Initial electroporation trials tested a range of voltages between 1.8 and 2.2 kV, keeping the cuvette gap at 0.2 cm. Early experiments showed inconsistent colony formation, particularly at higher voltages where arcing occasionally occurred. Through systematic testing, an optimal pulse strength of 2.5 kV was identified, which consistently yielded transformants while maintaining high cell survival rates. Recovery was performed in SOC medium at 29°C with gentle shaking (200 rpm) for 3–4 hours, allowing expression of antibiotic resistance markers prior to plating. For selection, LB agar plates were supplemented with 50 µg/mL kanamycin, 25 µg/mL rifampicin, and 100 µg/mL streptomycin, enabling co-selection of both pGreenII and pSoup-containing clones. Colonies typically appeared within 48 hours. To confirm the presence of the correct construct, multiple colonies from each electroporation batch were selected and validated by miniprep plasmid extraction and restriction digestion to assess plasmid integrity.



**Figure 40.** Schematic representation of the electroporation process to transform *A. tumefaciens* cells. (1) Incubation of plasmid DNA with electrocompetent cells. (2) Application of an electric field shock using an electroporator. (3) Uptake of plasmid DNA facilitated by transient pore formation in the bacterial membrane.

The optimized protocol significantly improved transformation reproducibility, reducing failed electroporation observed in early trials. Once established, this workflow became the standard for generating *A. tumefaciens* inoculum for plant infiltration experiments in this study. The optimization not only ensured reliable delivery of the eGFP construct but also minimized time lost in repeated unsuccessful transformations, which had been a bottleneck during the early stages of the project.

### 6.3.3. Agroinfiltration of *Nicotiana benthamiana* plants

Following successful transformation of *Agrobacterium tumefaciens* LBA4404 with the pGreenII-eGFP construct and the pSoup helper plasmid, plant infiltration experiments were carried out to deliver the expression cassette into *Nicotiana benthamiana* tissues. Vacuum-assisted agroinfiltration was selected over syringe infiltration due to its superior reproducibility, ability to uniformly treat large sections of plant tissue, and suitability for scaling up in controlled environments (228). This method enables penetration of bacterial suspensions deep into the leaf apoplast, increasing the number of transformed cells and ultimately enhancing transgene expression (229). Bacterial cultures of transformed *A. tumefaciens* LBA4404 were grown overnight in LB medium supplemented with the appropriate antibiotics at 28 °C with shaking until reaching  $OD_{600} \cong 2.0$ . Cells were harvested by centrifugation at  $4000 \times g$  for 10 min at room temperature, and the resulting pellet was washed once with infiltration buffer (10 mM MES, 10 mM  $MgCl_2$ , pH 5.7) to remove residual growth medium and antibiotics. The pellet was then gently resuspended in fresh infiltration buffer and homogenized by slowly pipetting to avoid cell clumping. The suspension was diluted to a final  $OD_{600}$  of 0.4, a value previously optimized for transient expression in *N. benthamiana* to balance high transformation efficiency with minimal risk of tissue necrosis



(87). This standardization step ensured reproducibility across infiltration trials. The bacteria suspension was used within 30 minutes of final OD adjustment to preserve bacterial viability and maintain consistent transformation efficiency.

Infiltrations were performed using five-week-old *N. benthamiana* plants grown hydroponically under controlled greenhouse conditions. Plants were fully immersed in the bacterial suspension contained within an 8 L capacity desiccator connected to a Sartorius vacuum pump. A vacuum of approximately -600 mbar was applied for 3 minutes, which was sufficient to remove air from the intercellular spaces without causing tissue collapse. Rapid restoration of atmospheric pressure forced the bacterial suspension deep into the leaf apoplast, facilitating uniform delivery of the *Agrobacterium* cells across multiple leaves and even into smaller veins. Visual inspection immediately after infiltration confirmed that the protocol did not induce leaf wilting, chlorosis, or other visible stress responses. Plants were allowed to drain briefly before being returned to the greenhouse under the same controlled conditions to recover and initiate transgene expression.

Two sets of controls were included in each infiltration experiment:

1. Negative control — un-infiltrated plants grown under identical conditions, serving as a baseline for fluorescence and physiological state.
2. Agroinfiltrated leaves — plants agroinfiltrated with an *A. tumefaciens* suspension carrying a previously validated GFP expression construct.

These controls allowed discrimination between background signal and genuine GFP expression resulting from delivery of the experimental construct.

#### 6.3.4 mRNA Expression Quantification

Following infiltration, plants were monitored for signs of GFP expression. Qualitative assessment was initially performed by visual inspection under a DARKBEAM UV Light Torch equipped with a 395 nm emission filter, allowing rapid identification of infiltrated areas exhibiting green fluorescence. This approach enabled non-destructive tracking of expression onset and distribution across the plant canopy. In most experiments, detectable fluorescence appeared peaked around day 5 after agroinfection. Once peak expression was reached, leaves from both experimental and control plants were harvested for downstream analysis. Immediately after collection, leaves were placed in sealed bags and stored at – 80 °C until

extraction. This workflow preserved RNA integrity and prevented degradation prior to processing.

The extraction workflow aimed to isolate high-quality mRNA from *N. benthamiana* leaf tissue for subsequent quantification and characterization. All steps were conducted using certified RNase-free consumables and freshly prepared buffers. For each extraction, leaf material was weighed and extraction buffer (10 mM phosphate, 2 mM EDTA, 150 mM NaCl, pH 7.4) was added in a 1:2 ratio (g fresh weight: mL buffer). Tissue homogenization was performed using a mechanical blender pre-chilled for 2 minutes. The homogenate was clarified by centrifugation at  $15\,000 \times g$  for 15 min at 4 °C, and the supernatant was collected and filtered through 0.65 µm filters to obtain clarified lysates for purification trials.

Multiple purification strategies were tested, including:

- Anion exchange chromatography to capture RNA based on its negative charge.
- Oligo(dT)-based chromatography to selectively isolate polyadenylated mRNA.
- Salting-out precipitation using ammonium sulphate to separate the mRNA based on hydrophobicity characteristics.
- Silica membrane-based extraction (RNeasy Plant Mini Kit) as a commercial benchmark method.
- Size exclusion chromatography (SEC) for separation of RNA species by size.

Each method was evaluated for yield, purity ( $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  ratios), and integrity through agarose gel electrophoresis.

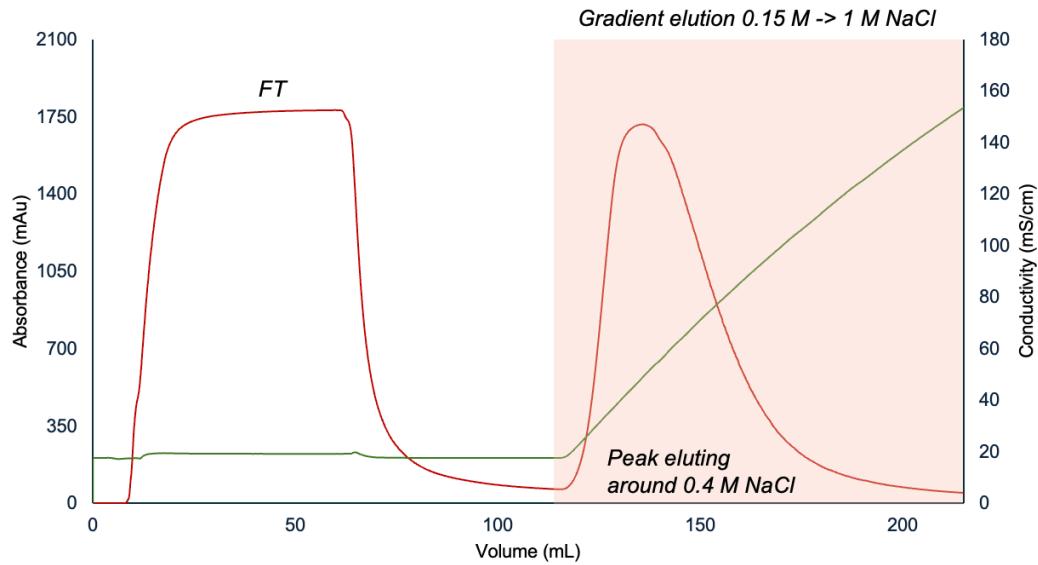
Given the polyanionic nature of RNA, anion exchange chromatography was selected as the first purification strategy (230), with CaptoQ ImpRes chosen for its high binding capacity, mechanical stability, and documented compatibility with plant-derived extracts. The resin is functionalized with quaternary ammonium ligands, which enable efficient capture of nucleic acids under low-salt conditions and controlled release through a salt gradient. This property made CaptoQ particularly suitable for an initial capture step aimed at enriching mRNA from complex plant lysates. The first trials were performed using clarified extracts from agroinfiltrated leaves and, as negative controls, from wild-type plants processed in parallel. Columns were pre-equilibrated in extraction buffer (10 mM phosphate, 2 mM EDTA, 150 mM NaCl, pH 7.4) and loaded at flow rates of 0.5–1.0 mL/min. Early chromatograms showed pronounced absorbance peaks at 280 nm in the flow through and early fractions, reflecting the elution of proteins and chlorophyll-derived pigments. Subsequent peaks at higher

conductivity indicated the release of nucleic acids. Notably, the agroinfiltrated extracts consistently yielded higher  $A_{260}$  signals compared to wild-type samples, supporting the presence of RNA species associated with transgene expression. Elution was performed using a NaCl gradient ranging from 0.15 M to 1 M, with fractions collected at each step. In the first elution gradients, nucleic acid-rich fractions began to appear at intermediate salt concentrations (0.4–0.6 M NaCl), where distinct  $A_{260}$  peaks were reproducibly detected (Figure 41). These observations established CaptoQ ImpRes as a promising platform for nucleic acid capture from plant extracts and guided further optimization of gradient design and sample preparation. To further validate the performance of the CaptoQ ImpRes resin, comparative runs were performed using extracts from agroinfiltrated plants and wild-type controls processed under identical conditions. Chromatograms were overlaid to directly compare the absorbance profiles (Figure 42). In every case, the agroinfiltrated samples exhibited higher overall signals than wild-type extracts, with maxima reaching ~1800 mAu compared to ~1600 mAu in negative controls. This difference was consistent across independent runs and strongly suggested the presence of additional nucleic acid species in agroinfiltrated samples. Beyond the difference in total absorbance, the shape and distribution of elution peaks also varied between conditions. Agroinfiltrated extracts displayed pronounced  $A_{260}$  peaks emerging at intermediate salt concentrations (approximately 0.4–0.6 M NaCl), whereas wild-type controls yielded weaker and less defined signals in the same region. These peaks corresponded to the fractions later confirmed by agarose gel electrophoresis to contain RNA species, supporting the conclusion that agroinfiltrated samples were enriched in nucleic acids relative to background levels in non-infiltrated plants. This comparative analysis not only validated the ability of CaptoQ to discriminate RNA-containing fractions but also provided the first chromatographic evidence that transgene-derived transcripts were present in agroinfiltrated material. These results motivated subsequent optimization of the gradient design and sample handling to improve resolution and recovery of the target mRNA. Following the initial runs, it became evident that further optimization was required to improve the resolution between contaminating plant components and RNA-containing fractions. One key adjustment was the dilution of the crude extracts prior to loading. Trials were conducted using 10× and 20× dilutions of clarified lysates in low-salt binding buffer, which reduced the ionic strength at the point of loading and enhanced the interaction between RNA and the quaternary ammonium ligands. These diluted samples produced cleaner chromatographic baselines and sharper transitions between early protein-rich peaks and the nucleic acid elution zone. In parallel, the design of the elution gradient was

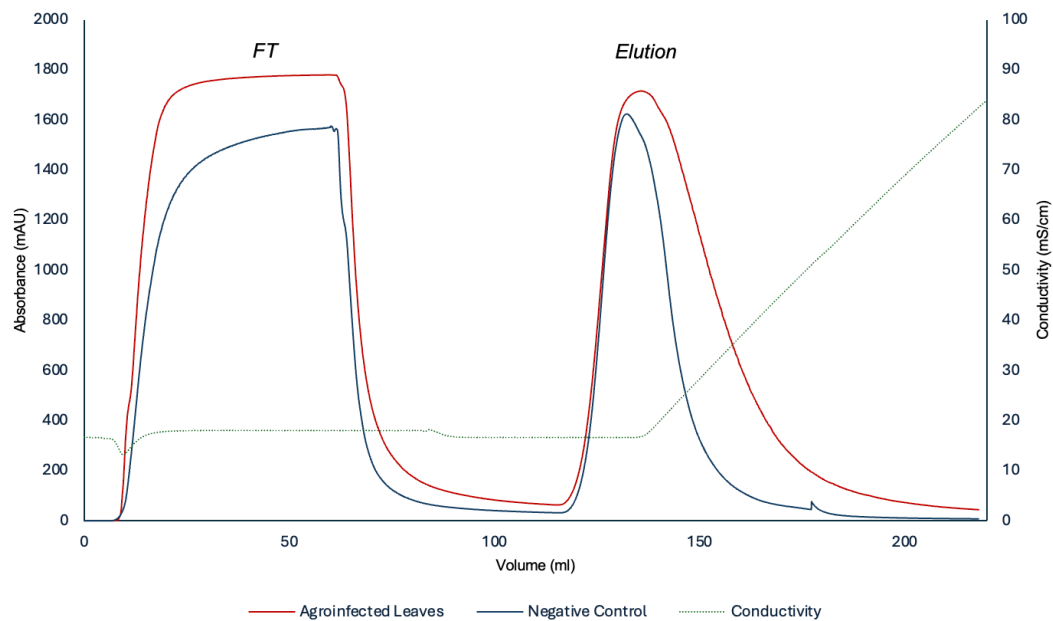
refined. Initial gradients of 20 column volumes (c.v.) proved too shallow, resulting in broad and overlapping elution profiles. By reducing the gradient length to 10–15 c.v., distinct peaks emerged more clearly at intermediate salt concentrations. The shorter gradients also improved reproducibility between runs, with nucleic acid peaks consistently appearing between 0.4–0.6 M NaCl. This concentration range was identified as optimal for selectively releasing RNA while leaving proteinaceous impurities bound to the matrix or eluted at lower salt steps. Together, the combination of sample dilution and shorter gradients substantially improved the separation efficiency of the CaptoQ method, yielding better defined RNA-containing fractions suitable for downstream validation. These optimizations also reduced processing times and buffer consumption, making the method more practical for repeated trials.

The chromatographic observations were corroborated by agarose gel electrophoresis of fractions collected across the runs. Both flow-through (FT) and eluted fractions were analysed to confirm RNA binding and release. As expected, the FT contained minimal RNA, demonstrating that the majority of nucleic acids were captured by the resin under the chosen loading conditions. Eluted fractions from wild-type plants did reveal the presence of RNA, reflecting the capture of abundant endogenous species such as rRNA and tRNA; however, these signals were relatively weak and diffuse compared to those obtained from agroinfiltrated samples. In contrast, fractions from agroinfiltrated plants showed stronger and more distinct RNA bands (Figure 43). A reproducible banding pattern was observed in fractions eluting at ~0.5 M NaCl, which correlated with the  $A_{260}$  peaks recorded on the chromatograms. In several experiments, a discrete band corresponding to the expected size of the eGFP transcript could be distinguished, supporting the conclusion that the method was successfully enriching transgene-derived mRNA from the plant extract. The difference in band intensity between agroinfiltrated and wild-type samples reinforced the specificity of the approach, despite the presence of co-eluting background RNAs.

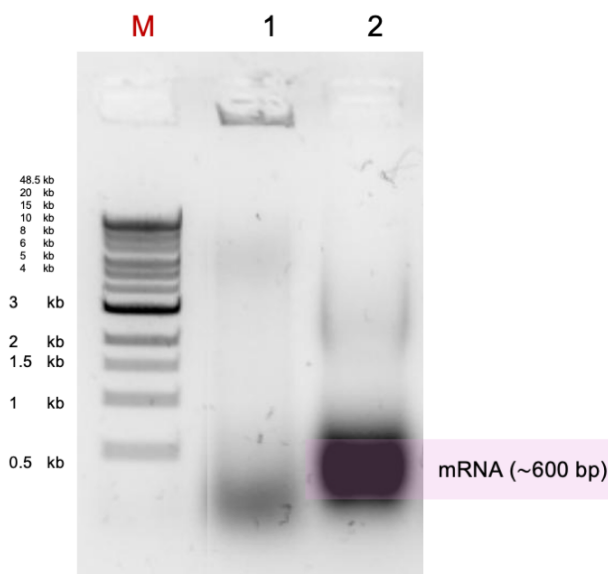
These results marked the first direct evidence of successful capture of plant-produced mRNA transcripts by anion exchange chromatography. While the CaptoQ ImpRes method provided good binding and recovery of RNA, co-elution of rRNA and other plant RNAs was observed, as expected for charge-based separation. Therefore, in some trials, RNA-containing fractions were subjected to a secondary poly(A)-specific capture step using oligo(dT) chromatography to improve specificity for mRNA.



**Figure 41.** CaptoQ ImpRes chromatography for mRNA capture. Agroinfiltrated extracts displayed pronounced peaks eluting at intermediate salt concentrations ( $\sim 0.4\text{--}0.5\text{ M NaCl}$ ), consistent with the retention and recovery of nucleic acid species.



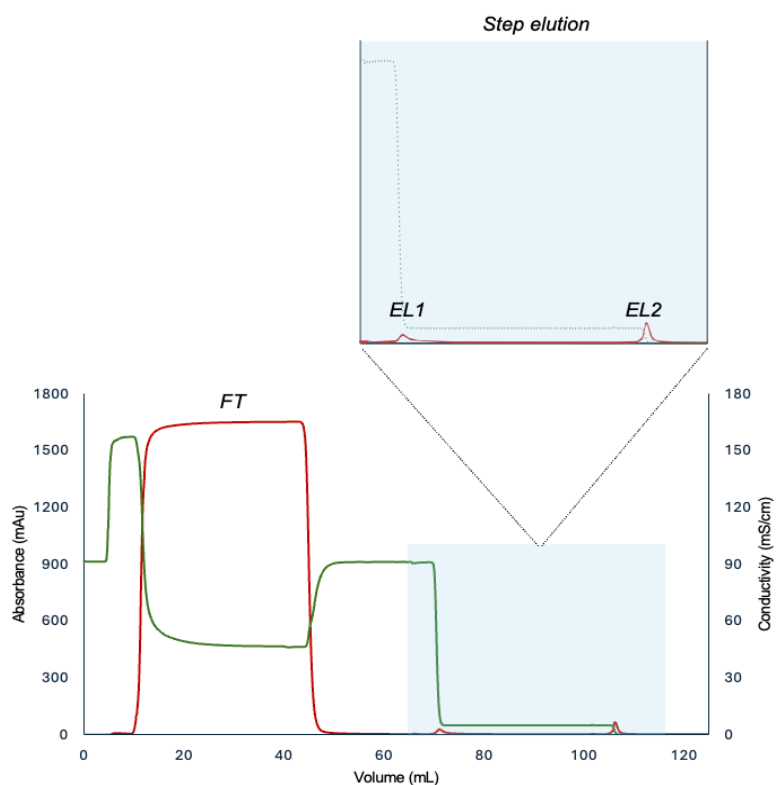
**Figure 42.** Overlay of CaptoQ ImpRes chromatography profiles. The agroinfiltrated plant extract (red) shows higher  $A_{260}$  absorbance compared to the negative control (dark blue), indicating the presence of additional nucleic acid species produced following agroinfection.



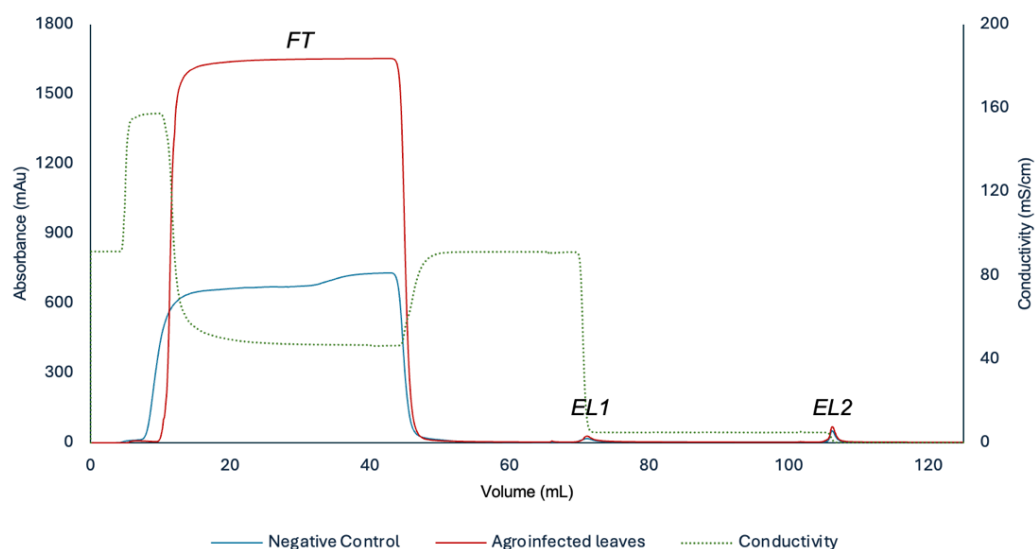
**Figure 43.** Agarose gel electrophoresis (1%) of fractions from CaptoQ ImpRes chromatography of agroinfiltrated leaf extracts. Lane M: Quick-Load® 1 kb Extend DNA Ladder; Lane 1: flow-through (FT) from CaptoQ; Lane 2: eluted fraction from CaptoQ. The gel shows differential band distribution indicative of potential separation between DNA and RNA species.

Given the promising performance of CaptoQ ImpRes, a second strong anion exchange resin, Nuvia HP-Q (Bio-Rad), was also evaluated in a single comparative trial. The rationale was to assess whether an alternative quaternary ammonium-based resin, marketed for its high binding capacity and broad applicability to nucleic acid purification, could provide improved performance or greater robustness when handling crude plant extracts. The Nuvia HP-Q is a strong anion exchanger functionalized with quaternary ammonium groups, designed for high-capacity binding of negatively charged biomolecules. The same binding and elution conditions optimized for CaptoQ were applied, using clarified plant lysates in low-salt buffer to promote RNA adsorption, followed by a linear salt gradient. However, the behaviour of the Nuvia HP-Q column diverged significantly from that observed with the CaptoQ. The elution profile was irregular and poorly resolved, with broad overlapping peaks rather than discrete nucleic acid elution. In addition, column backpressure increased rapidly during sample loading, which restricted the operational flow rate and raised concerns about the mechanical stability of the resin under plant lysate conditions. Fractions collected across the gradient were analysed by Nanodrop and agarose gel electrophoresis. Both analyses confirmed low RNA recovery and limited separation between nucleic acids and protein impurities. In light of these limitations and the technical issues encountered, no further experiments were performed with this resin. Instead, subsequent work focused on exploring affinity-based methods such as oligo(dT) chromatography to increase selectivity for polyadenylated mRNAs.

To enhance the selectivity for polyadenylated transcripts, clarified extracts were subjected to oligo(dT) affinity chromatography using the GoPure column (Bio-Rad). This method exploits the specific hybridization between the poly(A) tail of eukaryotic mRNAs and immobilized oligo(dT) sequences on the resin (231). The approach is designed to retain polyadenylated mRNAs while non-polyadenylated species, such as rRNA and tRNA, are removed during the washing steps. The first trials involved direct loading of clarified plant lysates onto the pre-equilibrated oligo(dT) column under binding conditions containing salt to stabilize poly(A)–oligo(dT) interactions. Extensive washing was performed to clear unbound material. Bound material was then eluted in a two-step protocol (EL1 and EL2), at different concentrations of nuclease-free water (buffer B) (Figure 44). The absence of salt destabilized the poly(A)–oligo(dT) hybridization, resulting in the release of bound mRNAs. This design was intended to distinguish weakly bound or partially hybridized RNA from transcripts with full-length poly(A) tails. Nanodrop analysis of elution fractions revealed variable outcomes. In many runs, EL2 contained the majority of the  $A_{260}$  signal and consistently displayed a higher  $A_{260}/A_{280}$  ratio compared to EL1, suggesting a purer nucleic acid composition in the second elution. However, the total RNA recovery was modest, and considerable batch-to-batch variability was observed. Control experiments using wild-type extracts yielded substantially weaker signals in both EL1 and EL2, confirming that agroinfiltration increased the abundance of polyadenylated RNA available for capture (Figure 45). Concentration of these fractions by isopropanol precipitation produced small but visible RNA pellets, which were successfully resuspended in nuclease-free water. Agarose gel electrophoresis confirmed the presence of RNA in the eluates, with agroinfiltrated samples showing stronger bands compared to wild-type controls, although the resolution between target mRNA and background species remained incomplete. Together these experiments demonstrated that oligo(dT) chromatography provided specific enrichment of polyadenylated RNA species, but the method was constrained by low overall yield and susceptibility to resin saturation when applied directly to crude plant lysates. From a process development perspective, this approach is best suited as a secondary polishing step after bulk capture by anion exchange chromatography, rather than as a standalone method. Further optimization would be required to improve reproducibility and scalability for preparative applications.

