European Journal of Biology,
EJB 2025, 84 (1): 132–142

# European Journal of Biology

https://doi.org/10.26650/EurJBiol.2025.1594126
Submitted: 01.12.2024
Revision Requested: 17.03.2024
Last Revision Received: 28.03.2025
Accepted: 04.04.2025
Published Online 09.05.2025

Review Article
Open Access

# Enhancing Recombinant Protein Production by Optimising Nutrient Replenishment, Light, and Humidity in a *Nicotiana benthamiana* Bioreactor

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**Abstract** In the past three decades, plant expression systems have been used and found efficient for the production of a variety of recombinant proteins, such as vaccines, therapeutic proteins, enzymes, and antibodies. They have several advantages over other expression systems, such as bacterial, mammalian, and yeast expression systems, in terms of cost-efficiency, speed of the process, ability to make complex proteins, safety (low risk of human contamination), high productivity, and scalability. Plants as bioreactors are capable of producing the desired recombinant proteins with complex structures. High levels of target protein expression in plants are critical for cost-effective pharmaceutical production. Therefore, it is important to develop strategies to increase the expression of target proteins. The expression of recombinant proteins in plants can be influenced by a variety of environmental factors, including nutrients, light, and humidity; therefore, a strategy to optimise their performance efficiency is required. In addition, the amount of different types of nutrients in the medium significantly affects the productivity of the expression process. In this review, we briefly discuss the effects of nutrients, humidity, and light on recombinant protein expression in *Nicotiana benthamiana*.

**Keywords** Recombinant protein production · Transient expression system · Humidity · Light · Nutrient

Citation: Yuksel, D., Yusifova, G., Hasanova, A. S. & Mamedov, T. Enhancing Recombinant Protein Production by Optimising Nutrient Replenishment, Light, and Humidity in a *Nicotiana benthamiana Bioreactor*. Eur J Biol. 2025; 84(1): 132-142. DOI: 10.26650/EurJBiol.2025.1594126



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## INTRODUCTION

The production of pharmaceuticals (vaccines, monoclonal antibodies, allergens, etc.) in plants for the prevention of various human and animal diseases is a growing area with great potential. Various expression systems, such as bacterial, yeast, insect, and mammalian systems, are currently used for the expression of recombinant proteins for different applications in health, agriculture, and industry. Plant expression systems offer several advantages over other expression systems, including safety, scalability, and low production cost.[1,2] In addition, plants, as eukaryotic organisms, have eukaryotic-type post-translational modifications, such as glycosylation, disulphide bridge formation, which are important for the functionality of most complex proteins.[2] In other words, plants are able to produce recombinant complex proteins that are difficult to produce using bacterial or mammalian cell expression systems. Plants do not naturally produce harmful or toxic substances, as some bacteria do.[3]

Several plants are used as heterologous expression hosts to produce a variety of recombinant proteins, including *Nicotiana benthamiana*, a wild relative of tobacco.[4,5] *N. benthamiana* is a plant species that belongs to the Solanaceae family and is a small, fast-growing plant that is easy to maintain in the laboratory. This plant is the current core production host for diverse recombinant proteins and various pharmaceuticals, such as vaccines[6–17], monoclonal antibodies[18–23], viral antigens[12–14,24–32], human therapeutic proteins[11], enzymes with complex structure[11,13], industrial enzymes[33], and research reagents[34] . All of these biomolecules were successfully produced in *the N. benthamiana* plant. Recently, the plant has been used as a bioreactor for the successful production of safe and cost-effective COVID-19 vaccine candidates and therapeutics.[12,13,32,35–37]

The production of desired recombinant proteins in plants is a complex and multi-step process. To produce a desired target protein of interest using the transient expression system[1,38,39], the target gene sequence encoding the desired protein of interest needs to be designed, engineered, and codon optimized[40,41], *de novo* synthesized[42], and cloned into an appropriate plant expression vector for expression in plants. The plant expression plasmid containing the gene sequence encoding a particular protein is then introduced into a soil bacterium called *Agrobacterium tumefaciens*[43], a bacterial vector capable of transferring genetic material to plants. Several methods are available for achieving transient gene expression in plant cells, including biolistic bombardment, agroinfiltration, and electroporation. Transient gene expression is often used as a preliminary step in plant genetic engineering because it allows researchers to test the function of genes before introducing them permanently into the plant's genome permanently.[1,38,39] It is also useful for producing large quantities of specific proteins, such as antibodies or vaccines, without the need for transgenic plants. Plant transient expression systems have several advantages over stable transformation systems: (i) Speed: Transient expression systems are much faster than stable transformation systems. The expression of the desired protein can be achieved in a few days, whereas stable transformation may take weeks or even months. (ii) High expression levels: Transient expression systems more often produce high levels of the target protein, which is beneficial for applications such as protein purification and structural studies. (iii) Flexibility: Transient expression systems are more flexible than stable transformation systems because they allow the expression of multiple genes in a single experiment. This flexibility allows for rapid screening of gene expression levels and the optimisation of expression conditions. (iv) Reduced risk of gene silencing: Transient expression systems carry a lower risk of gene silencing than stable transformation systems. In stable transformation, the introduced genes can integrate into the host genome. Transient expression systems avoid this risk by not integrating the gene into the genome. (v) Cost-effectiveness: Transient expression systems can be more cost-effective than stable transformation systems, especially for small-scale experiments. This is because they do not require the use of selectable markers or extensive screening to integrate the target gene.