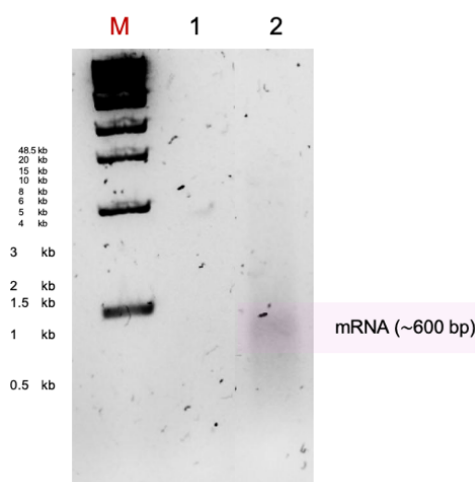


**Figure 44.** Chromatographic profile obtained using an Oligo(dT) column. The optimized salt gradient enabled selective retention and elution of mRNA species from agroinfiltrated plant extracts.



**Figure 45.** Overlay of Oligo(dT) chromatography profiles of agroinfiltrated leaf extracts (red) and negative control plants (dark blue). The conductivity profile (green, dotted line) indicates the optimized step elution performed.

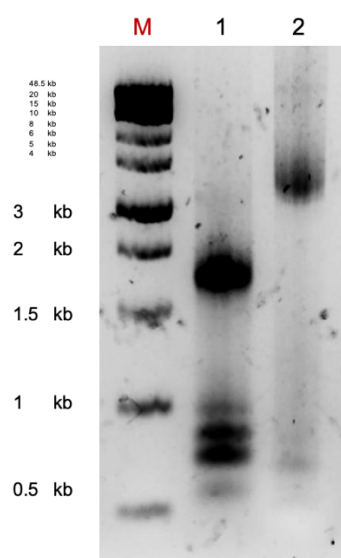




**Figure 46.** Agarose gel electrophoresis (1%) of Oligo(dT) chromatography fractions. Lane M: Quick-Load® 1 kb Extend DNA Ladder; Lane 1: flow-through fraction; Lane 2: eluted fraction showing the expected mRNA band at ~600 bp.

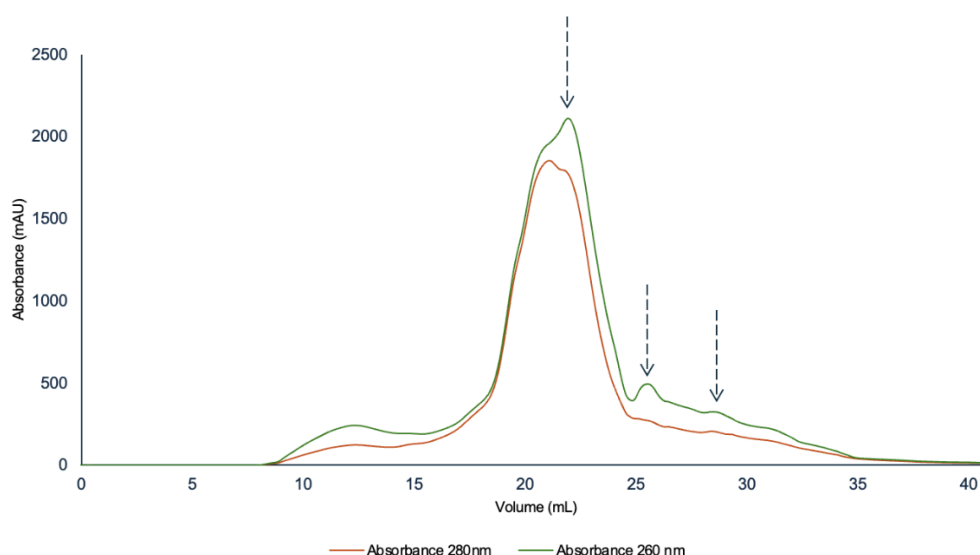
Having established that CaptoQ allowed broad enrichment of RNA and that oligo(dT) chromatography could increase specificity for polyadenylated species, the next step was to test whether it was possible to directly isolate the transgene-derived transcript. While oligo(dT) chromatography provided enrichment of polyadenylated RNA species, it lacked specificity for the transgene-derived mRNA of interest. To address this limitation, a series of trials were conducted using magnetic beads functionalized with a gene-specific oligonucleotide complementary to the GFP coding sequence. This approach was designed to enable sequence-specific enrichment of the target transcript by hybridization to a synthetic DNA probe immobilized on the bead surface, thus discriminating the transgene product from the background of host RNAs (232). The capture probe was synthesized with a 5' amino-C6 linker and covalently coupled to carboxy-functionalized Sera-Mag magnetic beads (Cytiva) via EDC-mediated amide bond formation in the presence of imidazole buffer. Following the coupling reaction, beads were subjected to sequential washing and blocking steps using sodium bicarbonate to remove unreacted reagents and minimise nonspecific adsorption. Functionalized beads were stored in nuclease-free PBS at 4 °C until use. The coupling chemistry and probe immobilization were successfully achieved, and control assays with *in vitro*-transcribed RNA confirmed that the oligo was functional. This was a crucial check, because it ruled out the possibility that the failure of capture in crude lysates was due to probe design or coupling efficiency. In simple systems the beads worked exactly as intended, binding and releasing the GFP transcript. HPLC analysis showed a retention peak consistent with the RNA–oligo complex. Binding trials were then performed by incubating clarified plant

lysates with the functionalized beads under gentle rotation at room temperature, allowing the target mRNA to hybridize to the immobilized probe. After binding, the beads were washed extensively to remove non-specifically bound material. Elution was achieved using nuclease-free water preheated to 80 °C, immediately followed by cooling on ice to reduce thermal degradation. However, the behaviour was very different compared to the one showed with IVT RNA product. Despite extensive washing and high-stringency elution, RNA recovery was consistently close to the detection limit. Isopropanol precipitation was attempted to concentrate the eluates, but the resulting pellets were barely visible and insufficient for characterization. These results suggested that in crude lysates the hybridization efficiency was strongly compromised, most likely due to competition from abundant non-target RNAs, secondary structures of the transcript, or probe saturation by background nucleic acids. Comparative trials with wild-type and agroinfiltrated samples did indicate a slightly higher RNA signal in the agroinfiltrated extracts, implying that some level of selective binding to the transgene transcript was occurring. Nevertheless, the magnitude of the effect was too small to be of practical use. The limiting factor was not the concept itself – validated by the IVT control – but rather the challenge of applying such a precise capture method directly to complex plant matrices. These experiments demonstrated that sequence-specific magnetic bead capture from crude plant extract is feasible in principle but not yet robust in practice. They provided important insight into the limitations of applying hybridization-based enrichment to crude extracts: low yield, strong matrix effects, and poor reproducibility. At the same time, they pointed the way forward for future optimization, including probe redesign, modulation of hybridization kinetics, and application to pre-cleared or partially purified RNA fractions rather than to whole lysates.



**Figure 47.** Agarose gel electrophoresis (1%) of RNA samples. Lane M: Quick-Load® 1 kb Extend DNA Ladder; Lane 1: IVT mRNA control sample. Lane 2: plant mRNA eluted from functionalized magnetic beads. The increase in size in the plant extract could be given by the presence of double poly(A) tail.

In parallel with ion exchange and affinity-based trials, size exclusion chromatography (SEC) was evaluated as an orthogonal method to probe the composition of crude plant extracts. The rationale for testing SEC was not to achieve preparative purification, but rather to gain insight into the molecular size distribution of RNA-containing species and to assess whether RNA could be separated from abundant protein contaminants based on hydrodynamic volume. For this purpose, clarified lysates were injected directly onto a Superose 6 Increase 10/300 column and eluted under isocratic conditions using the buffer (10 mM phosphate, 2 mM EDTA, 150 mM NaCl, pH 7.4). Chromatograms monitored at 260 nm and 280 nm revealed broad, overlapping peaks, with nucleic acid and protein signals largely co-eluting (Figure 48). This outcome reflected the intrinsic complexity of crude plant extracts, where ribosomal RNAs, mRNAs, and fragmented nucleic acids overlap in size with soluble proteins and pigment-associated complexes. Therefore, SEC did not provide the resolution required for selective isolation of mRNA. Nevertheless, the experiment fulfilled its analytical purpose. The elution profiles confirmed the high degree of sample heterogeneity and highlighted the presence of multiple nucleic acid species distributed across a wide size range. In addition, SEC allowed approximate comparison of RNA and protein co-elution patterns, which supported the decision to prioritize ion exchange and affinity-based methods for enrichment while retaining SEC as a complementary tool for analytical characterization. Although insufficient as a standalone strategy, SEC contributed with valuable information about the complexity of the plant extract matrix and demonstrated potential utility in downstream polishing steps once bulk enrichment of RNA had been achieved.

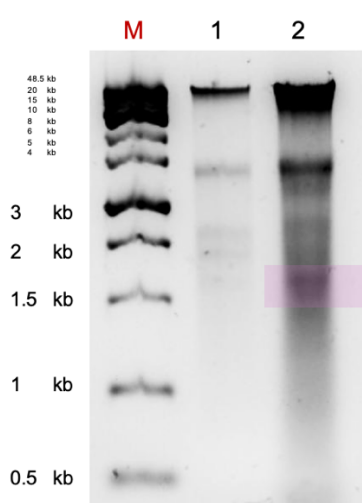


**Figure 48.** Size exclusion chromatography (SEC) profile of crude agroinfiltrated plant extract. Absorbance at 260 nm (green) highlights nucleic acid content, while absorbance at 280 nm (orange) reflects the presence of protein species. The indicated fractions (arrows) correspond to regions with higher  $A_{260}$  signals, consistent with nucleic acid enrichment.

In addition to chromatographic methods, ammonium sulphate precipitation was evaluated as a classical salting-out approach for the enrichment of total RNA from crude plant lysates. The rationale was to test whether a simple, non-chromatographic method could provide bulk concentration of nucleic acids prior to more selective steps. Stepwise additions of solid ammonium sulphate were made directly to clarified lysates, with gentle mixing to ensure dissolution. Increasing amounts of solid ammonium sulphate were added directly to clarified extracts, and concentrations up to 2.0 M were tested. Precipitation of nucleic acids became evident above 1.5 M ammonium sulphate, where visible pellets formed reproducibly after incubation on ice and centrifugation. Samples were incubated on ice to promote precipitation, followed by centrifugation to pellet the precipitated material. Visible pellets formed reproducibly at higher ammonium sulphate concentrations, confirming successful precipitation of macromolecules. These pellets were resuspended in nuclease-free water and analysed on SDS-PAGE gel. While the method was straightforward, the precipitate was heterogeneous and required further purification to enrich specifically for mRNA, as expected from a non-selective precipitation strategy.

To provide a benchmark against which alternative purification strategies could be evaluated, the RNeasy Plant Mini Kit (QIAGEN) was purchased to be tested on agroinfiltrated *N. benthamiana* leaves. The rationale for including this method was not only because it represents the most widely used commercial protocol for total plant RNA extraction, but also

because it provides a well-established baseline for assessing the efficiency and selectivity of experimental workflows. The kit relies on silica membrane adsorption under chaotropic conditions, enabling rapid isolation of total RNA with minimal equipment requirements. The extractions yielded clean and reproducible eluates, with consistent  $A_{260}/A_{280}$  ratios around ~2.0 and stable  $A_{260}/A_{230}$  values, confirming low levels of protein and polysaccharide contamination – two common challenges when working with plant tissues. Importantly, the reproducibility of this purity indices contrasted with the greater variability observed in chromatographic methods, underlining the robustness of the kit. However, a key limitation was the total RNA yield. The concentrations recovered, even though reproducible, were generally lower than those obtained with some of the column-based anion exchange approaches, reflecting the kit's design for analytical-scale nucleic acid preparation rather than preparative enrichment. Furthermore, while the method efficiently captured total RNA, it did not provide any enrichment for transgene mRNA from host RNAs, meaning that additional steps would still be required for applications dependent on transcript-specific purification. The RNeasy kit proved to be a reliable and reproducible baseline method, offering clean total RNA suitable for analytical assays and comparative benchmarking. Its inclusion in this study highlighted both the advantages of standardized commercial protocols and their inherent limitations for preparative mRNA purification. When viewed alongside the exploratory chromatography and precipitation trials, the kit results reinforced the conclusion that effective workflows for plant-derived mRNA will likely require a combination of complementary strategies—balancing capture efficiency, selectivity, and scalability.



**Figure 49.** 1% agarose gel analysis of RNA extracted with the RNeasy Plant Mini Kit. Lane M: Quick-Load® 1 kb Extend DNA Ladder; Lane 1: negative control leaves; Lane 2: agroinfiltrated samples expressing the mRNA construct at the expected size as highlighted in pink.

## 6.4 Conclusions

This study demonstrated the feasibility of adapting an mRNA sequence originally optimised for cell-free in vitro transcription to a plant-based transient expression system in *Nicotiana benthamiana*. The construct, designed to encode eGFP and equipped with regulatory elements to enhance stability and translation, was successfully integrated into the binary vector pGreenII and delivered via *Agrobacterium tumefaciens* LBA4404 using vacuum-assisted infiltration. Peak GFP expression was typically observed 4–5 days post-infiltration, confirming that the adapted cassette was transcriptionally active in planta. The experimental workflow – vector propagation, *Agrobacterium* co-transformation with pSoup, and infiltration under controlled greenhouse conditions – proved reproducible once critical parameters such as transformation voltage, culture OD, and infiltration timing were standardised.

Despite these advances, several challenges impacted the efficiency and throughput of the platform. A major constraint was the discovery of a persistent backbone insertion in both pGreenII 0579-1 and pGreenII Tfs-494 stocks. This sequence anomaly had the potential to influence plasmid topology, bacterial replication, and transient expression performance. Multiple attempts to reconstruct a clean backbone via Gibson Assembly were unsuccessful, indicating that the contaminant sequence was present in all available stocks. Consequently, infiltration experiments were conducted with vectors containing the insertion, which represented an intrinsic limitation of this proof-of-concept study.

mRNA extraction and purification trials revealed that while plant-derived transcripts could be recovered and enriched, method-dependent variations in yield, purity, and specificity were significant. CaptoQ ImpRes chromatography achieved good binding and recovery of RNA but co-eluted abundant rRNA and other plant RNAs. Oligo(dT) affinity chromatography provided selective enrichment of polyadenylated transcripts, though overall yields remained low. Sequence-specific magnetic beads demonstrated proof-of-principle capture of the GFP transcript but suffered from low recovery. Additional techniques such as ammonium sulphate precipitation and size exclusion chromatography provided complementary information or intermediate enrichment, while the RNeasy Plant Mini Kit served as a reliable benchmark for reproducible RNA recovery. These findings combined indicated that a multi-step purification workflow, integrating charge-based, affinity, and size-based separations, is likely required to achieve high-purity plant-derived mRNA suitable for downstream applications. The results clearly demonstrated that, while mRNA can be successfully expressed in *N. benthamiana*, its direct recovery from crude plant extracts remains technically challenging. The complexity of

the plant matrix, coupled with the intrinsic instability of naked mRNA, imposes significant constraints on both yield and purity when using conventional purification methods. These observations directly inspired the design of the next phase of this research, in which a plant-produced virus-like particle (VLP) system was developed to encapsulate the mRNA in planta. By physically protecting the transcript from nucleolytic degradation and enabling affinity-based downstream recovery, the VLP strategy offers a dual advantage: improved stability during extraction and a simplified, more selective purification process. The subsequent chapter will therefore focus on the design, production, and characterization of this VLP-based delivery platform, evaluating its potential as an integrated solution for mRNA stabilization and recovery in plant molecular farming workflows.

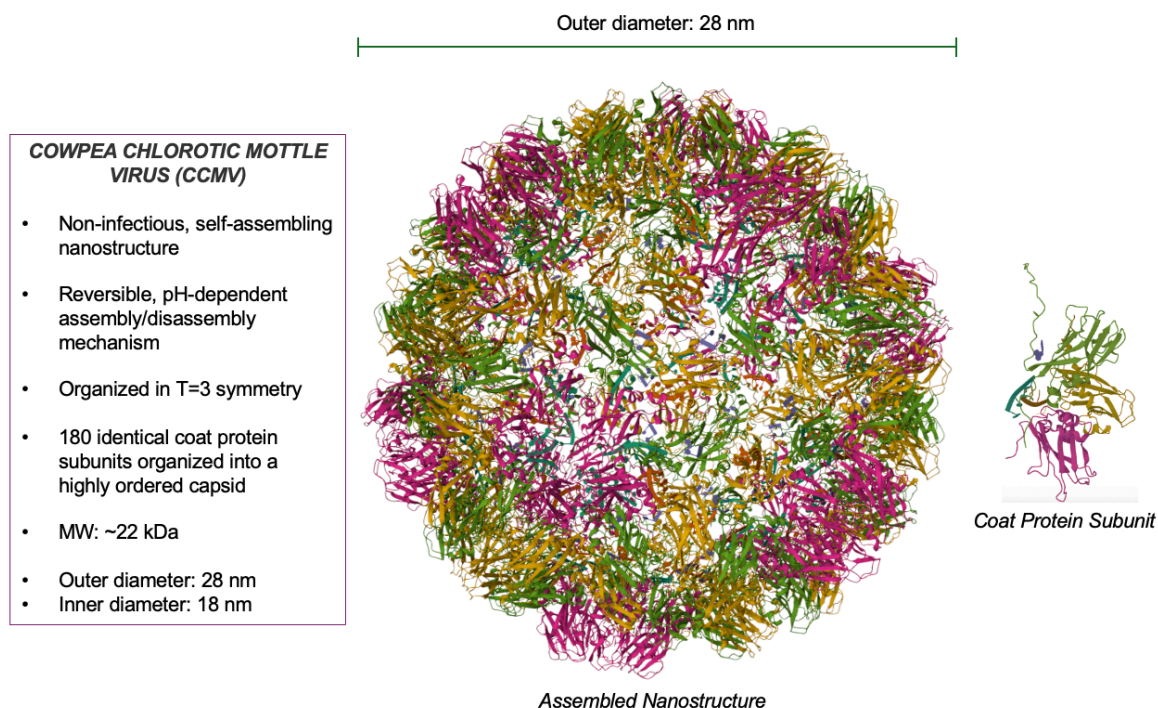
## 7. CCMV: a VLP Produced in Plants

### 7.1 Introduction and Scientific Background

Linking directly to the outcomes described in Chapter 6, where plant-derived mRNA was successfully produced and characterized in *Nicotiana benthamiana*, the present chapter focuses on the development of a complementary delivery platform based on cowpea chlorotic mottle virus (CCMV) virus-like particles (VLPs).

VLPs are non-infectious, self-assembling nanostructures that mimic the native architecture of their parent viruses while lacking the viral genome, thereby combining safety with the capacity to elicit robust immune responses and to efficiently deliver nucleic acids or other biomolecular cargo (233, 234). Due to their well-defined capsid symmetry, nanoscale dimensions, and repetitive antigenic surfaces, VLPs have been extensively explored as vaccine scaffolds and nanocarriers for mRNA delivery (235). CCMV, a member of the *Bromoviridae* family, is one of the best-studied plant viruses for nanotechnology applications. It is a non-enveloped, icosahedral virus of ~28 nm in diameter, organized in T=3 symmetry and composed of 180 identical coat protein subunits organized into a highly ordered capsid (236) (Figure 50). Structural and biophysical studies have revealed that CCMV is distinguished by its reversible, pH-dependent assembly/disassembly mechanism: at neutral to basic pH the capsid disassembles into dimers, whereas acidic pH promotes spontaneous reassembly into highly uniform particles (237, 238). This property provides a powerful tool for encapsulating heterologous RNA molecules under controlled physicochemical conditions, enabling the production of RNA-loaded VLPs with high efficiency (239). The assembly process is not random: packaging signals encoded in viral RNAs, as demonstrated in *Bromoviridae* such as BMV and CCMV, guide efficient encapsulation (240). Moreover, heterologous RNAs carrying artificial packaging signals (e.g., TMV origin-of-assembly sequences) have been successfully encapsulated in plant virus capsids both in vitro and in planta (241). Recent experimental work has also shown that engineered CCMV particles can encapsulate reporter mRNAs such as EYFP or self-amplifying RNAs and deliver them into mammalian cells where they remain translation-competent (242, 243). These results highlight the potential of CCMV VLPs as a natural nanocapsule for mRNA delivery. CCMV particles retain structural integrity during storage and handling, and their plant origin minimizes the risk of pre-existing immunity in human populations (244). The ability to engineer the CCMV coat protein, for example by introducing affinity tags or surface epitopes, further expands its versatility for therapeutic and diagnostic applications (245).





**Figure 50.** Representation of Cowpea chlorotic mottle virus (CCMV) with its main characteristics.

Image adapted from RCSB Protein Data Bank (252).

Plant-based expression systems, as previously extensively discussed, offer a rapid, scalable, and safe production platform for recombinant proteins, including viral capsid proteins (38). Co-production of both the mRNA cargo and the CCMV coat protein within the same plant host could streamline manufacturing workflows, reduce downstream processing steps, and align with good manufacturing practice (GMP) standards for large-scale production (146). This integrated approach represents a promising route toward the development of next-generation vaccine platforms and nucleic acid delivery systems that are cost-effective, adaptable, and suitable for deployment in both high- and low-resource settings.

## 7.2 Experimental Design

The experimental design for the production of cowpea chlorotic mottle virus (CCMV) virus-like particles (VLPs) was conceived as a two-stage process: an initial production in *Escherichia coli* to optimise expression and purification parameters, followed by heterologous expression in *Nicotiana benthamiana* plants to enable co-production with the target mRNA. This sequential approach was adopted to allow rapid method development and troubleshooting in a bacterial system before scaling to a plant-based platform, where production timelines are inherently longer and vector optimisation can be more challenging.

For bacterial expression, the CCMV CP sequence was cloned into pSB1C3 under a T7 promoter, enabling IPTG-inducible expression in *E. coli* BL21(DE3) cells. This construct was used for rapid production and optimization of purification protocols. Following induction with 0.5 mM IPTG at OD<sub>600</sub> ~0.4–0.6 and optimized prolonged incubation at 29 °C, cells were harvested by centrifugation and pellets stored at –20 °C pending protein extraction and purification.

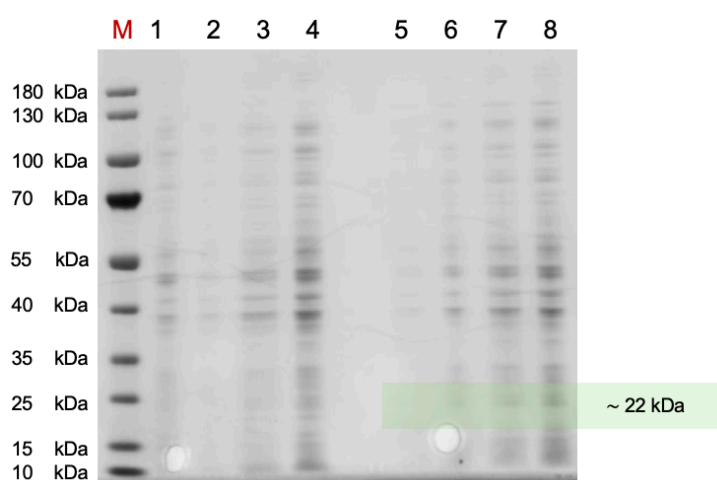
For plant expression, the binary vector pGreenII Tfs-494::LUC was selected for insertion of the CCMV coat protein coding sequence under the CaMV 35S promoter. The pGreen system is compact and flexible but requires the helper plasmid pSoup, which supplies the repA gene necessary for autonomous replication in *Agrobacterium tumefaciens*. The CCMV CP sequence was synthesized and cloned into pGreenII Tfs-494 via Gibson Assembly, with overlapping primers designed for precise insertion. Transformation efficiency was determined by colony counts per microgram of plasmid DNA, with co-transformation yielding around  $5 \times 10^6$  CFU/μg DNA. *A. tumefaciens* transformants were grown in selective LB medium (kanamycin 50 μg mL<sup>-1</sup>, streptomycin 100 μg mL<sup>-1</sup>, rifampicin 25 μg mL<sup>-1</sup>) in preparation for agroinfiltration. Bacterial suspensions were adjusted to a final OD<sub>600</sub> = 0.4 in infiltration buffer (10 mM MES, 10 mM MgCl<sub>2</sub>, pH 5.7) prior to vacuum infiltration of *N. benthamiana* plants. Quality control (QC) of all constructs was performed at multiple stages. At each stage, quality control included restriction digestion, agarose gel electrophoresis and sequencing, to ensure stability and correctness prior to expression experiments.

### 7.3 CCMV Expression and Purification from *E. coli*

Initial expression trials in *Escherichia coli* were designed to provide a fast, controllable system for optimising lysis, purification, and assembly conditions for CCMV VLPs. Although *E. coli* lacks the post-translational machinery of eukaryotic hosts, it remains one of the most widely used systems for producing recombinant viral capsid proteins due to its fast growth, ease of manipulation, and high yields under controlled fermentation conditions (246). The aim was to test a rapid and cost-effective platform for preliminary protein production and purification trials. This allowed evaluation of pH-triggered assembly/disassembly, chromatographic binding behaviours, and precipitation profiles prior to plant expression, where biomass availability is more limited. By characterising CCMV in a bacterial system first, it was possible to define

suitable buffer compositions, ionic strengths, and chromatographic strategies to be directly applied to plant-derived material.

The CP coding sequence was cloned under a T7 promoter and transformed into *E. coli* BL21(DE3), a strain optimized for inducible protein expression. Cultures were induced with 1 mM IPTG at OD<sub>600</sub> ~0.5 and incubated at 29 °C for approximately 20 hours post-induction. This relatively low temperature and extended induction period were chosen to enhance protein solubility and to reduce the formation of inclusion bodies, in line with strategies commonly employed for viral structural proteins (247). Cell pellets were harvested by centrifugation and subjected to lysis by sonication. Optimization experiments indicated that 20 sonication cycles (10 sec on – 10 sec off) provided more efficient disruption compared to the initial 10 cycles, resulting in higher recovery of soluble CP. Lysis was tested in two extraction buffers with distinct physicochemical conditions: (1) 50 mM Tris, 150 mM NaCl, 2 mM DTT, 10 mM MgCl<sub>2</sub>, 1% Triton X-100, pH 7.4; and (2) 50 mM Sodium Acetate, 150 mM NaCl, 2 mM DTT, 10 mM MgCl<sub>2</sub>, 1% Triton X-100, pH 5.0. These conditions were selected to assess whether the pH-dependent stability and assembly properties of CCMV CP, previously reported in vitro (237), could influence solubility and recovery at the extraction stage. Following lysis, soluble fractions and pellets were collected and analysed by SDS-PAGE to monitor expression and solubility of the recombinant protein (Figure 51). Initial observations indicated that a band corresponding to the expected molecular weight of CCMV CP (~ 22 kDa) was present in the soluble fraction under both extraction conditions, although showing differences in yield and purity. This confirmed successful expression in *E. coli* and established a baseline for subsequent purification trials.

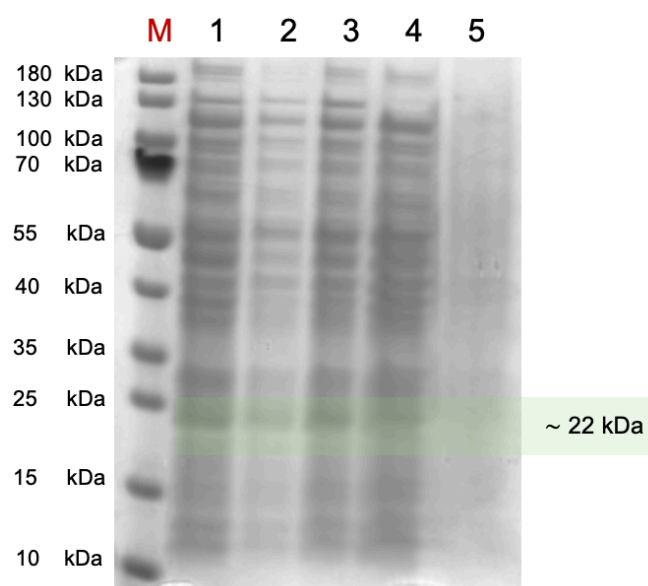


**Figure 51.** SDS–PAGE comparison between *E. coli* BL21 negative control (not expressing CCMV) and *E. coli* expressing CCMV capsid protein. Lane M: Page Ruler pre-stained protein ladder; Lane 1-4: negative control lysates; Lane 5-8: BL21 cells expressing CCMV lysates. A distinct band is visible at the

expected molecular weight of ~22 kDa in expressing samples (lanes 5-8), corresponding to the CCMV coat protein.

Following successful expression of the CCMV coat protein (CP) in *E. coli* BL21(DE3), several purification strategies were explored with the objective of identifying a robust purification workflow that could later be adapted to plant-derived protein extracts. Purification of viral structural proteins poses inherent challenges, as they often exhibit high aggregation propensity and can co-purify with host proteins of similar physicochemical properties (248). To address these challenges, three chromatographic strategies were systematically tested: hydrophobic interaction chromatography (HIC), immobilized metal affinity chromatography (IMAC), and size exclusion chromatography (SEC). Each method was evaluated under neutral (pH 7.4) and mildly acidic (pH 5.0) conditions, reflecting the pH-sensitive structural dynamics of CCMV CP.

The first purification trials were based on hydrophobic interaction chromatography, a technique reported for plant and bacterial virus-like particles due to its ability to discriminate proteins based on surface hydrophobicity (249). Hydrophobic interaction chromatography (HIC) was investigated as a potential method to purify CCMV coat protein (CP), given the protein's known hydrophobic character and tendency to undergo pH-dependent structural rearrangements. Before loading onto columns, preliminary salting-out experiments were conducted using ammonium sulphate to evaluate whether CCMV CP exhibited differential precipitation behaviour across increasing ionic strengths. A range of concentrations up to 2 M was systematically tested to identify the threshold at which CP became insoluble. The experiments showed that CP progressively precipitated as the concentration of ammonium sulphate increased, confirming the strong contribution of hydrophobic forces to its solubility profile (Figure 52). Importantly, these trials established that CCMV CP is highly sensitive to ionic strength. This behaviour supported the hypothesis that hydrophobic interactions could be exploited in a chromatographic setting, and that column based HIC would be a suitable next step for controlled separation.

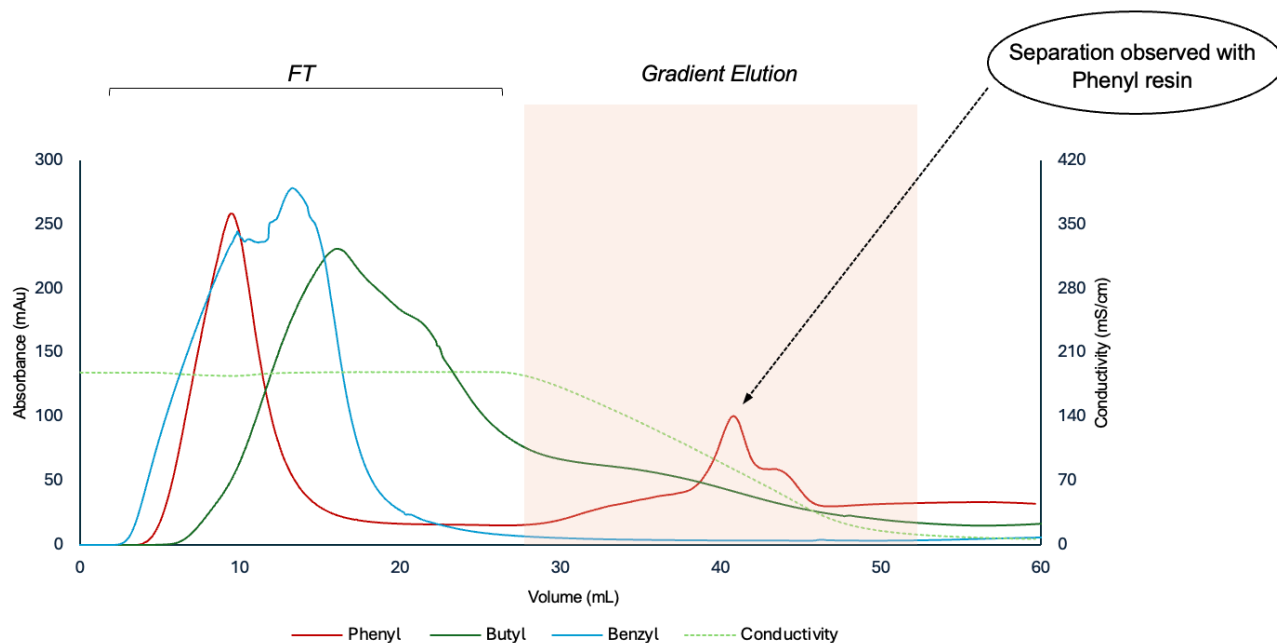


**Figure 52.** SDS-PAGE analysis of CCMV protein fractions obtained after ammonium sulphate precipitation. Lane M: Page Ruler pre-stained protein ladder; Lanes 1-5: lysates expressing CCMV at different saturation levels (20–60%). Distinct protein bands around 22 kDa are enriched between 20% and 40% saturation, indicating selective precipitation of the target protein.

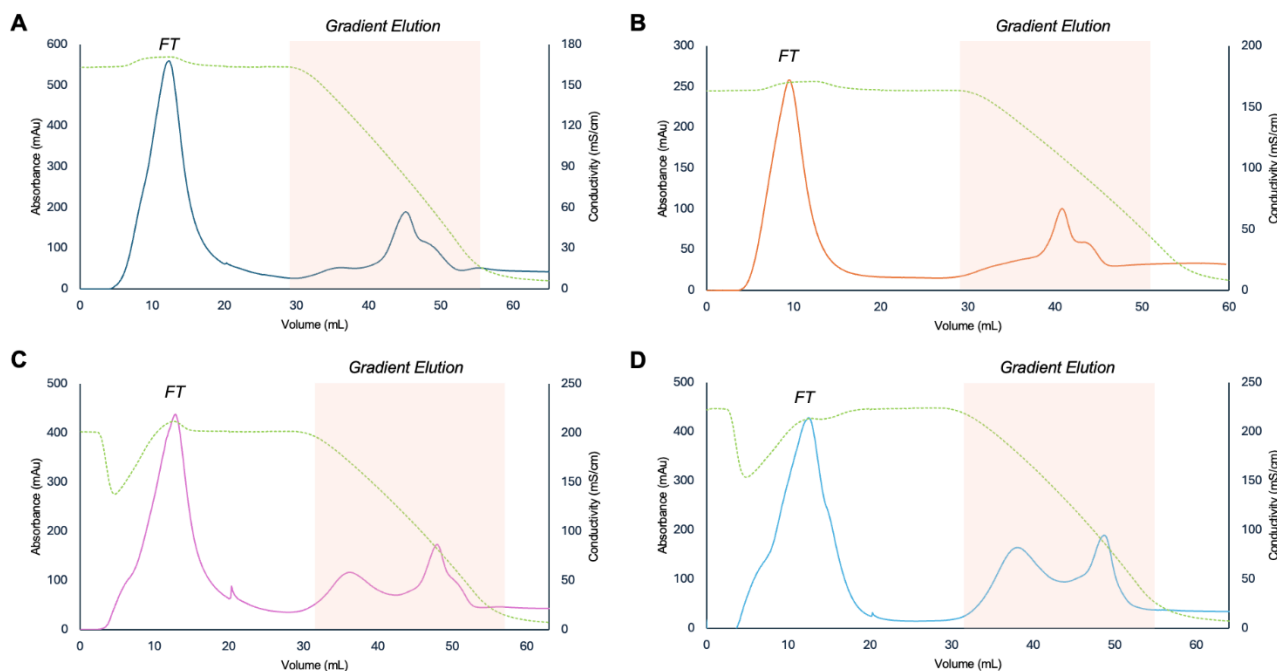
Building on the precipitation results, small-scale HIC trials were conducted using resins with different ligand chemistries – butyl, benzyl, and phenyl – to systematically evaluate their ability to capture CCMV CP. Each resin was tested under both neutral (pH 7.0) and acidic (pH 5.0) extraction conditions, since the conformational state of the protein is known to be strongly pH dependent. The comparative screening highlighted clear differences. The butyl resin, characterised by the weakest hydrophobic ligand, exhibited minimal retention of CP: a substantial portion of the protein passed directly into the flow-through, and elution profiles were broad and poorly resolved. In contrast, the benzyl resin, which provides a stronger hydrophobic surface, bound CP very tightly, but this strong interaction hindered efficient elution. Protein recovery was incomplete, and SDS-PAGE analysis of the eluates showed diffuse and smeared bands, consistent with protein being trapped or co-eluting with impurities. Between these two extremes, the phenyl resin proved to be the most effective. Its intermediate hydrophobicity provided a balance between strong binding under high salt condition and controlled release during elution (Figure 53). As a result, CP could be reproducibly recovered as discrete fractions, with sharper bands on SDS-PAGE compared to the other ligands. These observations indicated that CCMV CP exposes a moderate level of surface hydrophobicity, sufficient for stable retention without the irreversible interactions observed with benzyl ligands. Importantly, the phenyl resin supported reproducible capture

across repeated runs, laying the groundwork for subsequent optimization of salt concentrations and gradient elution conditions.

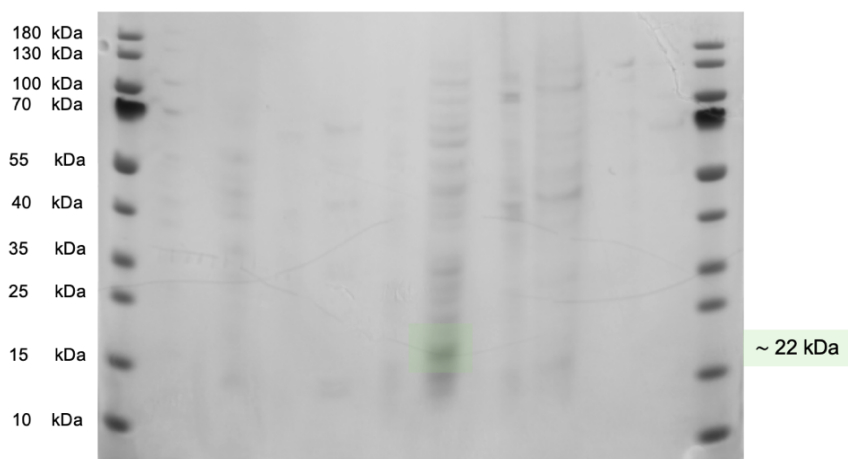
After identifying phenyl resin as the most suitable matrix, a series of optimization trials were performed to define binding and elution conditions that maximised recovery and selectivity of CCMV CP. Binding was tested at increasing concentrations of ammonium sulphate, and systematic elution gradients were applied to determine the salt concentration at which the protein would reproducibly desorb from the resin (Figure 54). Optimal binding was carried out at high ammonium sulphate concentration of 1.5 M, and elution was optimized using a stepwise approach with three steps: 45%, 75%, and 100% buffer B with no salt. This design allowed early removal of impurities at 45% B, followed by a single sharp elution peak of CP at 75% B, corresponding to approximately 0.38 M ammonium sulphate. The 100% B step served as a final wash, ensuring that no residual protein or tightly bound impurities remained on the column (Figure 56). Under these conditions, CP eluted as a single, well-defined peak at 0.38 M ammonium sulphate, while no detectable protein of interest was observed in either the flow-through or earlier fractions. This behaviour demonstrated that the phenyl resin could provide highly selective retention and release of the target protein. A practical challenge at this stage was the limited bed volume of the phenyl column, which restricted the total amount of sample that could be processed per run. To overcome this, eluates were concentrated prior to analysis by precipitation with four volumes of cold acetone at  $-20^{\circ}\text{C}$ . This step improved recovery and facilitated SDS-PAGE analysis, which consistently confirmed the presence of a strong band at the expected molecular weight of CCMV CP (Figure 55). The reproducible elution at an intermediate salt concentration indicated that CP binding to the phenyl resin is not only robust but also finely tuned, consistent with the protein's moderate surface hydrophobicity. This provided confidence that the method could be scaled up without major loss of resolution, and that HIC would be a valuable component of a multi-step purification workflow.



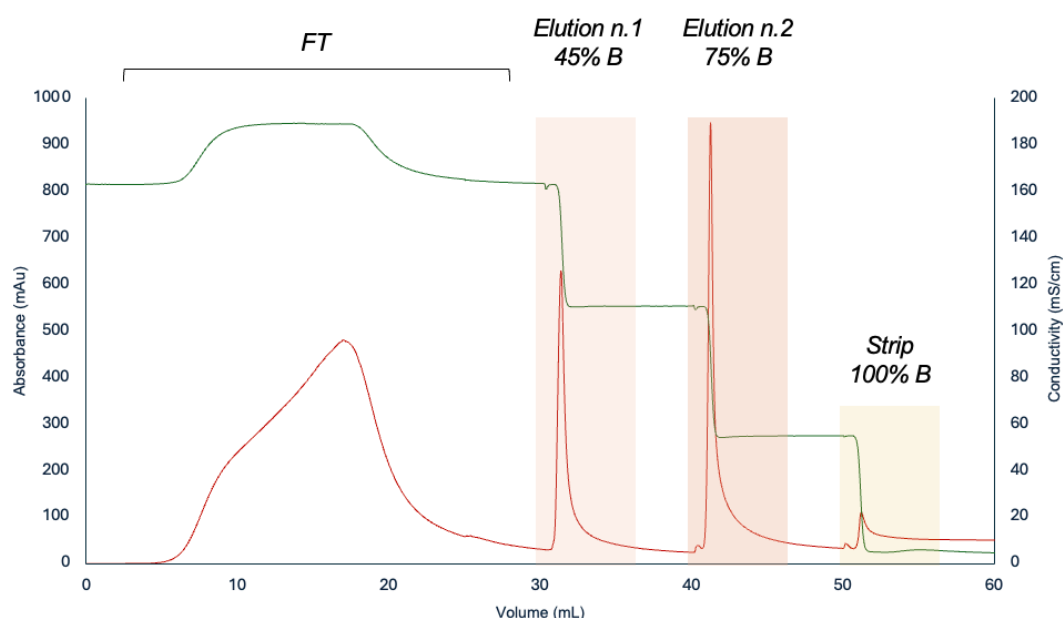
**Figure 53.** Hydrophobic Interaction Chromatography (HIC) resin screening for CCMV coat protein. Three resins were compared – Phenyl (red), Butyl (green), and Benzyl (blue) – under identical loading and gradient elution conditions. Distinct binding and elution behaviours were observed, guiding the selection of the most suitable matrix.



**Figure 54.** Hydrophobic Interaction Chromatography (HIC) salt concentration screening on Phenyl resin for CCMV coat protein. Binding and elution profiles were evaluated at different ammonium sulphate concentrations (A: 1.25 M; B: 1.5 M; C: 1.75 M; D: 2 M). The chromatograms highlight the influence of salt concentration on protein–resin interactions, with variations in peak intensity and resolution.



**Figure 55.** SDS–PAGE analysis of CCMV purification using hydrophobic interaction chromatography (HIC) on Phenyl column at pH 7.0, binding with 1.5 M ammonium sulphate. The gel bands corresponding to the CCMV capsid protein at ~22 kDa are evident only in the eluted fraction.



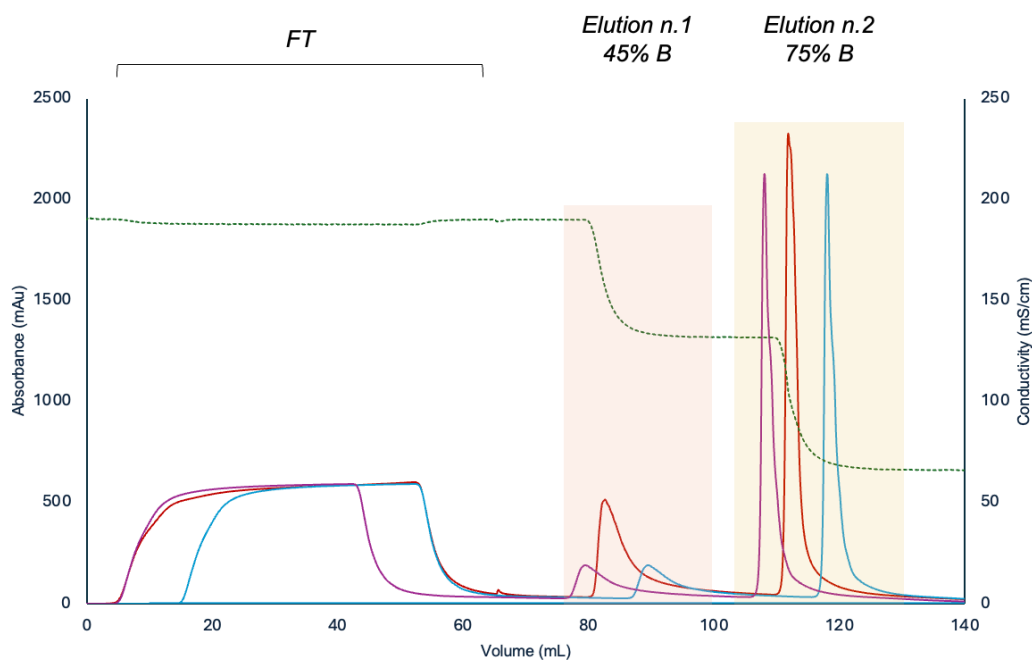
**Figure 56.** Optimized hydrophobic interaction chromatography (HIC) on Phenyl HS column. Chromatographic profile shows the flow-through (FT), two main elution peaks obtained at 45% B and 75% B, and a final strip at 100% B.

To overcome the inherent limitations of small packed-bed resins, the process was subsequently scaled up using a Sartorius CIMmultus OH monolithic column. This column combines hydrophobic and mixed-mode interactions. Unlike conventional bead-based matrices, monoliths consist of a continuous porous structure with convective flow channels that eliminate diffusional mass transfer limitations (250). This architecture allows significantly higher sample loading while maintaining high resolution and sharp peak separation. When the optimized binding and elution parameters established on the phenyl resin were applied to the monolith, the chromatography runs consistently displayed sharply resolved and well-

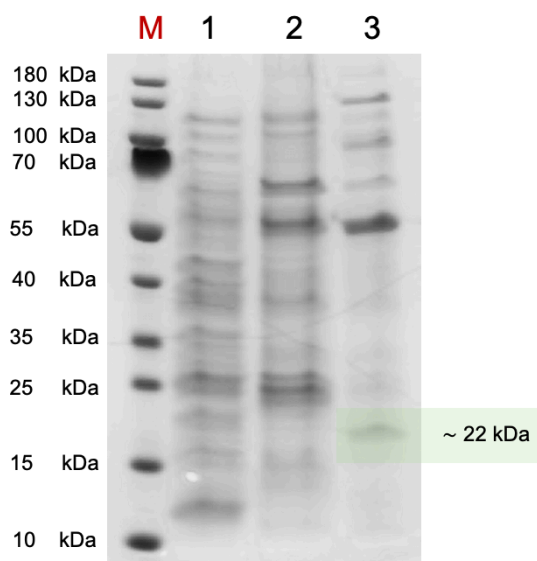


separated peaks, with CP eluting reproducibly at ~0.38 M ammonium sulphate, mirroring the profile previously identified on the phenyl column (Figure 57). The chromatography runs displayed sharply resolved and well-separated peaks, enabling clear discrimination between the target protein and the impurities. The major difference was that much larger sample volumes could be processed without compromising resolution, effectively addressing the throughput bottleneck encountered with small-scale resins. SDS-PAGE analysis of monolith-derived fractions further confirmed the presence of CP as a clear and intense band, with reduced background compared to earlier bead-based trials (Figure 58). The improved performance of the monolithic support validated the optimization work performed at small scale and confirmed that hydrophobicity could be exploited for reproducible purification of CCMV CP. Importantly, the scalability of the method established a practical route towards producing sufficient material for downstream characterization, while preserving selectivity and recovery efficiency.