Generally, recombinant protein expression is a complex process of gene expression that involves various methods, techniques, and considerations. It is a powerful tool for producing large quantities of specific proteins that can be used in various applications, including basic research, drug development, and biotechnology. After protein expression, host cells (plant leaves) are harvested, and the protein of interest is purified using a variety of techniques, such as chromatography and filtration. The purification process is critical for obtaining highly pure and active proteins. Finally, the purified protein is analysed to ensure that it is of the desired quality, including factors such as purity, identity, and activity. This may involve various analytical techniques, such as gel electrophoresis, mass spectrometry, chromatography, and functional assays.[44]

High levels of target protein expression in plants are critical for cost-effective pharmaceutical production. Therefore, it is important to develop strategies to increase the expression of target proteins expressed in plants. The expression levels of target proteins are affected by many processes, including plant growth conditions. Plants are highly dependent on environmental factors, such as nutrition, temperature, pH, humidity, and light, as these factors can significantly affect the different stages of plant growth and development.[45] For example, nutrition is critical for plant growth, as plants require a variety of macronutrients (such as nitrogen, phosphorus, and potassium) and micronutrients (such as iron, zinc, and manganese) to support their metabolic processes. Soil fertility and composition can greatly affect the availability of these nutrients, and deficiencies or excesses of certain nutrients can cause stunted growth or other physiological problems in plants.[46]

The pH of the soil or growing medium can greatly affect plant growth because plants have specific requirements for the acidity or alkalinity of their environment. Different plants have different optimal pH ranges, and outside of these ranges, nutrient uptake may be impaired, and the plant's ability to grow and develop may be reduced.[46]

Overall, environmental factors play a crucial role in determining plant growth and development. Understanding these factors is essential for successfully cultivating crops and other plant-based products. Thus, plants are highly dependent on environmental factors, such as nutrition, temperature, pH, humidity, and light, which affect different stages of plant growth. It is essential to understand these factors and their effects on plant growth and development. In this review, the effects of nutrients, light, and humidity on recombinant protein production in *N. benthamiana* are discussed.

## EFFECTS OF NUTRIENT REPLENISHMENT ON RECOMBINANT PROTEIN EXPRESSION IN *N. benthamiana*

Plants require various nutrients to support growth, metabolism, and other vital functions. Essential nutrients for plants are divided into two broad categories: macronutrients and micronutrients. Macronutrients are required in relatively large quantities and include nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S).[47] These nutrients are essential for plant growth and development, including the formation of proteins, nucleic acids, and other important molecules.

Micronutrients, also known as trace elements, are required in smaller quantities and include iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), boron (B), molybdenum (Mo), and chlorine (Cl).[47] These nutrients play crucial roles in plant metabolism and other physiological processes, such as photosynthesis, respiration, and enzyme activation. Plants are capable of obtaining these essential nutrients from the soil through their root systems, and the availability and accessibility of these nutrients can vary depending on the soil type and environmental conditions. In some cases, plants may require additional supplementation with specific nutrients to overcome deficiencies or improve growth and yield.

Overall, the proper balance and availability of essential nutrients are critical for maintaining the health and vitality of plants and supporting their various biological processes and functions.[47] These factors increased cell growth, enhanced protein productivity, improved protein quality, reduced stress response, and increased metabolic burden. Nutrient replenishment can provide the necessary energy and enhance cell growth, leading to increased cell density and protein expression. Nutrient replenishment promotes efficient protein synthesis by providing the necessary precursors and cofactors required for recombinant protein expression. The availability of nutrients and energy sources can support proper folding and post-translational modifications that enhance the quality of recombinant proteins. Adequate nutrient replenishment can reduce stress responses in cells, thereby improving protein expression and increasing the chances of obtaining properly folded and functional recombinant proteins. Oversupplying certain nutrients or energy sources can result in metabolic imbalances and toxicity, ultimately reducing recombinant protein expression.[47]

Nutrient deficiency or excess intake of nutrients more than the required amount affects plants negatively. In this case, excess nutrients can have toxic effects in plants, and one nutrient can interfere with the uptake of another nutrient.[48] Therefore, nutrient replenishment is crucial for efficient recombinant protein expression, but it is important to maintain a balance between the supply of nutrients and the metabolic capacity of cells. Optimisation of nutrient and energy sources is critical for the economic production of recombinant proteins. Although there are studies in the literature investigating the effects of nutrients on pre- and postagroinfiltration treatments on *N. benthamiana*, more research is needed, especially on the effect of nutrient intake and amount on recombinant protein production.[49]