A critical part of the HIC optimization was the evaluation of binding behaviour under different pH conditions. According to literature, stronger retention of CCMV CP would be expected at neutral pH (7.0), where the protein is disassembled and hydrophobic domains are more exposed on the protein surface. In contrast, at acidic pH (5.0), CP tends to reassemble into higher-order structures, a state in which hydrophobic patches are thought to be less accessible, leading to reduced binding. While the experiments confirmed efficient binding at pH 7.0, the results obtained at pH 5.0 were particularly noteworthy. Contrary to conventional expectations, CP also showed excellent retention at pH 5.0, and elution at 75% B produced a strong, well-defined peak comparable to that obtained at neutral pH. This finding suggests that even in the assembly-prone acidic state, sufficient hydrophobic regions remain exposed to permit robust interaction with the resin. From a process perspective, this represents a significant advantage, as it demonstrates that HIC purification can be successfully performed directly at pH 5.0, thereby aligning the purification environment with the conditions required for downstream assembly and encapsulation studies.



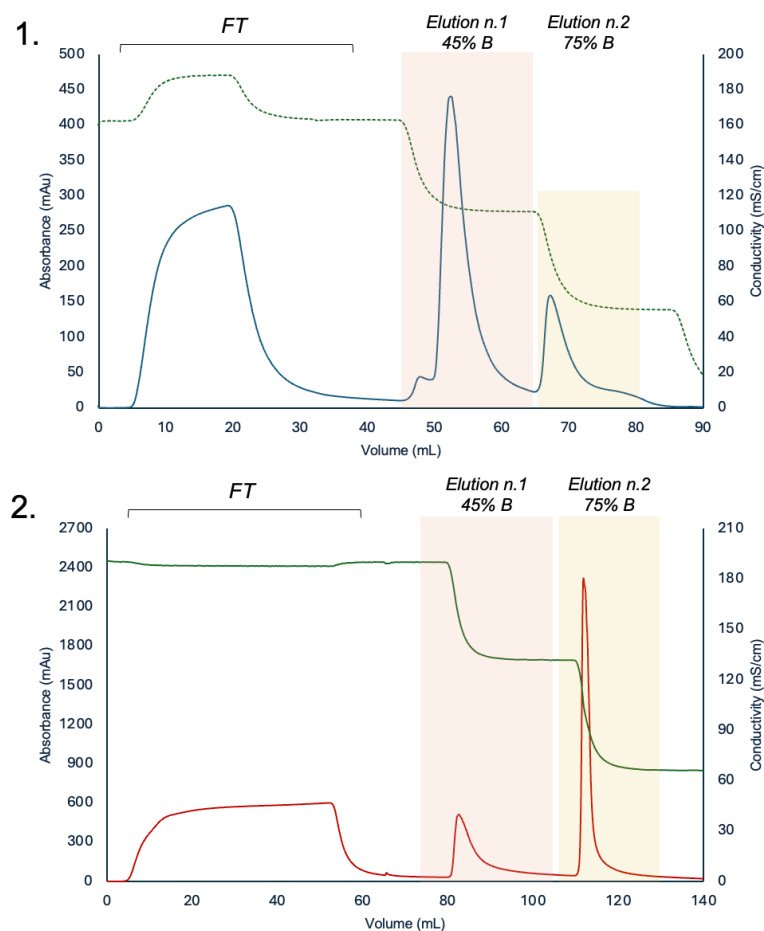
**Figure 57.** Overlay of hydrophobic interaction chromatography (HIC) runs performed with the optimized method at pH: 5.00 on the monolith column CIMmultus OH. The chromatograms demonstrate the method reproducibility, with reproducible elution peaks at 45% B and 75% B across independent experiments.



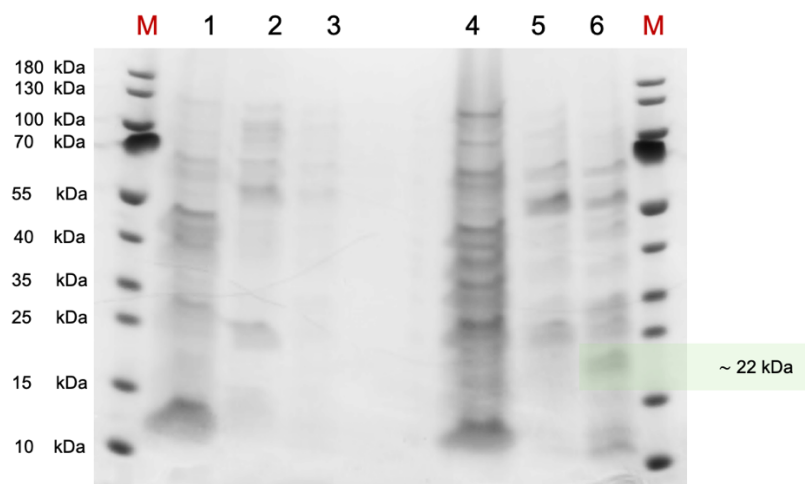
**Figure 58.** SDS-PAGE analysis of CCMV purification using a Monolith CIMmultus OH column. Lane M: Page Ruler pre-stained protein ladder; Lane 1: flow-through (FT); Lane 2: elution fraction n.1 45%B; Lane 3: elution fraction n.2 75%B. A distinct band at ~22 kDa, corresponding to the CCMV capsid protein, is visible in the elution fraction n.2 (Lane 3).

To confirm the specificity of these results, identical purification steps were performed on negative control lysates not expressing CCMV (Figure 59). In these samples, no bands at 22 kDa were detected, whereas the signal was consistently observed in the CCMV-expressing samples (Figure 60). This direct comparison provided the most conclusive evidence that the

CCMV coat protein was successfully expressed in *E. coli*, validating the expression system and the downstream workflow.



**Figure 59.** HIC chromatographic runs under optimized conditions. (1) Negative control; (2) *E. coli* cells expressing CCMV, which display a pronounced peak in the Elution n.2 (75% B), corresponding to the fraction where the CCMV coat protein band was consistently observed by SDS-PAGE at ~22 kDa. This comparison highlights the specificity of the target protein recovery.



**Figure 60.** SDS-PAGE analysis of HIC optimized method on both negative and positive samples. Lane M: Page Ruler pre-stained protein ladder; Lanes 1: FT from negative control; Lane 2: EL1 from negative

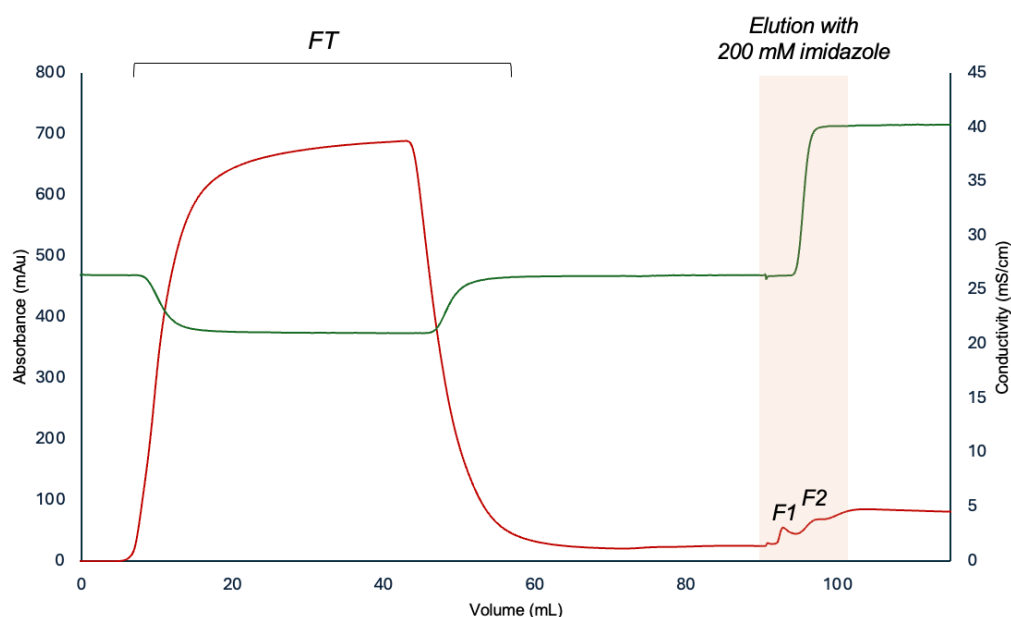
control; Lane 3: EL2 from negative control cells, showing no detectable band at ~22 kDa in any of the fractions. Lanes 4: FT from positive control; Lane 5: EL1 from positive control; Lane 6: EL2 from positive control, with a clear band at ~22 kDa corresponding to the viral coat protein in the elution fraction expected.

Together, these results demonstrated that HIC is a powerful tool for the selective purification of CCMV CP. The systematic progression from precipitation assays, through ligand screening, to the optimized stepwise elution protocol on phenyl resin, and finally the successful scale-up on a monolith column, established a reproducible method that combines efficiency with scalability. Importantly, the unexpected but robust retention of CP at pH 5.0 provided an opportunity to align purification directly with assembly-favouring conditions, simplifying integration with downstream encapsulation workflows. While HIC alone is not sufficient to achieve homogeneity, its ability to deliver well-resolved fractions with a dominant CP band makes it a strong candidate as part of a multi-step purification strategy. In particular, the combination of HIC with IMAC for capture and SEC for polishing offers a rational workflow that balances yield, selectivity, and analytical resolution. The knowledge gained from these optimization trials not only clarified the biochemical properties of CCMV CP but also provided a practical framework for developing a scalable purification pipeline.

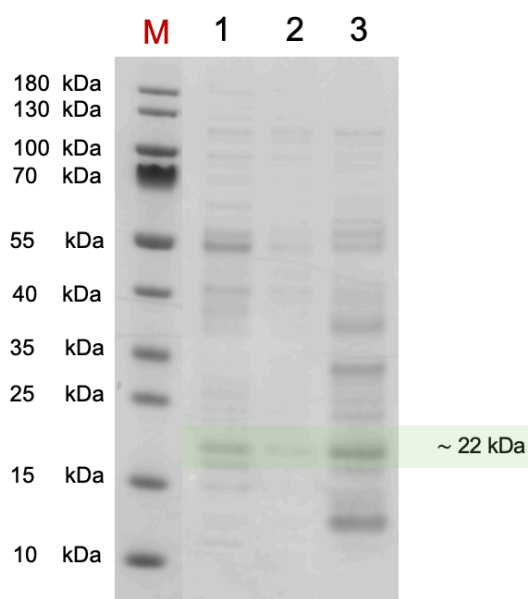
Immobilized metal affinity chromatography (IMAC) was evaluated as a capture method for CCMV CP, exploiting the engineered polyhistidine tag (6xHis) included in the construct. IMAC represents one of the most straightforward and widely applied strategies for recombinant protein purification, and its use here was intended to provide a high-yield first enrichment step. Crude lysates were loaded onto Ni<sup>2+</sup>-NTA HisTrap column in the presence of moderate salt NaCl concentrations, a condition reported to reduce non-specific electrostatic interactions with host proteins while maintaining efficient binding of histidine-tagged proteins. Initial trials confirmed that CCMV CP bound efficiently to Ni<sup>2+</sup>-NTA resin under the chosen conditions, but it rapidly became evident that background binding of host proteins was a major issue. To mitigate this, washing protocols were optimized by introducing low concentrations of imidazole (10–20 mM) into the wash buffer. This strategy reduced weak and non-specific interactions while preserving the strong affinity of the polyhistidine tag for the nickel ions. Despite these efforts, analysis of the wash and elution fractions by SDS-PAGE revealed that multiple contaminant proteins persisted. The target CP was consistently detected as a clear band at the expected molecular weight, but additional bands were present across fractions. To improve separation between the target protein and background contaminants, a stepwise

elution strategy with increasing imidazole concentrations was adopted. Fractions were collected at 30 mM and 200 mM imidazole, followed by a final high-strength elution at 500 mM. SDS-PAGE analysis showed that low concentrations of imidazole were sufficient to remove loosely bound impurities, while the CCMV CP eluted predominantly in a single fraction at intermediate concentrations ( $\approx 200$  mM) (Figure **61**). In these fractions, a strong band corresponding to the expected molecular weight of CP was consistently observed, confirming efficient recovery. However, additional bands of varying intensity persisted, indicating the co-elution of bacterial proteins with comparable affinity for  $\text{Ni}^{2+}$  (Figure **62**). Western blotting further highlighted this limitation: while CP was clearly detectable as the dominant signal, several background proteins were also strongly stained, consistent with reports of co-purification of *E. coli* proteins such as SlyD and ArnA. These findings underscored the inherent challenge of applying IMAC directly to crude lysates, as the presence of endogenous histidine-rich proteins reduces the selectivity of the method even when stepwise elution is employed.

Interestingly, pH-dependent trials revealed a behaviour distinct from what is generally reported in literature. According to literature, efficient interaction between the histidine tag and  $\text{Ni}^{2+}$  resin is expected at neutral to mildly basic pH, since histidine side chains must be deprotonated to coordinate the metal ion (251). Conversely, under acidic conditions such as pH 5.0, protonation of histidine residues is generally assumed to hinder binding and reduce recovery. However, the behaviour of CCMV CP contradicted these expectations. While efficient binding was observed at pH 7.4, as anticipated, the protein also displayed excellent retention and recovery at pH 5.0. In fact, the elution profiles suggested that binding under mildly acidic conditions was at least as selective, if not superior, compared to neutral pH. SDS-PAGE analysis of acidic extractions revealed a well-defined CP band in the imidazole-eluted fractions, and the relative intensity of contaminant bands was reduced compared to neutral conditions. These results indicate that despite the theoretical protonation of histidine residues, the His-tagged CCMV CP maintains strong affinity for  $\text{Ni}^{2+}$  resin at pH 5.0. From a process development standpoint, this unexpected robustness is highly advantageous, as it enables purification to be performed directly under assembly-favouring acidic conditions, simplifying integration with downstream workflows.



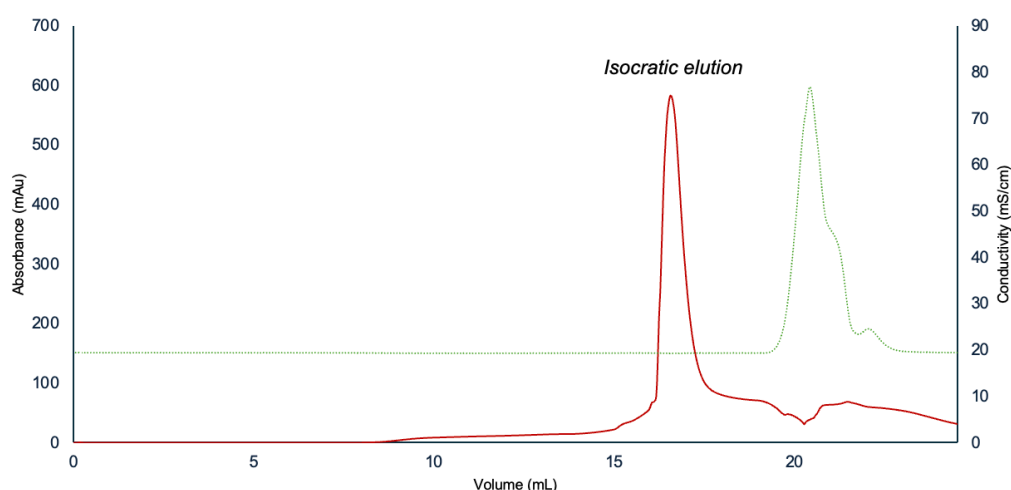
**Figure 61.** IMAC chromatography of *E. coli* lysates expressing CCMV-His. The chromatogram shows the flow-through (FT) and two fractions eluted with 200 mM imidazole, corresponding to the capture of His-tagged CCMV coat protein.



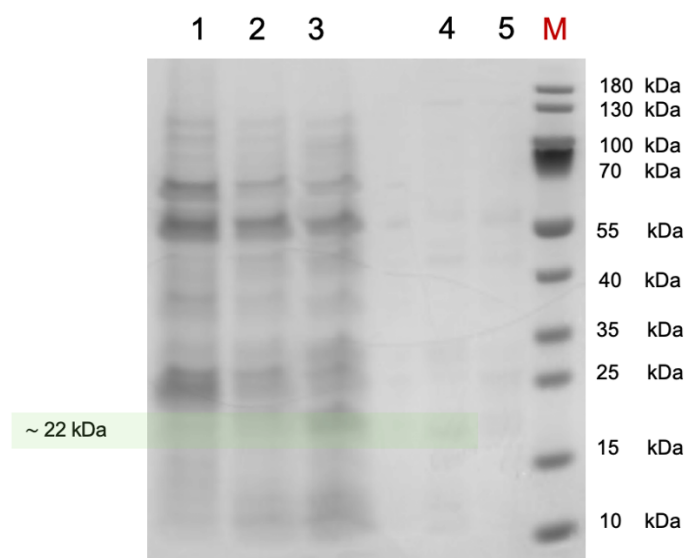
**Figure 62.** SDS-PAGE analysis of fractions from IMAC purification of CCMV-His. Lane M: Page Ruler pre-stained protein ladder; Lane 1: flow-through (FT); Lane 2: elution fraction 1 (F1); Lane 3: elution fraction 2 (F2). A distinct band at ~22 kDa, consistent with the CCMV coat protein, is visible in the elution fractions but also in the FT.

Size exclusion chromatography (SEC) was applied as a polishing step to improve the purity of CCMV CP obtained from IMAC and HIC chromatography and to characterize its oligomeric state under different conditions. Purification was performed using a Superose 6 Increase column (Cytiva), which provides high resolution in the range of 5 kDa–5 MDa and is therefore suitable for monitoring both monomeric CP and higher-order assemblies. Chromatographic

profiles revealed multiple peaks, consistent with the heterogeneous nature of CP in solution. At pH 7.4, fractions corresponding to lower molecular weight species eluted later in the chromatogram, compatible with monomeric and dimeric CP. A secondary, broader peak at earlier retention times indicated the presence of oligomeric species and partial aggregates. In contrast, at pH 5.0 the elution profile shifted markedly, with a single dominant peak. This behaviour reflects the pH-dependent assembly of CCMV CP: under mildly acidic conditions, the protein tends to form larger oligomers or capsid-like intermediates, whereas at neutral pH it remains predominantly in the disassembled form. SDS-PAGE analysis of collected fractions confirmed the presence of CP in both conditions, with the band intensity correlating with the main chromatographic peaks. However, protein recovery from SEC was modest, as expected for this technique, which is primarily designed for polishing and characterization rather than bulk capture. Importantly, SEC provided valuable insight into the conformational state of CP following purification: the predominance of high-molecular-weight assemblies at pH 5.0 confirmed that the protein retained its intrinsic capacity to undergo reversible assembly, while the separation of low-molecular-weight species at pH 7.4 was consistent with a disassembled state. Together, these observations highlighted SEC as a useful analytical and polishing step. Although not suitable as a primary purification method due to limited capacity, SEC was essential to obtain fractions enriched in either disassembled or assembled forms, which are critical for downstream encapsulation studies.



**Figure 63.** Size-exclusion chromatography (SEC) profile of CCMV after purification with the HIC monolith column. The chromatogram shows a single well-defined peak, consistent with a homogeneous particle population.



**Figure 64.** SDS–PAGE analysis of CCMV purification with Monolith CIMmultus OH and subsequent size exclusion SEC. Lane M: Page Ruler pre-stained protein ladder; Lanes 1–3: HIC Monolith run (FT, EL1, EL2); Lane 4: SEC peak (from EL2 of Monolith run); Lane 5: smaller SEC peak. A band at ~22 kDa, corresponding to the CCMV coat protein, is enriched in HIC elution fractions and confirmed in the SEC-purified sample.

### 7.3.1 Conclusions on *E. coli* as expression system

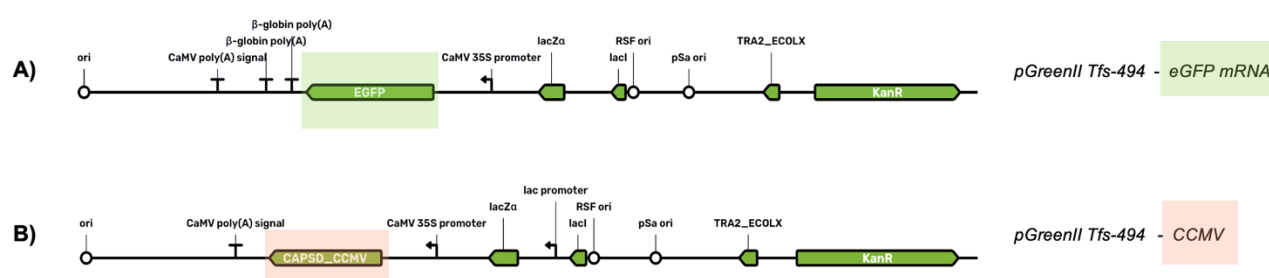
The comparative evaluation of the three chromatographic strategies underscored both the opportunities and the limitations of producing CCMV CP in *E. coli*. Hydrophobic interaction chromatography confirmed the significant hydrophobic contribution to CP behaviour and allowed the identification of optimized elution conditions. Immobilized metal affinity chromatography provided the highest overall yield, demonstrating that the engineered His-tag is functional and robust even under acidic conditions; however, persistent co-purification of *E. coli* proteins with intrinsic metal-binding properties significantly reduced selectivity. Size exclusion chromatography proved valuable as a polishing and analytical step, revealing the intrinsic pH-responsive assembly behaviour of the protein, but its low capacity and modest recovery precluded its use as a primary purification method. Collectively, these results confirmed the biochemical properties of CCMV CP, particularly its capacity to undergo reversible, pH-dependent transitions between disassembled and assembled states. At the same time, they highlighted the intrinsic drawbacks of *E. coli* as a production host: the high background of bacterial proteins, the propensity of CP to aggregate, and the difficulty of obtaining homogeneous, assembly-competent material suitable for encapsulation assays. For these reasons, *E. coli* trials were considered an essential proof-of-concept stage, useful for optimizing purification parameters and validating the structural behaviour of CCMV CP.



Building on these insights, the project transitioned to the plant-based expression system, where the co-production of CP and target mRNA could be pursued within a more favourable folding and assembly environment. This strategic shift was motivated not only by the potential to overcome the purification bottlenecks observed in bacteria, but also by the opportunity to exploit plants as integrated biofactories for the simultaneous generation and encapsulation of nucleic acid vaccines.

#### 7.4 CCMV Expression and Purification from *N. benthamiana*

To enable plant-based expression of CCMV CP, the coding sequence was cloned into a binary vector (pGreenII Tfs-494) under the control of a strong constitutive promoter (CaMV 35S). The choice of pGreenII as vector was motivated by its modularity, high compatibility with plant expression systems, and extensive validation in transient expression studies (178). It was finally possible to isolate a colony of pGreenII, which was affected by the presence of an IS10 insertion, without the insertion. After extensive screening of multiple colonies, a plasmid of the correct size and integrity was finally obtained and confirmed through sequencing. This breakthrough allowed the preparation of two fully validated constructs: pGreenII-CCMV, encoding the capsid protein, and pGreenII-mRNA, encoding the model RNA sequence. QC of the constructs was performed through restriction digestion and sequencing (Figure 65) to confirm the correct insertion of the genes of interest.



**Figure 65.** Schematic representation of the pGreenII Tfs-494 vector combined with the two sequences of interest to be expressed in *Nicotiana benthamiana*. A) pGreenII Tfs-494 with eGFP mRNA sequence; B) pGreenII Tfs-494 with CCMV capsid protein. No insertion was detected.

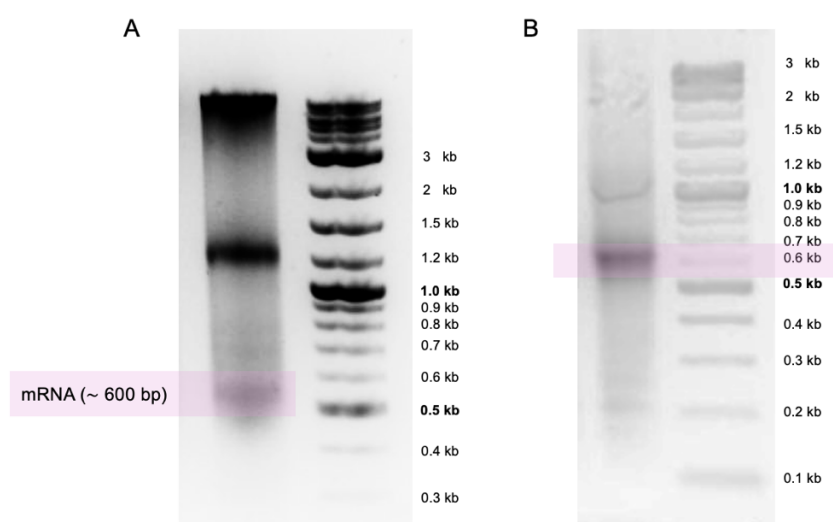
The validated recombinant plasmids were introduced into *Agrobacterium tumefaciens* via electroporation using the optimized protocol developed, and transformation efficiency was calculated by colony counting after antibiotic selection. The pSoup helper plasmid was co-transformed to ensure vector replication. Colonies appeared within three days and were grown in selective liquid cultures. Pre-inoculums were expanded into large-scale cultures and

both strains showed robust growth, with OD<sub>600</sub> values around 7.00 after overnight culture for both constructs. Following centrifugation, the cells were resuspended in infiltration buffer and adjusted to a final OD<sub>600</sub> of 0.2. Equalized suspensions were combined to get a final OD<sub>600</sub> of 0.4 and used for vacuum agroinfiltration of *Nicotiana benthamiana* plants. Each plant was immersed upside-down in the bacterial suspension, and vacuum was applied for four minutes. Infiltrated plants were then returned to greenhouse conditions for transient expression. Leaves were harvested at five days post-infiltration (dpi) and stored at -80 °C to preserve RNA and protein integrity for subsequent analyses.

This represented the first successful attempt within the project to combine the in-planta expression of a model mRNA and its encapsulating nanocage, paving the way for testing whether in vivo encapsulation could occur directly in the plant host. The establishment of dual constructs and their stable agroinfection marked an essential step toward realizing a plant-based co-expression platform.

Initial extractions were performed to assess whether both components of the system had been successfully expressed in planta.

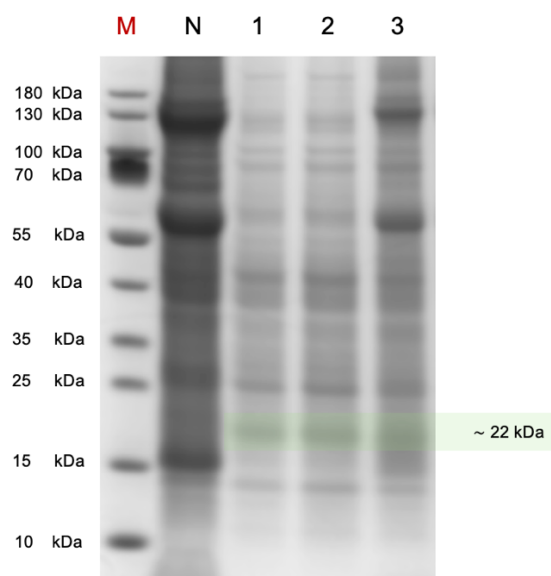
Total RNA was isolated from agroinfiltrated leaves with commercially purchased kit (RNeasy Plant Mini Kit) using two different lysis buffers, as this step had previously been shown to influence yield and purity. Nanodrop analysis revealed the conditions that promoted more efficient solubilisation of nucleic acids and optimal purity identified by ratio A<sub>260</sub>/A<sub>280</sub> value around 2.0. To confirm the identity of the transcripts, the extracts were resolved on agarose gel. A clear band of approximately 600 bp was visible in the infiltrated samples, matching the expected size of the eGFP construct. Furthermore, the comparison with an in vitro transcribed (IVT) positive control confirmed that the observed band corresponded to the desired transcript size (Figure 66). The presence of this distinct signal provided strong evidence for successful transcription of the target mRNA in planta.



**Figure 66.** Agarose gel electrophoresis (2%) comparing plant-expressed mRNA with an in vitro transcription (IVT) control. (A) mRNA extracted from agroinfiltrated *N. benthamiana* leaves using the RNeasy Plant Mini Kit, showing a distinct band at ~600 bp. (B) IVT-derived positive control mRNA of corresponding size, used as reference standard.

In parallel, crude leaves extracts were prepared from infiltrated leaves to verify expression of the CCMV coat protein. Given the known pH-dependent assembly properties of CCMV, extractions were carried out under both neutral and acidic conditions to favour solubility of the disassembled and assembled states, respectively. Leaves were homogenised either in buffer 50 mM Tris, 150 mM NaCl, 2 mM DTT, 10 mM MgCl<sub>2</sub>, 1% Triton X-100, pH 7.4 to favour solubilisation of the disassembled form, or in buffer 50 mM Sodium Acetate, 150 mM NaCl, 2 mM DTT, 10 mM MgCl<sub>2</sub>, 1% Triton X-100, pH 5.0 to promote the assembled state. Leaves were mechanically homogenized at a 1:2 (w/v) ratio with the extraction buffer. Following clarification through centrifugation, the extracts were analysed by SDS-PAGE in direct comparison with negative controls from wild-type plants. A distinct band at approximately ≈ 22 kDa, corresponding to the expected molecular weight of the CCMV CP, was consistently observed in the agroinfiltrated samples but not in the negative controls. The reproducibility of this signal across both pH conditions confirmed the successful expression of CCMV CP in planta.

Taken together, these preliminary results demonstrated that *N. benthamiana* was capable of simultaneously expressing the mRNA construct and the viral coat protein. The detection of both components in the same tissues provided the first experimental indication that in vivo encapsulation could be explored and laid the groundwork for subsequent optimisation of purification strategies to recover intact VLP–RNA complexes.



**Figure 67.** SDS-PAGE analysis of crude extracts from *N. benthamiana* plants expressing CCMV. Lane M: Page Ruler pre-stained protein ladder; Lane N: negative control (crude extract from non-agroinfiltrated plants); Lane 1-2: agroinfiltrated leaves extracted at pH: 5.0; Lane 3: agroinfiltrated leaves extracted at pH: 7.4. A band at ~22 kDa, consistent with the CCMV coat protein, is observed in the agroinfiltrated samples.

## 7.5 Conclusions

The work presented in this chapter established the foundation for producing CCMV virus-like particles as an RNA delivery platform. Initial studies in *E. coli* provided a rapid proof-of-concept system that enabled the optimisation of extraction, lysis and chromatographic purification strategies. These experiments confirmed the pH-responsive behaviour of CCMV coat protein and demonstrated that hydrophobic interaction chromatography, complemented by IMAC and SEC, can reproducibly recover the protein. However, persistent co-purification of bacterial proteins and the propensity of CCMV CP to aggregate highlighted the limitations of microbial hosts as production platforms.

A major breakthrough was the successful isolation of a pGreenII clone free from IS10 insertion, which enabled the generation of two validated plant constructs. For the first time within this project, *Nicotiana benthamiana* plants were co-infiltrated with both an mRNA construct and the CCMV coat protein. Analyses confirmed expression of the target mRNA, and simultaneous detection of the coat protein as a distinct ~22 kDa band on SDS-PAGE. These results demonstrated that both components of the encapsulation system can be

produced in planta, providing the first experimental indication that in vivo RNA packaging could be explored directly in the host plant.

Together, these findings position plants not only as a source of recombinant mRNA, as shown in Chapter 6, but also as integrated biofactories capable of producing both nucleic acid cargo and viral nanocages in a single system. Future work will need to address the recovery and purification of intact VLP–RNA complexes from plant tissue, optimise the ratio between RNA and capsid protein, and assess stability and functionality in downstream delivery assays. Nevertheless, the results obtained here represent a critical step towards the development of a fully plant-based platform for mRNA vaccine production and delivery.

## 8. CONCLUSIONS

This doctoral thesis explored plant molecular farming as a comprehensive strategy for vaccine production, ranging across upstream cultivation, downstream purification, and the prototyping of next-generation nucleic acid vaccine platforms. The project was articulated across two complementary contexts: an industrial development research focused on a recombinant subunit antigen (TB002) at Special Product's Line and an academic project conducted at UCL focused to the expression of mRNA and its potential packaging into CCMV virus-like particles. By exploring both these fronts, it was possible to balance the stringent constraints of process development in an industrial regulated environment with the flexibility to test novel and riskier concepts in a laboratory setting, ultimately showing how plants can operate as biofactories for producing both protein and nucleic acid vaccines.

The work carried out on upstream cultivation and infiltration clearly demonstrated how process parameters established at production scale determine product quality before purification even begins. The optimization of vacuum agroinfiltration defined a robust set of operating conditions, 4 minutes at 700 mbar, that reproducibly maximized expression while avoiding tissue collapse. These data confirmed that infiltration is a decisive determinant of yield, homogeneity, and stability of the product of interest. At the same time, the comparison between lab-scale and industrial-scale systems highlighted their respective advantages: while compact laboratory modules offered rapid prototyping and parameter screening, the industrial vertical farms provided higher biomass, environmental stability, and the reproducibility required for large-scale and GMP-oriented production. This dual analysis pointed out how laboratory systems and industrial platforms can be strategically combined, with small-scale rapid development converging into standardized industrial platforms where automation ensures consistency.

Within the industrial framework, the TB002 project served as a case study to construct a reproducible downstream pipeline adapted to the peculiarities of plant extracts. The main technical barrier was the tendency of TB002 to aggregate and co-purify with abundant host proteins. Systematic buffer screening showed that introducing a strong chaotropic agent – 7 M urea – was decisive for better solubilisation, enabling efficient capture on immobilized metal affinity columns. Subsequent steps, including thrombin tag removal and polishing by size exclusion, provided a reproducible process that consistently delivered the protein in a tag-free, analytically confirmed form. Across repeated runs, the pipeline proved robust, although one main limitation was consistently encountered. The inherent complexity of the plant matrix – phenolics, proteases, and complex host proteins – complicates purification and increases

costs compared to microbial systems. Nevertheless, the process reproducibly generated material of sufficient quality for preclinical studies. The TB002 study showed that, once expression levels are high enough and the extraction conditions are suited to the protein's properties, downstream becomes feasible with conventional unit operations, but will remain the main driver of cost.

Building on this foundation, the academic phase explored the feasibility of producing mRNA directly in *Nicotiana benthamiana*. Transient expression yielded the expected eGFP transcript, confirmed by agarose electrophoresis. This was a significant milestone, proving that plants can serve as hosts for nucleic acid production. At the same time, several limitations became evident. The downstream purification consistently revealed the difficulty of separating the target transcript from abundant host RNAs. Various strategies were tested, including anion exchange, oligo(dT) affinity, magnetic bead capture, size exclusion, and kit-based methods, each of which provided partial recovery but none of which achieved high purity in a single step. These results confirmed that while plant-made mRNA expression is technically feasible, efficient recovery requires multi-modal approaches and, ideally, protective strategies that shield the transcript from degradation and co-purification during extraction. This observation led to the decision to explore a packaging system based on virus-like particles.

The CCMV coat protein was first expressed and purified in *E. coli* to provide rapid proof of concept and to optimise purification workflows. These trials confirmed the protein's reversible pH-dependent assembly behaviour and defined suitable purification protocols with hydrophobic interaction chromatography, affinity capture, and size exclusion. However, the purification outcome was limited by the high background of host proteins and by the tendency of the coat protein to aggregate, highlighting the inherent drawbacks of microbial systems for producing structurally demanding proteins. A decisive breakthrough was achieved when a pGreenII clone with no insertion was isolated and used to generate two validated constructs, one carrying the eGFP mRNA and the other the CCMV CP. Co-infiltration into *Nicotiana benthamiana* established, for the first time in this project, simultaneous expression in planta of both the nucleic acid cargo and its viral nanocapsule. Analyses confirmed the presence of the eGFP transcript ( $\approx 600$  bp) and of a distinct band at 22 kDa corresponding to the coat protein. The detection of both components in the same tissue provided the first experimental indication that in vivo encapsulation could be attempted, opening the door to the direct production of RNA-loaded VLPs in plants.

Together, these complementary approaches yielded three main contributions. First, they established that plant infiltration can be precisely controlled to reduce variability and secure

expression levels compatible with downstream processing. Second, they demonstrate that downstream purification of plant-made proteins, while inherently challenging, can yield high-quality material when extraction is carefully tuned to product properties and multi-step strategies are applied. Third, they show that plants can be harnessed not only for recombinant proteins and mRNA separately, but also as integrated systems capable of producing both cargo and VLP platform in a single host. This last point is particularly significant, as it points to the possibility of simplifying purification encapsulating the RNA as it is produced and thereby shielding it from degradation and host contaminants.

Several limitations must be acknowledged. The TB002 process was developed at research scale and requires GMP validation to confirm robustness under industrial conditions, particularly regarding sterilization and formulation. The CCMV project, while demonstrating co-expression, still lacks quantitative evidence of packaging efficiency and stability, which will require advanced analytical methods such as RNase protection assays, DLS, TEM, and sequencing of encapsulated RNA. Despite these limitations, the work provides a realistic and detailed picture of both the potential and the constraints of plant-based vaccine production.

The results achieved in this thesis support a vision with plants as flexible biofactories capable of producing antigens, nucleic acids, and viral nanocapsule. The platforms tested align with the distinctive advantages of plant molecular farming – safety, speed, scalability, and sustainability – and extends them into the domain of nucleic acid medicines. As global health systems increasingly demand decentralized and surge-ready production capacity, the ability to produce both the therapeutic payload and its protective carrier within the same plant system represents a significant scientific advance with important practical implications for the future work.



## 9. BIBLIOGRAPHY

1. World Health Organization. *Immunization Coverage*; <https://www.who.int/news-room/fact-sheets/detail/immunization-coverage> (accessed Aug 2, 2025).
2. Centers for Disease Control and Prevention. *Ten Great Public Health Achievements—United States, 2001–2010*. *MMWR Morb. Mortal. Wkly. Rep.* **2011**, 60 (19), 619–623.
3. Fenner, F.; Henderson, D. A.; Arita, I.; Ježek, Z.; Ladnyi, I. D. *Smallpox and Its Eradication*; World Health Organization: Geneva, 1988.
4. Plotkin, S.; Robinson, J. M.; Cunningham, G.; Iqbal, R.; Larsen, S. The Complexity and Cost of Vaccine Manufacturing – An Overview. *Vaccine* **2017**, 35 (33), 4064–4071. DOI: 10.1016/j.vaccine.2017.06.003.
5. Minor, P. D. Live Attenuated Vaccines: Historical Successes and Current Challenges. *Virology* **2015**, 108 (10), 395–400. DOI: 10.1016/j.virol.2015.03.032.
6. Moyle, P. M.; Toth, I. Modern Subunit Vaccines: Development, Components, and Research Opportunities. *ChemMedChem* **2013**, 8(3), 360–376. DOI: 10.1002/cmdc.201200487.
7. Pardi, N.; Hogan, M. J.; Porter, F. W.; Weissman, D. mRNA Vaccines – A New Era in Vaccinology. *Nat. Rev. Drug Discov.* **2018**, 17 (4), 261–279. DOI: 10.1038/nrd.2017.243.
8. Krammer, F. SARS-CoV-2 Vaccines in Development. *Nature* 2020, 586 (7830), 516–527. <https://doi.org/10.1038/s41586-020-2798-3>.
9. Ura, T.; Yamashita, A.; Mizuki, N.; Okuda, K.; Shimada, M. New Vaccine Production Platforms Used in Developing SARS-CoV-2 Vaccine Candidates. *Vaccine* **2021**, 39 (2), 197–201. DOI: 10.1016/j.vaccine.2020.11.054.
10. Pardi, N.; Weissman, D. Nucleoside Modified mRNA Vaccines for Infectious Diseases. *Methods Mol. Biol.* **2017**, 1499, 109–121. DOI: 10.1007/978-1-4939-6481-9\_6.
11. Baden, L. R.; El Sahly, H. M.; Essink, B.; Kotloff, K.; Frey, S.; Novak, R.; Diemert, D.; Spector, S. A.; Rouphael, N.; Creech, C. B.; McGettigan, J.; Khetan, S.; Segall, N.; Solis, J.; Brosz, A.; Fierro, C.; Schwartz, H.; Neuzil, K.; Corey, L.; Gilbert, P.; Janes, H.; Follmann, D.; Marovich, M.; Mascola, J.; Polakowski, L.; Ledgerwood, J.; Graham, B. S.; Bennett, H.; Pajon, R.; Knightly, C.; Leav, B.; Deng, W.; Zhou, H.; Han, S.; Ivarsson, M.; Miller, J.; Zaks, T. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N. Engl. J. Med.* **2021**, 384 (5), 403–416. DOI: 10.1056/NEJMoa2035389.
12. Sahin, U.; Karikó, K.; Türeci, Ö. mRNA-Based Therapeutics – Developing a New Class of Drugs. *Nat. Rev. Drug Discov.* **2014**, 13(10), 759–780. DOI: 10.1038/nrd4278.
13. Liu, M. A. A Comparison of Plasmid DNA and mRNA as Vaccine Technologies. *Vaccines* **2019**, 7 (2), 37. DOI: 10.3390/vaccines7020037.

14. Kushnir, N.; Streatfield, S. J.; Yusibov, V. Virus-Like Particles as a Highly Efficient Vaccine Platform: Diversity of Targets and Production Systems and Advances in Clinical Development. *Vaccine* **2012**, *31* (1), 58–83. DOI: 10.1016/j.vaccine.2012.10.083.
15. Shariati, A.; Khezarpour, A.; Shariati, F.; Afkhami, H.; Yarahmadi, A.; Alavimanesh, S.; Kamrani, S.; Modarressi, M. H.; Khani, P. DNA Vaccines as Promising Immuno-therapeutics against Cancer: A New Insight. *Front. Immunol.* **2025**, *15*, 1498431. DOI: 10.3389/fimmu.2024.1498431.
16. Ozawa, S.; Clark, S.; Portnoy, A.; Grewal, S.; Brenzel, L.; Walker, D Return On Investment From Childhood Immunization In Low- And Middle-Income Countries. *Health Aff.* **2011**, *35* (2), 199–207. DOI: 10.1377/hlthaff.2015.1089.
17. Bärnighausen, T.; Bloom, D. E.; Cafiero, E. T.; O'Brien, J. C. Economic Evaluation of Vaccination: Capturing the Full Benefits, with an Application to Human Papillomavirus. *Clin. Microbiol. Infect.* **2012**, *18* (Suppl. 5), 70–76. DOI: 10.1111/j.1469-0691.2012.03977.
18. Bloom, D. E.; Canning, D.; Weston, M. The Value of Vaccination, Fighting the Disease of Poverty. *World Econ.* **2017**, 214-238. DOI: 10.4324/9780203791950-8.
19. World Health Organization. *Immunization Agenda 2030: A Global Strategy to Leave No One Behind*; WHO: Geneva, 2020. <https://www.who.int/publications/i/item/9789240015702>.
20. Wouters, O. J.; Shadlen, K. C.; Salcher-Konrad, M.; Pollard, A. J.; Larson, H. J.; Teerawattananon, Y.; Jit, M. Challenges in Ensuring Global Access to COVID-19 Vaccines: Production, Affordability, Allocation, and Deployment. *Lancet* **2021**, 397 (10278), 1023–1034. DOI: 10.1016/S0140-6736(21)00306-8.
21. Gavi, The Vaccine Alliance. *Cold Chain Equipment Optimization Platform (CCEOP) Annual Report 2021*; Gavi: Geneva, 2022. <https://www.gavi.org>.
22. Excler, J. L.; Saville, M.; Berkley, S.; Kim, J. H. Vaccine Development for Emerging Infectious Diseases. *Nat. Med.* **2021**, 27 (4), 591–600. DOI: 10.1038/s41591-021-01301-0.
23. Rydland, H. T.; Friedman, J.; Stringhini, S.; Link, B. G.; Eikemo, T. A. The Radically Unequal Distribution of COVID-19 Vaccinations: A Predictable yet Avoidable Symptom of the Fundamental Causes of Inequality. *Humanit. Soc. Sci. Commun.* **2022**, *9*, 61. DOI: 10.1057/s41599-022-01073-z.
24. World Health Organization; UNICEF. *WHO/UNICEF Estimates of National Immunization Coverage (WUENIC)*, **2024**; World Health Organization: Geneva, 2024. Available online: <https://www.who.int/teams/immunization-vaccines-and-biologicals/immunization-analysis-and-insights/global-monitoring/immunization-coverage/who-unicef-estimates-of-national-immunization-coverage> (accessed Aug 2, 2025).
25. Pagliusi, S.; Jarrett, S.; Hayman, B.; Kreysa, U.; Pridgeon, C.; Aguado, M. T. Emerging Manufacturers Engagement in the COVID-19 Vaccine Research, Development and Supply. *Vaccine* **2020**, *38* (34), 5418-5423 DOI: 10.1016/j.vaccine.2020.06.022.

26. Li, X.; Jin, S.; Guo, S.; Yang, D.; Sai, W.; Qiu, X.; Zhao, X.; Wang, L.; Wang, T.; Li, M. Platform Technology in Global Vaccine Regulation: Development, Applications, and Regulatory Strategies with Insights from China. *Vaccines* **2024**, *12* (12), 1436. DOI: 10.3390/vaccines12121436.
27. Sachs, J. D.; Karim, S. S. A.; Akinin, L.; Allen, J.; Brosbøl, K.; Colombo, F.; Barron, G. C.; Binagwaho, A.; Bustreo, F.; Buse, K.; Cyranoski, D.; Desai, I.; Florêncio, O.; Girum, T.; Gupta, S.; Haines, A.; Leung, G. M.; Maani, N.; Marcisevic, A.; Mokdad, A. H.; Nsanzimana, S.; Nzabarinda, L. E.; Ribeiro, I.; Serag, H.; Thwaites, J.; Weerasinghe, M.; Wouters, O.; Finacier, M.; Guzman, C.; Mallard, C.; Mate, K. S.; Rida, W.; Santos, I. S.; Watson, R.; Yadav, U.; Daar, A. S.; Singer, P. A.; Daar, R.; Moorthy, V. The Lancet Commission on Lessons for the Future from the COVID-19 Pandemic. *Lancet* **2022**, *400* (10359), 1224–1280. DOI: 10.1016/S0140-6736(22)01585-9.
28. Moorthy, V.; Henao-Restrepo, A. M.; Preziosi, M. P.; Swaminathan, S. Data Sharing for Novel Coronavirus (COVID-19). *Bull World Health Organ.* **2020**, *98* (3), 150. DOI: 10.2471/BLT.20.251561.
29. Shimabukuro, T. T.; Cole, M.; Su, J. R. Reports of Anaphylaxis after Receipt of mRNA COVID-19 Vaccines in the US – December 14, 2020–January 18, 2021. *N. Engl. J. Med.* **2021**, *325* (11), 1101–1102. DOI: 10.1001/jama.2021.1967.
30. World Health Organization. *Vaccine Quality*. <https://www.who.int/teams/health-product-and-policy-standards/standards-and-specifications/vaccines-quality> (accessed Aug 2, 2025).
31. Pardi, N.; Muramatsu, H.; Weissman, D.; Karikó, K. In Vitro Transcription of Long RNA Containing Modified Nucleosides. *Methods Mol Biol.* **2013**, *969*, 29–42. DOI: 10.1007/978-1-62703-260-5\_2.
32. Chen, D.; Kristensen, D. Opportunities and Challenges of Developing Thermostable Vaccines. *Expert Rev. Vaccines* **2009**, *8* (5), 547–557. DOI: 10.1586/erv.09.20.
33. Matrajt, L.; Eaton, J.; Leung, T.; Brown, E. R. Vaccine Optimization for COVID-19: Who to Vaccinate First? *Sci. Adv.* **2021**, *7* (6), eabf1374. DOI: 10.1126/sciadv.abf1374.
34. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). *E6(R2) Good Clinical Practice: Integrated Addendum to ICH E6(R1)*; 2016. <https://www.ich.org/page/efficacy-guidelines>.
35. Sallusto, F.; Lanzavecchia, A.; Araki, K.; Ahmed, R. From Vaccines to Memory and Back. *Immunity* **2010**, *33* (4), 451–463. DOI: 10.1016/j.immuni.2010.10.008.
36. Masignani, V.; Palacios, R.; Martin, M.-T.; Tibaldi, F.; Vadivelu, V. K. De-risking Vaccine Development: Lessons, Challenges, and Prospects. *npj Vaccines* **2025**, *10*, 177. DOI: 10.1038/s41541-025-01211-z.
37. Buyel, J. F. Plant Molecular Farming—Integration and Exploitation of Side Streams to Achieve Sustainable Biomanufacturing. *Front. Plant Sci.* **2019**, *9*, 1893. DOI: 10.3389/fpls.2018.01893.

38. Fischer, R.; Schillberg, S.; Hellwig, S.; Twyman, R. M.; Drossard, J. GMP Issues for Recombinant Plant-Derived Pharmaceutical Proteins. *Biotechnol. Adv.* **2012**, *30* (2), 434–439. DOI: 10.1016/j.biotechadv.2011.08.007.
39. Yusibov, V.; Streatfield, S. J.; Kushnir, N. Clinical Development of Plant-Produced Recombinant Pharmaceuticals: Vaccines, Antibodies and Beyond. *Hum. Vaccin.* **2011**, *7* (3), 313–321. DOI: 10.4161/hv.7.3.14207.
40. Fischer, R.; Schillberg, S.; Buyel, J. F.; Twyman, R. M. Commercial Aspects of Pharmaceutical Protein Production in Plants. *Curr. Pharm. Des.* **2013**, *19* (31), 5471–5477. DOI: 10.2174/1381612811319310006.
41. Ward, B. J.; Gobeil, P.; Séguin, A.; Atkins, J.; Boulay, I.; Charbonneau, P.-Y.; Couture, M.; D'Aoust, M.-A.; Dhaliwall, J.; Finkle, C.; et al. Phase 1 Randomized Trial of a Plant-Derived Virus-Like Particle Vaccine for Covid-19. *Nat. Med.* **2021**, *27* (6), 1071–1078. DOI: 10.1038/s41591-021-01370-1.
42. Sharma, M.; Sharma, A. K. Plants as Bioreactors: Recent Developments and Emerging Opportunities. *Biotechnol. Adv.* **2009**, *27* (6), 811–832. DOI: 10.1016/j.biotechadv.2009.06.004.
43. Qiu, X.; Fernando, L.; Melito, P. L.; Audet, J.; Feldmann, H.; Kobinger, G.; Alimonti, J. B.; Jones, S. M. MAbs and Adjuvants Targeting Ebola GP Rapidly Protect Mice against Lethal Challenge. *PLoS Negl. Trop. Dis.* **2012**, *6* (3), e1575. DOI: 10.1371/journal.pntd.0001575.
44. Tacket, C. O.; Mason, H. S.; Losonsky, G.; Clements, J. D.; Levine, M. M.; Arntzen, C. J. Immunogenicity in Humans of a Recombinant Bacterial Antigen Delivered in a Transgenic Potato. *Nat. Med.* **1998**, *4* (5), 607–609. DOI: 10.1038/nm0598-607.
45. Liu, H.; Ding, Y.; Zhou, Y.; Jin, H.; Huang, Y.; Yan, H.; Zhao, X.; Wang, H.; Zhang, L. Improving Protein Quantity and Quality – The Next Level of Plant Molecular Farming. *Int. J. Mol. Sci.* **2022**, *23* (3), 804026. DOI: 10.3390/ijms23031326.
46. Ma, J. K.-C.; Drake, P. M. W.; Christou, P. The Production of Recombinant Pharmaceutical Proteins in Plants. *Nature Reviews* **2003**, *4* (10), 794–805. DOI: 10.1038/nrg1177.
47. Horsch, R. B.; Fry, J. E.; Hoffmann, N. L.; Eichholtz, D.; Rogers, S. G.; Fraley, R. T. A Simple and General Method for Transferring Genes into Plants. *Science* **1985**, *227* (4691), 1229–1231. DOI: 10.1126/science.227.4691.1229.
48. Barta, A.; Sommergruber, K.; Thompson, D.; Hartmuth, K.; Matzke, M. A.; Matzke, A. J. M. The Expression of a Nopaline Synthase – Human Growth Hormone Chimeric Gene in Transformed Tobacco and Sunflower Callus Tissue. *Plant Mol. Biol.* **1986**, *6* (5), 347–357. DOI: 10.1007/BF00034942.
49. Gleba, Y.; Klimyuk, V.; Marillonnet, S. Magniffection—A New Platform for Expressing Recombinant Vaccines in Plants. *Vaccine* **2005**, *23* (17–18), 2042–2048. DOI: 10.1016/j.vaccine.2005.01.006.

50. Schillberg, S.; Raven, N.; Spiegel, H.; Rasche, S.; Buntru, M. Critical Analysis of the Commercial Potential of Plants for the Production of Recombinant Proteins. *Front. Plant Sci.* **2019**, *10*, 720. DOI: 10.3389/fpls.2019.00720.
51. Qiu, X.; Wong, G.; Audet, J.; Bello, A.; Fernando, L.; Alimonti, J. B.; Fausther-Bovendo, H.; Wei, H.; Aviles, J.; Hiatt, E.; et al. Reversion of Advanced Ebola Virus Disease in Nonhuman Primates with ZMapp. *Nature* **2014**, *514* (7520), 47–53. DOI: 10.1038/nature13777.
52. Government of Canada. *Health Canada Authorizes Medicago COVID-19 Vaccine*. Healthy Canadians, 24 February 2022. <https://healthycanadians.gc.ca/recall-alert-rappel-avis/hc-sc/2022/75149a-eng.php>.
53. Phoolcharoen, W.; Shanmugaraj, B.; Khorattanakulchai, N.; Sunyakumthorn, P.; Pichyangkul, S.; Taepavarapruk, P.; Praserthsee, W.; Malaivijitnond, S.; Manopwisedjaroen, S.; Thitithanyanont, A.; Srisutthisamphan, K.; Jongkaewwattana, A.; Tomai, M.; Fox, C. B.; Taychakhoonavudh, S. Preclinical Evaluation of Immunogenicity, Efficacy and Safety of a Recombinant Plant-Based SARS-CoV-2 RBD Vaccine Formulated with 3M-052-Alum Adjuvant. *Vaccine* **2023**, *41* (17), 2781–2792. DOI: 10.1016/j.vaccine.2023.03.027.
54. Schillberg, S.; Raven, N.; Fischer, R.; Twyman, R. M.; Schiermeyer, A. Molecular Farming of Pharmaceutical Proteins Using Plant Suspension Cell and Tissue Cultures. *Curr. Pharm. Des.* **2013**, *19* (31), 5531–5542. DOI: 10.2174/1381612811319310013.
55. Castilho, A.; Steinkellner, H. Glyco-Engineering in Plants to Produce Human-Like N-Glycan Structures. *Biotechnol. J.* **2012**, *7* (9), 1088–1098. DOI: 10.1002/biot.201200032.
56. Fischer, R.; Buyel, J. F. Molecular Farming — The Slope of Enlightenment. *Biotechnol. Adv.* **2020**, *40*, 107519. DOI: 10.1016/j.biotechadv.2020.107519.
57. Bally, J.; Jung, H.; Mortimer, C.; Naim, F.; Philips, J. G.; Hellens, R.; Bombarely, A.; Goodin, M. M.; Waterhouse, P. M. The Rise and Rise of *Nicotiana benthamiana*: A Plant for All Reasons. *Annu. Rev. Phytopathol.* **2018**, *56*, 405–426. DOI: 10.1146/annurev-phyto-080417-050141.
58. Chen, Q.; He, J.; Phoolcharoen, W.; Mason, H. S. Geminiviral Vectors Based on Bean Yellow Dwarf Virus for Production of Vaccine Antigens and Monoclonal Antibodies in Plants. *Hum. Vaccin.* **2011**, *7* (13), 331–338. DOI: 10.4161/hv.7.3.14262.
59. Sparks, C. A.; Jones, H. D. Biolistics Transformation of Wheat. *Methods Mol. Biol.* **2009**, *478*, 71–92. DOI: 10.1007/978-1-59745-379-0\_4.
60. Horsch, R. B.; Fry, J. E.; Hoffmann, N. L.; Eichholtz, D.; Rogers, S. G.; Fraley, R. T. A Simple and General Method for Transferring Genes into Plants. *Science* **1985**, *227* (4691), 1229–1231. DOI: 10.1126/science.227.4691.1229.
61. Daniell, H.; Lee, S.-B.; Panchal, T.; Wiebe, P. A. Expression of the Native Cholera Toxin B Subunit Gene and Assembly as Functional Oligomers in Transgenic Tobacco Chloroplasts. *J. Mol. Biol.* **2001**, *311* (5), 1001–1009. DOI: 10.1006/jmbi.2001.4921.

62. Daniell, H.; Lin, C. S.; Yu, M.; Chang, W. J. Chloroplast Genomes: Diversity, Evolution, and Applications in Genetic Engineering. *Genome Biol.* **2016**, *17*, 134. DOI: 10.1186/s13059-016-1004-2.
63. Bock, R. Engineering Plastid Genomes: Methods, Tools, and Applications in Basic Research and Biotechnology. *Annu. Rev. Plant Biol.* **2015**, *66*, 211–241. DOI: 10.1146/annurev-arplant-050213-040212.
64. Vulpe, C.-B.; Toplicean, I.-M.; Agachi, B.-V.; Datcu, A.-D. A Review on Uses of *Lemna minor*, a Beneficial Plant for Sustainable Water Treatments, in Relation to Bioeconomy Aspects. *Water* **2025**, *17*, 1400. DOI: 10.3390/w17091400.
65. Norkunas, K.; Harding, R.; Dale, J.; Dugdale, B. Improving Agroinfiltration-Based Transient Gene Expression in *Nicotiana benthamiana*. *Plant Methods* **2018**, *14*, 71. DOI: 10.1186/s13007-018-0343-2.
66. Buyel, JF.; Stöger, E.; Bortesi, L. Targeted genome editing of plants and plant cells for biomanufacturing. *Transgenic Res.* **2021**;30(4):401-426. DOI: 10.1007/s11248-021-00236-z.
67. Chen, F.; Chen, L.; Yan, Z.; Jingyuan, X.; Luoluo, F.; Na, H.; Mingli, G; Jiaxiong, Z.; Zhijun, C.; Huiqi, C.; Gengzhen, Y.; Chunping, L. Recent advances of CRISPR-based genome editing for enhancing staple crops. *Front Plant Sci.* **2024**, *15*. DOI: 10.3389/fpls.2024.1478398.
68. Moon, K.-B.; Park, J.-S.; Park, Y.-I.; Song, I.-J.; Lee, H.-J.; Cho, H.-S.; Jeon, J.-H.; Kim, H.-S. Development of Systems for the Production of Plant-Derived Biopharmaceuticals. *Plants* **2019**, *9* (1), 30. DOI: 10.3390/plants9010030.
69. Strasser, R. Plant protein glycosylation. *Glycobiology* **2016**, *26* (9), 926–939. DOI: 10.1093/glycob/cww074.
70. Srisangsung, T.; Phetphoung, T.; Manopwisedjaroen, S.; Rattanapisit, K.; Bulaon, C. J. I.; Thitithanyanont, A.; Limprasutr, V. The Impact of N-Glycans on the Immune Response of Plant-Produced SARS-CoV-2 RBD-Fc Proteins. *Biotechnol. Rep. (Amst.)* **2024**, *43*, e00847. DOI: 10.1016/j.btre.2024.e00847.
71. Kallolimath, S.; Castilho, A.; Strasser, R.; Grünwald-Gruber, C.; Altmann, F.; Strubl, S.; Galuska, S. P.; Zlatina, K.; Galuska, C. E.; Werner, S.; Thiesler, H.; Steinkellner, H. Engineering of Complex Protein Sialylation in Plants. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113* (34), 9498–9503. DOI: 10.1073/pnas.1604371113.
72. Singh, A. A.; Pillay, P.; Tsekoa, TL. Engineering Approaches in Plant Molecular Farming for Global Health. *Vaccines* **2021**, *9* (11), 1270. DOI: 10.3390/vaccines9111270.
73. Shin, Y. J.; Chong, Y. J.; Yang, M. S.; Kwon, T. H. Production of Recombinant Antibodies in Plants. *Front. Plant Sci.* **2016**, *7*, 1036. DOI: 10.3389/fpls.2016.01036.
74. Margolin, E.; Chapman, R.; Williamson, A.-L.; Rybicki, E. P.; Meyers, A. A Roadmap for the Molecular Farming of Viral Glycoprotein Vaccines. *Front. Plant Sci.* **2020**, *11*, 609207. DOI: 10.3389/fpls.2020.609207.

75. Wang, L.-X.; Lomino, J. V. Emerging Technologies for Making Glycan-Defined Glycoproteins. *ACS Chem. Biol.* **2012**, *7* (1), 110–122. DOI: 10.1021/cb200429n.
76. Lai, T.; Yang, Y.; Ng, SK. Advances in Mammalian Cell Line Development Technologies for Recombinant Protein Production. *Pharmaceuticals* **2013**, *6* (5), 579-603. DOI: 10.3390/ph6050579.
77. Krishna S, Jung ST, Lee EY. *Escherichia coli* and *Pichia pastoris*: microbial cell-factory platform for -full-length IgG production. *Crit Rev Biotechnol.* **2025**, *45*(1),191-213. DOI: 10.1080/07388551.2024.2342969.
78. Lebozec, K.; Jandrot-Perrus, M.; Avenard, G.; Favre-Bulle, O.; Billiald, P. Quality and Cost Assessment of a Recombinant Antibody Fragment Produced from Mammalian, Yeast and Prokaryotic Host Cells: A Case Study Prior to Pharmaceutical Development. *New Biotechnol.* **2018**, *44*, 31–40. DOI: 10.1016/j.nbt.2018.04.006.
79. Eidenberger, L.; Kogelmann, B.; Steinkellner, H. Plant-Based Biopharmaceutical Engineering. *Nat. Rev. Bioeng.* **2023**, *1* (6), 426–439. DOI: 10.1038/s44222-023-00044-6.
80. McNulty, M. J.; Xie, H.; Simpson, B. M.; Buyel, J. F.; Tusé, D.; McDonald, K. A. Techno-Economic Analysis of a Plant-Based Platform for Manufacturing Antimicrobial Proteins for Food Safety. *Biotechnol Prog.* **2020**, *36*(1). DOI: 10.1002/btpr.2896.
81. Nandi, S.; Kwong, A. T.; Holtz, B. R.; Erwin, R. L.; Marcel, S.; McDonald, K. A.; Tusé, D. Techno-Economic Analysis of a Transient Plant-Based Platform for Monoclonal Antibody Production. *MAbs* **2016**, *8* (8), 1456–1466. DOI: 10.1080/19420862.2016.1227901.
82. Walther, J.; Godawat, R.; Hwang, C.; Abe, Y.; Sinclair, A.; Konstantinov, K. The Business Impact of an Integrated Continuous Biomanufacturing Platform for Recombinant Protein Production. *J. Biotechnol.* **2015**, *213*, 3–12. DOI: 10.1016/j.jbiotec.2015.05.010.
83. Alam, A.; Jiang, L.; Kittleson, G. A.; Steadman, K. D.; Nandi, S.; Fuqua, J. L.; Palmer, K. E.; McDonald, K. A. Technoeconomic Modelling of Plant-Based Griffithsin Manufacturing. *Front. Bioeng. Biotechnol.* **2018**, *6*, 102. DOI: 10.3389/fbioe.2018.00102.
84. **Buyel, J. F.** Plant Molecular Farming – Integration and Exploitation of Side Streams to Achieve Sustainable Biomanufacturing. *Front. Plant Sci.* **2019**, *9*, 1893. DOI: 10.3389/fpls.2019.01893.
85. Karuppiyah, K.; Sankaranarayanan, B.; Ali, S. M.; Santibanez Gonzalez, E. D. R. Impact of Circular Bioeconomy on Industry's Sustainable Performance: A Critical Literature Review and Future Research Directions Analysis. *Sustainability* **2023**, *15*, 10759. DOI: 10.3390/su151410759.
86. Tschöfen, M.; Knopp, D.; Hood, E. Stöger E. Plant Molecular Farming: Much More than Medicines. *Annu Rev Anal Chem.* **2016**, *9* (1), 271-294. DOI: 10.1146/annurev-anchem-071015-041706.

87. Buyel, J. F.; Fischer, R. Predictive Models for transient protein expression in tobacco (*Nicotiana tabacum* L.) can optimize process time, yield, and downstream costs. *Biotechnol. Bioeng.* **2012**, *109* (10), 2575-2588. DOI: 10.1002/bit.24523.
88. **Buyel, J. F.** Product Safety Aspects of Plant Molecular Farming. *Front. Bioeng. Biotechnol.* **2023**, *11*, 1238917. DOI: 10.3389/fbioe.2023.1238917.
89. Casau, M.; Dias, M. F.; Matias, J. C. O.; Nunes, L. J. R. Residual Biomass: A Comprehensive Review on the Importance, Uses and Potential in a Circular Bioeconomy Approach. *Resources* **2022**, *11* (4), 35. DOI: 10.3390/resources11040035.
90. Gaobotse, G.; Venkataraman, S.; Mmereke, K.M.; Moustafa, K.; Hefferon, K.; Makhzoum, A. Recent Progress on Vaccines Produced in Transgenic Plants. *Vaccines* **2022**, *10*, 1861. DOI: 10.3390/vaccines10111861.
91. McHughen, A.; Smyth, S. J. US Regulatory System for Genetically Modified [genetically modified organism (GMO), rDNA or transgenic] Crops Cultivars. *Plant Biotechnol. J.* **2008**, *6* (1), 2-12. DOI: 10.1111/j.1467-7652.2007.00300.
92. Eckerstorfer, M. F.; Engelhard, M.; Heissenberger, A.; Simon, S.; Teichmann, H. Plants Developed by New Genetic Modification Techniques – Comparison of Existing Regulatory Frameworks in the EU and Non-EU Countries. *Front. Bioeng. Biotechnol.* **2019**, *7*, 26. DOI: 10.3389/fbioe.2019.00026.
93. Krzywonos, M.; Piwowar-Sulej, K. Plant-Based Innovations for the Transition to Sustainability: A Bibliometric and in-Depth Content Analysis. *Foods*. **2022**, *11* (19), 3137. DOI: 10.3390/foods11193137.
94. Tusé, D.; Tu, T.; McDonald, K. A. Manufacturing Economics of Plant-Made Biologics: Case Studies in Therapeutic and Industrial Enzymes. *Biomed Res Int.* **2014**, 256135. DOI: 10.1155/2014/256135.
95. Shanmugaraj, B.; Bulaon, C. J. I.; Phoolcharoen, W. Plant Molecular Farming: A Viable Platform for Recombinant Biopharmaceutical Production. *Plants* **2020**, *9* (7), 842. DOI: 10.3390/plants9070842.
96. Capell, T.; Twyman, R. M.; Armario-Nájera, V.; Ma, J. K.-C.; Schillberg, S.; Christou, P. Potential Applications of Plant Biotechnology against SARS-CoV-2. *Trends Plant Sci.* **2020**, *25* (7), 635–643. DOI: 10.1016/j.tplants.2020.04.009.
97. Vo, D.-K.; Trinh, K. T. L. Molecular Farming for Immunization: Current Advances and Future in Plant-Produced Vaccines. *Vaccines* **2025**, *13*, 191. DOI: 10.3390/vaccines13020191.
98. Muneer, S.; Bharathi, J. K. Exploring Recent Progress of Molecular Farming for Therapeutic and Recombinant Molecules in Plant Systems. *Heliyon* **2024**, *10* (18), e37634. DOI: 10.1016/j.heliyon.2024.e37634.



99. Buyel, J. F. Towards a Seamless Product and Process Development Workflow for Recombinant Proteins Produced by Plant Molecular Farming. *Biotechnol. Adv.* **2024**, *75*, 108403. DOI: 10.1016/j.biotechadv.2024.108403.
100. Vo, DK.; Trinh, KTL. Molecular Farming for Immunization: Current Advances and Future Prospects in Plant-Produced Vaccines. *Vaccines* **2025**, *13*(2), 191. DOI: 10.3390/vaccines13020191.
101. Raven, N.; Rasche, S.; Kuehn, C.; Anderlei, T.; Klöckner, W.; Schillberg, S.; Fischer, R. Scale-up Manufacturing of Recombinant Antibodies Produced by Plant Cells in a 200-L Orbital Shaken Disposable Bioreactor. *Biotechnol. Bioeng.* **2015**, *112* (2), 308-321. DOI: 10.1002/bit.25352.
102. Mathew, M.; Thomas, J. Tobacco-Based Vaccines, Hopes, and Concerns: A Systematic Review. *Mol Biotechnol.* **2023**, *65*(7), 1023-1051. DOI: 10.1007/s12033-022-00627-5.
103. Xu, J.; Dolan, M. C.; Medrano, G.; Cramer, C. L.; Weathers, P. J. Green Factory: Plants as Bioproduction Platforms for Recombinant Proteins. *Biotechnol. Adv.* **2012**, *30* (5), 1171–1184. DOI: 10.1016/j.biotechadv.2011.08.020.
104. Fischer, R.; Stoger, E.; Schillberg, S.; Christou, P.; Twyman, RM. Plant-based Production of Biopharmaceuticals. *Curr Opin Plant Biol.* **2004**, *7* (2), 152-158. DOI: 10.1016/j.pbi.2004.01.007.
105. Becker, T.; Bruns, B.; Lier, S.; Werners, B. Decentralized modular production to increase supply chain efficiency in chemical markets. *J Bus Econ* **2021**, *91*, 867–895. DOI: 10.1007/s11573-020-01019-4.
106. Hood, EE.; Requesens, DV. Recombinant Protein Production in Plants: Challenges and Colutions. *Methods Mol Biol.* **2012**, *824*, 469-481. DOI: 10.1007/978-1-61779-433-9\_25.
107. Vatistas, C. A Systematic Literature Review on Controlled-Environment Agriculture (CEA): Efficiency and Impact of Greenhouses and Vertical Farms. *Atmosphere* **2022**, *13* (8), 1258. DOI: 10.3390/atmos13081258.
108. Heuvelink, E.; Hemming, S.; Marcelis, L. F. M. Some Recent Developments in Controlled-Environment Agriculture: On Plant Physiology, Sustainability, and Autonomous Control. *J. Hortic. Sci. Biotechnol.* **2024**, *100*, 1–11. DOI: 10.1080/14620316.2024.2440592.
109. Guo, M.; Jin, Z.; Ma, L.; Ou, S. Application of Plant Factory with Artificial Lighting in Horticultural Production: Current Progress and Future Trends. *Hortic. Plant J.* **2025**. DOI: 10.1016/j.hpj.2025.04.005.
110. Nichols, M. Plant factories - The ultimate in controlled environment agriculture. *Acta Horticulturae* **2017**, *1176*, 17-22. DOI: 10.17660/ActaHortic.2017.1176.3.
111. Gupta, G.; Pal, S. K. Applications of AI in Precision Agriculture. *Discov. Agric.* **2025**, *3*, 61. DOI: 10.1007/s44279-025-00220-9.
112. Rathor, A.; Kumar, R.; Verma, P.; Singh, A.; Yadav, R.; Sharma, A.; Khan, M. A.; Gupta, V. Empowering Vertical Farming through IoT and AI-Driven Technologies: A Comprehensive Review. *Heliyon* **2024**, *10*, e34998. DOI: 10.1016/j.heliyon.2024.e34998.

113. Gerszberg, A.; Hnatuszko-Konka, K. Compendium on Food Crop Plants as a Platform for Pharmaceutical Protein Production. *Int. J. Mol. Sci.* **2022**, *23*, 3236. DOI: 10.3390/ijms23063236.
114. Santos, R. B.; Abranches, R.; Fischer, R.; Sack, M.; Holland, T. Putting the Spotlight Back on Plant Suspension Cultures. *Front. Plant Sci.* **2016**, *7*, 297. DOI: 10.3389/fpls.2016.00297.
115. Verdu-Navarro, F.; Moreno-Cid, J. A.; Weiss, J.; Egea-Cortines, M. The Advent of Plant Cells in Bioreactors. *Front. Plant Sci.* **2023**, *14*, 1310405. DOI: 10.3389/fpls.2023.1310405.
116. Tekoah, Y.; Shulman, A.; Kizhner, T.; Ruderfer, I.; Fux, L.; Nataf, Y.; Bartfeld, D.; Ariel, T.; Gingis-Velitski, S.; Hanania, U.; Shaaltiel, Y.; Aviezer, D. Large-Scale Production of Pharmaceutical Proteins in Plant Cell Culture—The Protalix Experience. *Plant Biotechnol. J.* **2015**, *13*(8), 1199–1208. DOI: 10.1111/pbi.12428.
117. Huang, T. K.; McDonald, K. A. Bioreactor Systems for In Vitro Production of Foreign Proteins Using Plant Cell Cultures. *Biotechnol. Adv.* **2012**, *30* (2), 398–409. DOI: 10.1016/j.biotechadv.2011.07.020.
118. Mirmazloum, I.; Slavov, A. K.; Marchev, A. S. The Untapped Potential of Hairy Root Cultures and Their Multiple Applications. *Int. J. Mol. Sci.* **2024**, *25* (23), 12682. DOI: 10.3390/ijms252312682.
119. Coates, R. J.; Young, M. T.; Scofield, S. Optimising expression and extraction of recombinant proteins in plants. *Front. Plant Sci.* **2022**, *13*, 1074531. DOI: 10.3389/fpls.2022.1074531.
120. Titova, M.; Mukherjee, A.; Firsov, A.; Erokhin, A.; Gogoleva, N.; Shaloiko, L.; Shchennikova, A.; Kochieva, E. Bioreactor Systems for Plant Cell Cultivation at the Institute of Plant Physiology of the Russian Academy of Sciences: 50 Years of Technology Evolution from Laboratory to Industrial Implications. *Plants* **2024**, *13* (3), 430. DOI: 10.3390/plants13030430.
121. Pan, Z.-W.; Zhong, J.-J.; Wu, J.-Y.; Takagi, M.; Yoshida, T. Fluid Mixing and Oxygen Transfer in Cell Suspensions of *Taxus chinensis* in a Novel Stirred Bioreactor. *Biotechnol. Bioprocess Eng.* **1999**, *4*, 269–272.
122. Donini, M.; Marušić, C. Current State-of-the-Art in Plant-Based Antibody Production Systems. *Biotechnol. Lett.* **2019**, *41*(3), 335–346. DOI: 10.1007/s10529-019-02651-z.
123. Mor, T. S. Molecular Pharming's Foot in the FDA's Door: Protalix's Trailblazing Story. *Biotechnol. Lett.* **2015**, *37* (11), 2147–2150. DOI: 10.1007/s10529-015-1908-z.
124. Ward, B. J.; Séguin, A.; Couillard, J.; Trépanier, S.; Landry, N. 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* **2021**, *39* (10), 1528–1533. DOI: 10.1016/j.vaccine.2021.01.004.
125. Lico, C.; Santi, L.; Baschieri, S.; Miozzi, L.; Favalli, C.; Certo, G.; Accotto, G. P.; Noris, E. Plant Molecular Farming as a Strategy against COVID-19 – The Italian Perspective. *Front. Plant Sci.* **2020**, *11*, 609910. DOI: 10.3389/fpls.2020.609910.

126. Buyel, J. F. Plant Molecular Farming as a Tool for Reducing Global Inequalities in Medicine Access and Supply Chain Dependency. *Science-Policy Brief, UN-SDG Multistakeholder Forum on STI for SDGs*, **2024**. Available online: <https://sdgs.un.org/sites/default/files/2024-05/BUYEL~1.PDF> (accessed Sep 3, 2025).
127. Venkataraman, S.; Khan, I.; Habibi, P.; Le, M.; Lippert, R.; Hefferon, K. Recent Advances in Expression and Purification Strategies for Plant-Made Vaccines. *Front. Plant Sci.* **2023**, *14*, 1273958. DOI: 10.3389/fpls.2023.1273958.
128. Michelfelder, S.; Parsons, J.; Bohlender, L. L.; Hoernstein, S. N. W.; Niederkrüger, H.; Busch, A.; Krieghoff, N.; Koch, J.; Fode, B.; Schaaf, A.; Frischmuth, T.; Pohl, M.; Zipfel, P. F.; Reski, R.; Decker, E. L. Moss-Produced, Glycosylation-Optimized Human Factor H for Therapeutic Application in Complement Disorders. *J. Am. Soc. Nephrol.* **2017**, *28* (5), 1462–1474. DOI: 10.1681/ASN.2015070745.
129. Rattanapisit, K.; Yusakul, G.; Shanmugaraj, B.; Kittirotruji, K.; Suwatsrisakul, P.; Prompetchara, E.; Taychakhoonavud, S.; Phoolcharoen, W. Plant-Produced Recombinant SARS-CoV-2 Receptor-Binding Domain; an Economical, Scalable Biomaterial Source for COVID-19 Diagnosis. *Biomater. Transl.* **2021**, *2* (1), 43–49. DOI: 10.3877/cma.j.issn.2096-112X.2021.01.006.
130. To, T. L.; McCoy, J. G.; Ostriker, N. K.; Farnung, L.; Melms, J. C.; Lee, J.; Rao, M.; Liu, Y.; Masutani, M.; Wang, A. L.; Kim, H. J.; Wilson, K. E.; Jones, K. T.; Turi, G. F.; Artyomov, M. N.; McKeown, A. N.; Deshmukh, M.; Mootha, V. K. PMF-seq: A Highly Scalable Screening Strategy for Linking Genetics to Mitochondrial Bioenergetics. *Nat. Metab.* **2024**, *6*, 687–696. DOI: 10.1038/s42255-024-00994-0.
131. One Nucleus. HotHouse BioEngineering and Leaf Expression Systems Sign Plant-Based Expression License Agreement. *One Nucleus News* **2021**, April 13, 2021. Available online: <https://onenucleus.com/hothouse-bioengineering-and-leaf-expression-systems-sign-plant-based-expression-license-agreement> (accessed Sep 3, 2025).
132. Menary, J.; Langford, K. H.; Headley, J. V.; Patel, B. Identifying Barriers and Facilitators to Scaling-Up Plant Molecular Farming in Europe: Qualitative Interviews with Experts. *PLoS One* **2020**, *15* (11), e0229952. DOI: 10.1371/journal.pone.0229952.
133. Chung, Y. H.; Church, D.; Koellhoffer, E. C.; Osota, E.; Shukla, S.; Rybicki, E. P.; Pokorski, J. K.; Steinmetz, N. F. Integrating Plant Molecular Farming and Materials Research for Next-Generation Vaccines. *Nat. Rev. Mater.* **2022**, *7* (5), 372–388. DOI: 10.1038/s41578-021-00399-5.
134. Daniell, H.; Streatfield, S. J.; Wycoff, K. Medical Molecular Farming: Production of Antibodies, Biopharmaceuticals and Edible Vaccines in Plants. *Trends Plant Sci.* **2001**, *6* (5), 219–226. DOI: 10.1016/s1360-1385(01)01922-7.

135. Fujiuchi, N.; Matoba, N.; Matsuda, R. Environment Control to Improve Recombinant Protein Yields in Plants Based on *Agrobacterium*-Mediated Transient Gene Expression. *Front. Bioeng. Biotechnol.* **2016**, *4*, 23. DOI: 10.3389/fbioe.2016.00023.
136. Vatistas, C.; Avgoustaki, D.D.; Bartzanas, T. A Systematic Literature Review on Controlled-Environment Agriculture: How Vertical Farms and Greenhouses Can Influence the Sustainability and Footprint of Urban Microclimate with Local Food Production. *Atmosphere* **2022**, *13*, 1258. DOI: 10.3390/atmos13081258.
137. Bhattarai, K.; Ogden, A. B.; Pandey, S.; Sandoya, G. V.; Shi, A.; Nankar, A. N.; Jayakodi, A.; Huo, N.; Jiang, D.; Tripodi, P.; Dardick, C. Improvement of Crop Production in Controlled Environment Agriculture through Breeding. *Front. Plant Sci.* **2025**, *15*, 1524601. DOI: 10.3389/fpls.2024.1524601.
138. Rajaseger, G.; Chan, K. L.; Tan, K. Y.; Goh, W. S.; Prasath, V. S.; Kumar, S. Hydroponics: Current Trends in Sustainable Crop Production. *Bioinformation* **2023**, *19* (9), 925–938. DOI: 10.6026/97320630019925.
139. He, L.; Ding, X.; Jin, H.; Zhang, H.; Cui, J.; Chu, J.; Li, R.; Zhou, Q.; Yu, J. Comparison of Rockwool and Coir for Greenhouse Cucumber Production: Chemical Element, Plant Growth, and Fruit Quality. *Heliyon* **2022**, *8* (9), e10593. DOI: 10.1016/j.heliyon.2022.e10593.
140. Palmitessa, O. D.; Signore, A.; Santamaria, P. Advancements and Future Perspectives in Nutrient Film Technique Hydroponic System: A Comprehensive Review and Bibliometric Analysis. *Front. Plant Sci.* **2024**, *15*, 1504792. DOI: 10.3389/fpls.2024.1504792.
141. Chowdhury, M.; Samarakoon, U. C.; Altland, J. E. Evaluation of Hydroponic Systems for Organic Lettuce Production in Controlled Environment. *Front. Plant Sci.* **2024**, *15*, 1401089. DOI: 10.3389/fpls.2024.1401089.
142. Lourenço, M. S. C.; Freitas, V.; Heuvelink, E.; Carvalho, S. M. P. Phenotypic Variability and Anticancer Alkaloid Profiles of *Catharanthus roseus* Cultivars Grown under a Vertical Farming System. *Plants* **2025**, *14* (16), 2576. DOI: 10.3390/plants14162576.
143. Wirz, H.; Sauer-Budge, A. F.; Briggs, J.; Sharpe, A.; Shu, S. D.; Sharon, A. Automated Production of Plant-Based Vaccines and Pharmaceuticals. *J. Lab. Autom.* **2012**, *17* (6), 449–457. DOI: 10.1177/2211068212458717.
144. Sack, M.; Rademacher, T.; Spiegel, H.; Boes, A.; Hellwig, S.; Drossard, J.; Stoger, E.; Fischer, R. From Gene to Harvest: Insights into Upstream Process Development for the GMP Production of a Monoclonal Antibody in Transgenic Tobacco Plants. *Plant Biotechnol. J.* **2015**, *13* (8), 1094–1105. DOI: 10.1111/pbi.12438.
145. Huebbers, J. W.; Buyel, J. F. On the Verge of the Market – Plant Factories for the Automated and Standardized Production of Biopharmaceuticals. *Biotechnol. Adv.* **2021**, *46*, 107681. DOI: 10.1016/j.biotechadv.2020.107681.

146. Goodin, M. M.; Zaitlin, D.; Naidu, R. A.; Lommel, S. A. *Nicotiana benthamiana*: Its History and Future as a Model for Plant–Pathogen Interactions. *Mol. Plant Microbe Interact.* **2008**, *21* (8), 1015–1026. DOI: 10.1094/MPMI-21-8-1015.
147. Lico, C.; Santi, L.; Baschieri, S.; Noris, E.; Marusic, C.; Donini, M.; Pedrazzini, E.; Maga, G.; Franconi, R.; Di Bonito, P.; Avesani, L. Plant Molecular Farming as a Strategy against COVID-19 – The Italian Perspective. *Front. Plant Sci.* **2020**, *11*, 609910. DOI: 10.3389/fpls.2020.609910.
148. Wylie, S.; Li, H. Historical and Scientific Evidence for the Origin and Cultural Importance to Australia’s First-Nations Peoples of the Laboratory Accession of *Nicotiana benthamiana*, a Model for Plant Virology. *Viruses* **2022**, *14* (4), 771. DOI: 10.3390/v14040771.
149. Ludman, M.; Anita S.; Fáyol, K. Deficiency of Multiple RNA Silencing-Associated Genes May Contribute to the Increased Susceptibility of *Nicotiana benthamiana* to Viruses. *Plant Cell Rep.* **2024**, *43*, 177. DOI: 10.1007/s00299-024-03262-3.
150. Liu, H.; Timko, M.P. Improving Protein Quantity and Quality—The Next Level of Plant Molecular Farming. *Int. J. Mol. Sci.* **2022**, *23*, 1326. DOI: 10.3390/ijms23031326.
151. Gutiérrez, S. P.; Saberianfar, R.; Kohalmi, S. E.; Menassa, R. Protein Body Formation in Stable Transgenic Tobacco Expressing Elastin-Like Polypeptide and Hydrophobin Fusion Proteins. *BMC Biotechnol.* **2013**, *13*, 40. DOI: 10.1186/1472-6750-13-40.
152. Takaiwa, F.; Wakasa, Y.; Takagi, H.; Hiroi, T. Rice Seed for Delivery of Vaccines to Gut Mucosal Immune Tissues. *Plant Biotechnol. J.* **2015**, *13* (8), 1041–1055. DOI: 10.1111/pbi.12423.
153. Suzaki, T.; Tsuda, M.; Ezura, H.; Day, B.; Miura, K. Agroinfiltration-Based Efficient Transient Protein Expression in Leguminous Plants. *Plant Biotechnol.* **2019**, *36* (2), 119–123. DOI: 10.5511/plantbiotechnology.19.0220b.
154. Singh, A. A.; Pillay, P.; Naicker, P.; Alexandre, K.; Malatji, K.; Mach, L.; Steinkellner, H.; Vorster, J.; Chikwamba, R.; Tsekoa, T. L. Transient Proteolysis Reduction of *Nicotiana benthamiana*-Produced CAP256 Broadly Neutralizing Antibodies Using CRISPR/Cas9. *Front. Plant Sci.* **2022**, *13*, 953654. DOI: 10.3389/fpls.2022.953654.
155. Beritza, K.; Watts, E. C.; van der Hoorn, R. A. L. Improving Transient Protein Expression in Agroinfiltrated *Nicotiana benthamiana*. *New Phytol.* **2024**, *243* (3), 846–850. DOI: 10.1111/nph.19894.
156. Norkunas, K.; Harding, R.; Dale, J.; Dugdale, B. Improving Agroinfiltration-Based Transient Gene Expression in *Nicotiana benthamiana*. *Plant Methods* **2018**, *14*, 71. DOI: 10.1186/s13007-018-0343-2.
157. Pogue, G. P.; Vojdani, F.; Palmer, K. E.; Hiatt, E.; Hume, S.; Phelps, J.; Long, L.; Bohorova, N.; Kim, D.; Pauly, M.; Velasco, J.; Whaley, K.; Zeitlin, L.; Garger, S. J.; White, E.; Bai, Y.; Haydon, H.; Bratcher, B. Production of Pharmaceutical-Grade Recombinant Aprotinin and a Monoclonal

- Antibody Product Using Plant-Based Transient Expression Systems. *Plant Biotechnol. J.* **2010**, 8 (5), 638–654. DOI: 10.1111/j.1467-7652.2009.00495.
158. Obembe, O. O.; Popoola, J. O.; Leelavathi, S.; Reddy, S. V. Advances in Plant Molecular Farming. *Biotechnol. Adv.* **2011**, 29 (2), 210–222. DOI: 10.1016/j.biotechadv.2010.11.004.
  159. Shawon, M. R. A. The Electrical Conductivity of Nutrient Solution Influenced Nutrient Uptake, Ion Toxicity, and Osmotic Pressure in Hydroponic Systems. *Agriculture* **2023**, 13 (12), Article 2236. DOI: 10.3390/agriculture13122236.
  160. Malik, A.; Iqbal, K.; Aziem, S.; Mahato, P.; Negi, A. A Review on the Science of Growing Crops without Soil (Soilless Culture) – A Novel Alternative for Growing Crops. *Int. J. Agric. Crop Sci.* **2014**, 7, 833–842.
  161. Fujiuchi, N.; Matoba, N.; Matsuda, R. Environmental control to improve recombinant protein yield in plant molecular farming. *Front. Bioeng. Biotechnol.* **2016**, 4, 23. DOI: 10.3389/fbioe.2016.00023.
  162. Salas-Sanjuán, M. C.; Ruíz-Zubiate, J. L.; Valenzuela, J. L.; Campos, A. X. Nutrient Solution from Aqueous Extracts as an Alternative to Fertigation in Hydroponic. *Horticulturae* **2023**, 9 (12), 1281. DOI: 10.3390/horticulturae9121281.
  163. Wong, C. Z. W. N.; Shen, L.; Yu, H. Seeing the Lights for Leafy Greens in Indoor Vertical Farming. *Trends Food Sci. Technol.* **2020**, 106, 48–63. DOI: 10.1016/j.tifs.2020.09.031.
  164. Franklin, K. A.; Whitelam, G. C. Phytochromes and Shade-Avoidance Responses in Plants. *Ann. Bot.* **2005**, 96 (2), 169–175. DOI: 10.1093/aob/mci165.
  165. Hogewoning, S. W.; Trouwborst, G.; Maljaars, H.; Poorter, H.; van Ieperen, W.; Harbinson, J. Blue Light Dose-Responses of Leaf Photosynthesis, Morphology, and Chemical Composition of *Cucumis sativus* Grown under Different Combinations of Red and Blue Light. *J. Exp. Bot.* **2010**, 61 (11), 3107–3117. DOI: 10.1093/jxb/erq132.
  166. Park, Y.; Runkle, E. S. Far-Red Radiation Promotes Growth of Seedlings by Increasing Leaf Expansion and Whole-Plant Net Assimilation. *Environ. Exp. Bot.* **2018**, 155, 112–121. DOI: 10.1016/j.envexpbot.2018.06.033.
  167. Barceló-Muñoz, A.; Barceló-Muñoz, M.; Gago-Calderón, A. Effect of LED Lighting on Physical Environment and Microenvironment on *In Vitro* Plant Growth and Morphogenesis: The Need to Standardize Lighting Conditions and Their Description. *Plants* **2021**, 11 (1), 60. DOI: 10.3390/plants11010060.
  168. Yao, X.; Wuzhang, K.; Peng, B.; Wu, H.; Zhang, Y.; Li, X.; Chen, W.; Chen, Z.; Xu, X.; Wang, H.; Zhu, Z.; Zheng, S.; Huang, J.; Yang, C.; Zhou, X. Engineering the Expression of Plant Secondary Metabolites—Genistein and Scutellarin—through an Efficient Transient Production Platform in *Nicotiana benthamiana* L. *Front. Plant Sci.* **2022**, 13, 994792. DOI: 10.3389/fpls.2022.994792.

169. Thenveettil, N.; Bheemanahalli, R.; Reddy, K. N.; Gao, W.; Reddy, K. R. Temperature and Elevated CO<sub>2</sub> Alter Soybean Seed Yield and Quality, Exhibiting Transgenerational Effects on Seedling Emergence and Vigor. *Front. Plant Sci.* **2024**, *15*, 1427086. DOI: 10.3389/fpls.2024.1427086.
170. Zupan, J. R.; Zambryski, P. Transfer of T-DNA from *Agrobacterium* to the Plant Cell. *Plant Physiol.* **1995**, *107* (4), 1041–1047. DOI: 10.1104/pp.107.4.1041.
171. Tyurin, A. A.; Suhorukova, A. V.; Kabardaeva, K. V.; Goldenkova-Pavlova, I. V. Transient Gene Expression Is an Effective Experimental Tool for the Research into the Fine Mechanisms of Plant Gene Function: Advantages, Limitations, Possibilities. *Plants* **2020**, *9* (9), 1187. DOI: 10.3390/plants9091187.
172. Karimi, M.; Inzé, D.; Depicker, A. GATEWAY Vectors for *Agrobacterium*-Mediated Plant Transformation. *Trends Plant Sci.* **2002**, *7* (5), 193–195. DOI: 10.1016/S1360-1385(02)02251-3.
173. Gelvin, S. B. *Agrobacterium*-Mediated Plant Transformation: The Biology behind the “Gene-Jockeying” Tool. *Microbiol. Mol. Biol. Rev.* **2003**, *67* (1), 16–37. DOI: 10.1128/MMBR.67.1.16-37.2003.
174. Wise, A. A.; Liu, Z.; Binns, A. N. Three Methods for the Introduction of Foreign DNA into *Agrobacterium*. *Methods Mol. Biol.* **2006**, *343*, 43–53. DOI: 10.1385/1-59745-130-4:43.
175. Hwang, H.-H.; Yu, M.; Lai, E.-M. *Agrobacterium*-Mediated Plant Transformation: Biology and Applications. *Arabidopsis Book* **2017**, *15*, e0186. DOI: 10.1199/tab.0186.
176. McCullen, C. A.; Binns, A. N. *Agrobacterium tumefaciens* and Plant Cell Interactions and Activities Required for Interkingdom Macromolecular Transfer. *Annu. Rev. Cell Dev. Biol.* **2006**, *22* (1), 101–127. DOI: 10.1146/annurev.cellbio.22.011105.102022.
177. Liu, X.; Miceli, J.F., III; Patton, S.; Murray, M.; Evans, J.; Wei, X.; Wang, P. *Agrobacterium* Transformation Enhancement by Improved Competent Cell Preparation and Optimized Electroporation. *Life* **2023**, *13*, 2217. DOI: 10.3390/life13112217.
178. Hellens, R. P.; Edwards, E. A.; Leyland, N. R.; Bean, S.; Mullineaux, P. M. pGreen: A Versatile and Flexible Binary Ti Vector for *Agrobacterium*-Mediated Transformation. *Plant Mol. Biol.* **2000**, *42* (6), 819–832. DOI: 10.1023/a:1006496308160.
179. Holtz, B. R.; Berquist, B. R.; Bennett, L. D.; Kommineni, V. J.; Munigunti, R. K.; White, E. L.; Wilkerson, D. C.; Wong, K. Y. I.; Ly, L. H.; Marcel, S. Commercial-Scale Biotherapeutics Manufacturing Facility for Plant-Made Pharmaceuticals. *Plant Biotechnol. J.* **2015**, *13* (9), 1180–1190. DOI: 10.1111/pbi.12469.
180. Schillberg, S.; Finner, R. Plant Molecular Farming for the Production of Valuable Proteins—Critical Evaluation of Achievements and Future Challenges. *J. Plant Physiol.* **2021**, *258–259*, 153359. DOI: 10.1016/j.jplph.2020.153359.

181. Fujiuchi, N.; Matoba, N.; Matsuda, R. Environment Control to Improve Recombinant Protein Yields in Plants Based on *Agrobacterium*-Mediated Transient Gene Expression. *Front. Bioeng. Biotechnol.* **2016**, *4*, 23. DOI: 10.3389/fbioe.2016.00023.
182. Norkunas, K.; Harding, R.; Dale, J.; Dugdale, B. Improving Agroinfiltration-Based Transient Gene Expression in *Nicotiana benthamiana*. *Plant Methods* **2018**, *14*, 71. DOI: 10.1186/s13007-018-0343-2.
183. Xu, J.; Towler, M.; Weathers, P. J. Platforms for Plant-Based Protein Production. In *Bioprocessing of Plant In Vitro Systems*; Pavlov, A., Bley, T., Eds.; Springer: Cham, 2018; pp 509–548. DOI: 10.1007/978-3-319-54600-1\_14.
184. Folta, K. M.; Koss, L. L.; McMorrow, R.; Kim, H.-H.; Kenitz, J. D.; Wheeler, R.; Sager, J. C. Design and Fabrication of Adjustable Red-Green-Blue LED Light Arrays for Plant Research. *BMC Plant Biol.* **2005**, *5* (1), 17. DOI: 10.1186/1471-2229-5-17.
185. Sulaiman, H.; Yusof, A. A.; Mohamed Nor, M. K. Automated Hydroponic Nutrient Dosing System: A Scoping Review of pH and Electrical Conductivity Dosing Frameworks. *AgriEngineering* **2025**, *7*, 43. DOI: 10.3390/agriengineering7020043.
186. McNulty, M. J.; Gleba, Y.; Tusé, D.; Hahn-Löbmann, S.; Giritch, A.; McDonald, K. A.; McDonald, K. A. Techno-Economic Analysis of a Plant-Based Platform for Manufacturing Antimicrobial Proteins for Food Safety. *Biotechnol. Prog.* **2020**, *36* (1), e2896. DOI: 10.1002/btpr.2896.
187. Rybicki, E. P. Plant Molecular Farming of Virus-Like Nanoparticles as Vaccines and Reagents. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2020**, *12* (2), e1587. DOI: 10.1002/wnan.1587.
188. Diamos, A. G.; Hunter, J. G. L.; Pardhe, M. D.; Rosenthal, S. H.; Sun, H.; Foster, B. C.; DiPalma, M. P.; Chen, Q.; Mason, H. S. High Level Production of Monoclonal Antibodies Using an Optimized Plant Expression System. *Front. Bioeng. Biotechnol.* **2020**, *7*, 472. DOI: 10.3389/fbioe.2019.00472.
189. Zhou, Y.; Xia, Q.; Zhang, Z.; Quan, M.; Li, H. Artificial intelligence and machine learning for the green development of agriculture in the emerging manufacturing industry in the IoT platform. *Acta Agric. Scand. Sect. B Soil Plant Sci.* **2022**, *72*, 284–299. DOI: 10.1080/09064710.2021.2006392.
190. Margolin, E.; Chapman, R.; Williamson, A. L.; Rybicki, E. P.; Meyers, A. E. Production of Complex Viral Glycoproteins in Plants as Vaccine Immunogens. *Plant Biotechnol. J.* **2018**, *16* (9), 1531–1545. DOI: 10.1111/pbi.12963.
191. Jansing, J.; Sack, M.; Augustine, S. M.; Fischer, R.; Bortesi, L. CRISPR/Cas9-Mediated Knockout of Six Glycosyltransferase Genes in *Nicotiana benthamiana* for the Production of Recombinant Proteins Lacking  $\beta$ -1,2-Xylose and Core  $\alpha$ -1,3-Fucose. *Plant Biotechnol. J.* **2019**, *17* (2), 350–361. DOI: 10.1111/pbi.12981.



192. World Health Organization. *Global Tuberculosis Report 2024*; World Health Organization: Geneva, 2024. <https://www.who.int/publications/i/item/9789240101531>.
193. Tait, D. R.; Hatherill, M.; Van Der Meeren, O.; Ginsberg, A. M.; Van Brakel, E.; Salaun, B.; Scriba, T. J.; Akite, E. J.; Ayles, H. M.; Bollaerts, A.; Demoitié, M.-A.; Diacon, A.; Landry, B. S.; Lempicki, M.; Malahleha, M.; Martinson, N.; Mesia Vela, D.; Muyoyeta, M.; Nduba, V.; Pascal, T. G.; Pau, M. G.; Tameris, M.; Thienemann, F.; Vingerhoets, J.; Weckx, L. Y.; Roman, F.; Voss, G. Final Analysis of a Trial of M72/AS01E Vaccine to Prevent Tuberculosis. *N. Engl. J. Med.* **2019**, *381* (25), 2429–2439. DOI: 10.1056/NEJMoa1909953.
194. Scriba, T. J.; Netea, M. G.; Ginsberg, A. M. Key Recent Advances in TB Vaccine Development and Understanding of Protective Immune Responses against *Mycobacterium tuberculosis*. *Semin. Immunol.* **2020**, *50*, 101431. DOI: 10.1016/j.smim.2020.101431.
195. Wang, M.; Jiang, S.; Wang, Y. Recent Advances in the Production of Recombinant Subunit Vaccines in *Pichia pastoris*. *Bioengineered* **2016**, *7* (3), 155–165. DOI: 10.1080/21655979.2016.1191707.
196. Sookhoo, J.R.V.; Schiffman, Z.; Ambagala, A.; Kobasa, D.; Pardee, K.; Babiuk, S. Protein Expression Platforms and the Challenges of Viral Antigen Production. *Vaccines* **2024**, *12*, 1344. DOI: 10.3390/vaccines12121344.
197. Takeyama, N.; Kiyono, H.; Yuki, Y. Plant-Based Vaccines for Animals and Humans: Recent Advances in Technology and Clinical Trials. *Ther. Adv. Vaccines* **2015**, *3* (5–6), 139–154. DOI: 10.1177/2051013615613272.
198. Su, H.; van Eerde, A.; Rimstad, E.; Popov, S.; Chandra, S.; Rueegg, C. L.; Xu, W.; Wirz, M.; Starzengruber, P.; Su, J. Plant-Made Vaccines against Viral Diseases in Humans and Farm Animals. *Front. Plant Sci.* **2023**, *14*, 1170815. DOI: 10.3389/fpls.2023.1170815.
199. Wilken, L. R.; Nikolov, Z. L. Recovery and Purification of Plant-Made Recombinant Proteins. *Biotechnol. Adv.* **2012**, *30*(2), 419–433. DOI: 10.1016/j.biotechadv.2011.07.020.
200. Buyel, J. F.; Twyman, R. M.; Fischer, R. Extraction and Downstream Processing of Plant-Derived Recombinant Proteins. *Biotechnol. Adv.* **2015**, *33* (6, Pt. 1), 902–913. DOI: 10.1016/j.biotechadv.2015.04.010.
201. Sathiyapriyan, P.; Mukherjee, S.; Vogel, T.; Chatterjee, S.; Bhutani, R.; Bhutani, H.; Walch, N.; Becker, T.; Rathore, A. S. Current PAT Landscape in the Downstream Processing of Biopharmaceuticals. *Anal. Sci. Adv.* **2025**, *6* (1), e70013. DOI: 10.1002/ansa.70013.
202. Frenzel, A.; Hust, M.; Schirrmann, T. Expression of Recombinant Antibodies. *Front. Immunol.* **2013**, *4*, 217. DOI: 10.3389/fimmu.2013.00217.
203. Pardi, N.; Hogan, M. J.; Porter, F. W.; Weissman, D. mRNA Vaccines — A New Era in Vaccinology. *Nat. Rev. Drug Discov.* **2018**, *17* (4), 261–279. DOI: 10.1038/nrd.2017.243
204. Sahin, U.; Karikó, K.; Türeci, Ö. mRNA-Based Therapeutics — Developing a New Class of Drugs. *Nat. Rev. Drug Discov.* **2014**, *13* (10), 759–780. DOI: 10.1038/nrd4278.

205. Schlake, T.; Thess, A.; Fotin-Mleczek, M.; Kallen, K.-J. Developing mRNA-Vaccine Technologies. *RNA Biol.* **2012**, *9*(11), 1319–1330. DOI: 10.4161/rna.22269.
206. Kowalski, P. S.; Rudra, A.; Miao, L.; Anderson, D. G. Delivering the Messenger: Advances in Technologies for Therapeutic mRNA Delivery. *Mol. Ther.* **2019**, *27* (4), 710–728. DOI: 10.1016/j.ymthe.2019.02.012
207. Chaudhary, N.; Weissman, D.; Whitehead, K. A. mRNA Vaccines for Infectious Diseases: Principles, Delivery and Clinical Translation. *Nat. Rev. Drug Discov.* **2021**, *20* (11), 817–838. DOI: 10.1038/s41573-021-00283-5.
208. Peixeiro, I.; Silva, A. L.; Romão, L. Control of Human  $\beta$ -Globin mRNA Stability and Its Impact on the  $\beta$ -Thalassemia Phenotype. *Haematologica* **2011**, *96* (6), 905–913. DOI: 10.3324/haematol.2010.039206.
209. Anderson, B. R.; Muramatsu, H.; Nallagatla, S. R.; Bevilacqua, P. C.; Sansing, L. H.; Weissman, D.; Karikó, K. Incorporation of Pseudouridine into mRNA Enhances Translation by Diminishing PKR Activation. *Nucleic Acids Res.* **2010**, *38* (17), 5884–5892. DOI: 10.1093/nar/gkq347.
210. Leong, K. Y.; Tham, S. K.; Poh, C. L. Revolutionizing Immunization: A Comprehensive Review of mRNA Vaccine Technology and Applications. *Virol. J.* **2025**, *22*, 71. DOI: 10.1186/s12985-025-02645-6.
211. Lenk, R.; Kleindienst, W.; Szabó, G. T.; Baiersdörfer, M.; Boros, G.; Keller, J. M.; Mahiny, A. J.; Vlatkovic, I. Understanding the Impact of In Vitro Transcription Byproducts and Contaminants. *Front. Mol. Biosci.* **2024**, *11*, 1426129. DOI: 10.3389/fmolb.2024.1426129.
212. Kato, H.; Takeuchi, O.; Mikamo-Satoh, E.; Hirai, R.; Kawai, T.; Matsushita, K.; Hiiragi, A.; Dermody, T. S.; Fujita, T.; Akira, S. Length-Dependent Recognition of Double-Stranded Ribonucleic Acids by Retinoic Acid–Inducible Gene-I and Melanoma Differentiation–Associated Gene 5. *J. Exp. Med.* **2008**, *205* (7), 1601–1610. DOI: 10.1084/jem.20080091.
213. Dousis, A.; Ravichandran, K.; Hobert, E. M.; Moore, M. J.; Rabideau, A. E. An Engineered T7 RNA Polymerase That Produces mRNA Free of Immunostimulatory Byproducts. *Nat. Biotechnol.* **2023**, *41* (4), 560–568. DOI: 10.1038/s41587-022-01525-6.
- Stepinski, J.; Waddell, C.; Stolarski, R.; Darzynkiewicz, E.; Rhoads, R. E. Synthesis and Properties of mRNAs Containing the Novel “Anti-Reverse” Cap Analogs. *RNA* **2001**, *7* (10), 1486–1495.
214. Grudzien-Nogalska, E.; Kiledjian, M. New Insights into Decapping Enzymes and Their Roles in mRNA Decay. *Wiley Interdiscip. Rev. RNA* **2017**, *8* (1), e1379. DOI: 10.1002/wrna.1379.
215. Henderson, J. M.; Ujita, A.; Hill, E.; Hager, C.; Singh, K.; Burmistrz, K.; Feldman, R. A.; Roth, C. M.; DeRosa, F.; Shattock, R. J.; Brito, L. A. Cap 1 Messenger RNA Synthesis with Co-Transcriptional CleanCap® Analog by *In Vitro* Transcription. *Curr. Protoc.* **2021**, *1* (2), e39. DOI: 10.1002/cpz1.39.

216. Silva-Santos, A. R.; Sousa Rosa, S.; Marques, M. P. C.; Azevedo, A. M.; Prazeres, D. M. F. Quantification of ssDNA Scaffold Production by Ion-Pair Reverse Phase Chromatography. *ACS Omega* **2024**, 9 (21), 22619–22624. DOI: 10.1021/acsomega.3c10533.
217. Holland, D. A.; Acevedo-Skrip, J.; Barton, J.; Thompson, R.; Bowman, A.; Dewar, E. A.; Miller, D. V.; Zhao, K.; Swartz, A. R.; Loughney, J. W. Development and Application of Automated Sandwich ELISA for Quantitating Residual dsRNA in mRNA Vaccines. *Vaccines* **2024**, 12 (8), 899. DOI: 10.3390/vaccines12080899.
218. Rosa, S. S.; Nunes, D.; Antunes, L.; Prazeres, D. M. F.; Marques, M. P. C.; Azevedo, A. M. Maximizing mRNA Vaccine Production with Bayesian Optimization. *Biotechnol. Bioeng.* **2022**, 119 (11), 3127–3139. DOI: 10.1002/bit.28216.
219. Perenkov, A. D.; Sergeeva, A. D.; Vedunova, M. V.; Krysko, D. V. In Vitro Transcribed RNA-Based Platform Vaccines: Past, Present, and Future. *Vaccines* **2023**, 11 (10), 1600. DOI: 10.3390/vaccines11101600.
220. Twyman, R. M.; Stoger, E.; Schillberg, S.; Christou, P.; Fischer, R. Molecular Farming in Plants: Host Systems and Expression Technology. *Trends Biotechnol.* **2003**, 21 (12), 570–578. DOI: 10.1016/j.tibtech.2003.10.002.
221. Sari, Y.; Sousa Rosa, S.; Jeffries, J.; Brabazon, H.; Drake, A. F.; Marques, M. P. C.; Prazeres, D. M. F.; Azevedo, A. M. Comprehensive Evaluation of T7 Promoter for Enhanced Yield and Quality in mRNA Production. *Sci. Rep.* **2024**, 14, 9655. DOI: 10.1038/s41598-024-59978-5.
222. Hellens, R. P.; Edwards, E. A.; Leyland, N. R.; Bean, S.; Mullineaux, P. M. pGreen: A Versatile and Flexible Binary Ti Vector for Agrobacterium-Mediated Plant Transformation. *Plant Mol. Biol.* **2000**, 42, 819–832. DOI: 10.1023/a:1006496308160.
223. Voinnet, O.; Rivas, S.; Mestre, P.; Baulcombe, D. An Enhanced Transient Expression System in Plants Based on Suppression of Gene Silencing by the p19 Protein of Tomato Bushy Stunt Virus. *Plant J.* **2003**, 33 (5), 949–956. DOI: 10.1046/j.1365-313x.2003.01676.x.
224. Sanders, P. R.; Winter, J. A.; Barnason, A. R.; Rogers, S. G.; Fraley, R. T. Comparison of Cauliflower Mosaic Virus 35S and Nopaline Synthase Promoters in Transgenic Plants. *Nucleic Acids Res.* **1987**, 15 (4), 1543–1558. DOI: 10.1093/nar/15.4.1543.
225. Chen, Q.; Lai, H.; Hurtado, J.; Stahnke, J.; Leuzinger, K.; Dent, M. Agroinfiltration as an Effective and Scalable Strategy of Gene Delivery for Production of Pharmaceutical Proteins. *Adv. Tech. Biol. Med.* **2013**, 1 (1), 103. DOI: 10.4172/atbm.1000103.
226. Henkel, C. V.; den Dulk-Ras, A.; Zhang, X.; Hooykaas, P. J. J. Genome sequence of the octopine-type *Agrobacterium tumefaciens* strain Ach5. *Genome Announc.* **2014**, 2 (2), e00225-14. DOI: 10.1128/genomeA.00225-14.
227. Leng, C.; Sun, B.; Liu, Z.; Li, C.; Chen, K.; Zhang, W.; Nie, Y.; Yang, D.; Liu, G.; Zhang, J. An Optimized Double T-DNA Binary Vector System for Improved Production of Marker-Free

- Transgenic Tobacco Plants. *Biotechnol. Lett.* **2020**, *42* (4), 641–655. DOI: 10.1007/s10529-020-02797-1.
228. Leuzinger, K.; Dent, M.; Hurtado, J.; Stahnke, J.; Lai, H.; Zhou, X.; Chen, Q. Efficient Agroinfiltration of Plants for High-Level Transient Expression of Recombinant Proteins. *J. Vis. Exp.* **2013**, (77), e50521. DOI: 10.3791/50521.
  229. Feng, Z.; Li, X.; Fan, B.; Zhu, C.; Chen, Z. Maximizing the Production of Recombinant Proteins in Plants: From Transcription to Protein Stability. *Int. J. Mol. Sci.* **2022**, *23* (21), 13516. DOI: 10.3390/ijms232113516.
  230. Feng, X.; Su, Z.; Cheng, Y.; Ma, G.; Zhang, S. Messenger RNA Chromatographic Purification: Advances and Challenges. *J. Chromatogr. A* **2023**, *1707*, 464321. DOI: 10.1016/j.chroma.2023.464321.
  231. Mencin, N.; Štepec, D.; Margon, A.; Vidič, J.; Dolenc, D.; Simčič, T.; Rotar, S.; Sekirnik, R.; Štrancar, A.; Černigoj, U. Development and Scale-Up of Oligo-dT Monolithic Chromatographic Column for mRNA Capture through Understanding of Base-Pairing Interactions. *Sep. Purif. Technol.* **2023**, *304*, 122320. DOI: 10.1016/j.seppur.2022.122320.
  232. Adams, N. M.; Bordelon, H.; Wang, K.-K. A.; Albert, L. E.; Wright, D. W.; Haselton, F. R. Comparison of Three Magnetic Bead Surface Functionalities for RNA Extraction and Detection. *ACS Appl. Mater. Interfaces* **2015**, *7* (11), 6062–6069. DOI: 10.1021/am506374t.
  233. Nooraei, S.; Bahrololum, H.; Hoseini, Z. S.; Katalani, C.; Hajizadeh, A.; Easton, A. J.; Ahmadian, G. Virus-Like Particles: Preparation, Immunogenicity and Their Roles as Nanovaccines and Drug Nanocarriers. *J. Nanobiotechnol.* **2021**, *19* (1), 59. DOI: 10.1186/s12951-021-00806-7.
  234. Travassos, R.; Martins, S.A.; Fernandes, A.; Correia, J.D.G.; Melo, R. Tailored Viral-like Particles as Drivers of Medical Breakthroughs. *Int. J. Mol. Sci.* **2024**, *25*, 6699. DOI: 10.3390/ijms25126699.
  235. Mohsen, M. O.; Zha, L.; Cabral-Miranda, G.; Bachmann, M. F. Major Findings and Recent Advances in Virus-Like Particle (VLP)-Based Vaccines. *Semin. Immunol.* **2017**, *34*, 123–132. DOI: 10.1016/j.smim.2017.08.014.
  236. Segovia-González, X. F.; Villagrana-Escareño, M. V.; Ríos-Ramírez, M.; González-González, E.; Gutiérrez-Urbe, J. A.; Ruiz-García, J.; Lozano-Durán, R.; Peña-Ramírez, Y. J.; Angulo, C.; Almanza-Reyes, H. An Observation of a Very High Swelling of Bromovirus Members at Specific Ionic Strengths and pH. *Viruses* **2023**, *15* (10), 2046. DOI: 10.3390/v15102046.
  237. Smith, T. J.; Chase, E.; Schmidt, T.; Perry, K. L. The Structure of Cucumber Mosaic Virus and Comparison to Cowpea Chlorotic Mottle Virus. *J. Virol.* **2000**, *74* (16), 7578–7586. DOI: 10.1128/JVI.74.16.7578-7586.2000.
  238. Tama, F.; Brooks, C. L., III. The Mechanism and Pathway of pH-Induced Swelling in Cowpea Chlorotic Mottle Virus. *J. Mol. Biol.* **2002**, *318* (3), 733–747. DOI: 10.1016/S0022-2836(02)00135-3.

239. Cadena-Nava, R. D.; Comas-Garcia, M.; Garmann, R. F.; Rao, A. L.; Knobler, C. M.; Gelbart, W. M. Self-Assembly of Viral Capsid Protein and RNA Molecules of Different Sizes: Requirement for a Specific High Protein/RNA Mass Ratio. *J. Virol.* **2012**, 86 (6), 3318–3326. DOI: 10.1128/JVI.06566-11.
240. Karan, S.; Durán-Meza, A. L.; Chapman, A.; Rivera-Serrano, E. E.; Danthi, P.; Jacobson, A. E.; Olsen, R. J.; Natrajan, G.; Finn, M. G. In Vivo Delivery of Spherical and Cylindrical In Vitro Reconstituted Virus-Like Particles Containing the Same Self-Amplifying mRNA. *Mol. Pharm.* **2024**, 21 (6), 2727–2739. DOI: 10.1021/acs.molpharmaceut.3c01105.
241. Saunders, K.; Thuenemann, E. C.; Peyret, H.; Lomonossoff, G. P. The Tobacco Mosaic Virus Origin of Assembly Sequence Is Dispensable for Specific Viral RNA Encapsidation but Necessary for Initiating Assembly at a Single Site. *J. Mol. Biol.* **2022**, 434 (24), 167873. DOI: 10.1016/j.jmb.2022.167873.
242. Nikitin, N.; Vasiliev, Y.; Kovalenko, A.; Ryabchevskaya, E.; Kondakova, O.; Evtushenko, E.; Karpova, O. Plant Viruses as Adjuvants for Next-Generation Vaccines and Immunotherapy. *Vaccines* **2023**, 11, 1372. DOI: 10.3390/vaccines11081372.
243. Fiedler, J. D.; Brown, S. D.; Lau, J. L.; Finn, M. G. RNA-Directed Packaging of Enzymes within Virus-Like Particles. *Angew. Chem. Int. Ed.* **2022**, 49 (50), 9648-9651. DOI: 10.1002/anie.201005243.
244. Villagrana-Escareño, M. V.; Reynaga-Hernández, E.; Galicia-Cruz, O. G.; Durán-Meza, A. L.; De la Cruz-González, V.; Hernández-Carballo, C. Y.; Ruíz-García, J. VLPs Derived from the CCMV Plant Virus Can Directly Transfect and Deliver Heterologous Genes for Translation into Mammalian Cells. *BioMed Res. Int.* **2019**, 2019, e4630891. DOI: 0.1155/2019/4630891.
245. Vervoort, D. F. M.; Heiringhoff, R.; Timmermans, S. B. P. E.; van Stevendaal, M. H. M. E.; van Hest, J. C. M. Dual Site-Selective Presentation of Functional Handles on Protein-Engineered Cowpea Chlorotic Mottle Virus-Like Particles. *Bioconjugate Chem.* **2021**, 32 (5), 958-963. DOI: 10.1021/acs.bioconjchem.1c00108.
246. Zhang, Z. X.; Nong, F. T.; Wang, Y. Z.; Chen, H.; Tang, Y.; Liu, Y. F.; Zhang, J. L.; Zhang, X. J.; Li, H. Y.; Yang, S. C.; Jiang, W. H.; Yang, J. Strategies for Efficient Production of Recombinant Proteins in *Escherichia coli*: Alleviating the Host Burden and Enhancing Protein Activity. *Microb. Cell Fact.* **2022**, 21 (1), 191. DOI: 10.1186/s12934-022-01917-y.
247. Atroshenko, D.L.; Sergeev, E.P.; Golovina, D.I.; Pometun, A.A. Additives for Soluble Recombinant Protein Expression in Cytoplasm of *Escherichia coli*. *Fermentation* **2024**, 10, 120. DOI: 10.3390/fermentation10030120.
248. Royster, A.; Mir, S.; Mir, M. A. A Novel Approach for the Purification of Aggregation Prone Proteins. *PLoS One* **2021**, 16 (11), e0260143. DOI: 10.1371/journal.pone.0260143.
249. Li, Z.; Wei, J.; Yang, Y.; Ma, X.; Teng, Y.; Wang, J.; Chen, Q.; Qi, J.; Gao, G. F.; Ma, Y. Strong Hydrophobicity Enables Efficient Purification of HBc VLPs Displaying Various Antigen Epitopes

through Hydrophobic Interaction Chromatography. *Biochem. Eng. J.* **2018**, *140*, 157–167. DOI: 10.1016/j.bej.2018.09.020.

250. Rocha, J. M. Aqueous Two-Phase Systems and Monolithic Chromatography as Alternative Technological Platforms for Virus and Virus-Like Particle Purification. *J. Chem. Technol. Biotechnol.* **2021**, *96* (2), 309–317. DOI: 10.1002/jctb.6595.
251. Bornhorst, J. A.; Falke, J. J. Purification of Proteins Using Polyhistidine Affinity Tags. *Methods Enzymol.* **2000**, *326*, 245–254. DOI: 10.1016/S0076-6879(00)26058-8.
252. <https://www.rcsb.org/3d-view/1CWP/1>
253. Chase, M. W.; Cauz-Santos, L. A.; Dodsworth, S.; Christenhusz, M. J. M. Taxonomy of the Australian *Nicotiana benthamiana* complex (*Nicotiana* section *Suaveolentes*; Solanaceae): five species, four newly described, with distinct ranges and morphologies. *Aust. Syst. Bot.* **2022**, *35* (5), 345–363. DOI: 10.1071/SB22009.

**Statement on Copyright and License**

*This doctoral thesis is distributed under the Creative Commons license CC BY-NC-ND (Attribution – Non-Commercial – No Derivatives).*

*This license allows others to download and share the work with proper attribution, but it may not be altered or used for commercial purposes.*