### Nitrogen

Nitrogen is an element in the composition of proteins, nucleic acids, and chlorophyll and is essential for the performance of many plant functions. Proteins are composed of amino acids, and nitrogen is a key component of the amino group ($-NH_2$) found in all amino acids. Proteins play an important role in many plant functions. Therefore, nitrogen plays a key role in the assimilation of nutrients such as potassium and phosphorus. Nucleic acids, such as DNA and RNA, are also composed of nitrogen-containing bases, including adenine, guanine, cytosine, and thymine. These nitrogen-containing bases are critical for the storage and transfer of genetic information necessary for cell division, growth, and development. Chlorophyll, the pigment responsible for capturing light energy during photosynthesis, also contains nitrogen. Nitrogen is an essential component of chlorophyll molecules. Without sufficient nitrogen, plants cannot produce enough chlorophyll to capture the light energy required for photosynthesis. Without sufficient nitrogen, plants cannot synthesise enough amino acids to form proteins, which are essential for cell growth and development, enzyme activity, and numerous other biological processes.[48]

Nitrogen is also important for other plant functions, such as nitrogen fixation, which is the process by which some bacteria convert atmospheric nitrogen gas into usable form for plants. In addition, nitrogen is important for plant defence against pests and diseases and for regulating plant water balance. Nitrogen deficiency negatively affects the vegetative growth of plants by reducing their growth rate of the plants.[48] In nitrogen deficiency, leaf and root development and branching are weakened, flower and fruit retention rates decrease, and fruits remain small. Smaller, lighter coloured leaves with mostly chlorosis are observed on the plants. When nitrogen deficiency increases, leaves turn brown and die. These are the physiological changes occurring in plants due to nitrogen deficiency. However, the expression of various proteins can increase or decrease depending on the amount of nitrogen. Nitrogen starvation occurs when plants grow in an environment that lacks an adequate supply of nitrogen. When plants are starved of nitrogen, they undergo a series of physiological and metabolic changes, including a decrease in photosynthesis and an increase in the accumulation of nitrogen-containing compounds such as amino acids and proteins. Nitrogen starvation can enhance the expression of recombinant proteins.[50] This phenomenon occurs because nitrogen starvation triggers a series of physiological and metabolic changes that can increase the efficiency of protein synthesis and folding.[51] For example, plants grown under nitrogen starvation conditions produce higher levels of recombinant proteins such as antibodies, enzymes, and vaccines.[52,53] However, since nitrogen starvation can also have negative effects on plant growth and development, it is important to carefully balance the benefits of enhanced protein expression with the potential negative impacts on plant health.[54]

Nevertheless, the effects of nitrogen deficiency or excess on recombinant protein production in *N. benthamiana* have not been extensively examined. In a limited number of studies, recombinant protein production increased with the application of nitrogenous fertilisers in plant nutrition.[45,55] Fujiuchi et al. investigated the effect of nitrate concentration on recombinant hemagglutinin production in *N. benthamiana.*[45] Leaf biomass decreased with increasing nitrate concentration. The application of elevated nitrate levels resulted in a 40% increase in the amount of hemagglutinin per leaf fresh weight.[45,49] In contrast, another studies by Shang demonstrated that an elevated ammonium concentration did not influence the yield of the hemagglutinin H1 protein produced in *N. benthamiana.*[55,56] The effects of nitrogen on protein expression in *N. benthamiana* remain poorly understood, representing a significant challenge in the pursuit of enhanced recombinant protein yield. Further research on the impact of nitrogen on recombinant protein production could unlock the potential for high protein yield per low biomass from *N. benthamiana* plants.

## Phosphorus

Phosphorus is another macronutrient vital to plants. Phosphate is an ionic and biologically active form of phosphorus and is an essential element for plant metabolism. It plays a role in many functions in membrane composition, photosynthesis, enzymatic regulation, respiration, energy transfer, signal transduction, nucleic acid synthesis, redox reactions, glycolysis, and nitrogen fixation. Chloroplasts exhibit phosphate homeostasis that regulates sugar transport across the membrane.[57] Phosphorus promotes seed formation, root growth and winter hardiness in plants. However, the responses of different plants to P deficiency or excess vary at the molecular level. A deficiency of this element significantly slows plant growth, maturity, and development. In general, the phosphorous needs of plants, the amount of phosphorus in the soil, and the use of fertilisers are determined. In one of these studies, scientists successfully established a visual reporter system for tobacco. They expressed a purple gene (Pr) isolated from cauliflower (*Brassica oleracea*) to track the amount of phosphorus and its movement inside the plant.[58] They used the rice Pi transporter gene OsPT6, which is expressed under Pi-starvation. This study showed that when the severity of phosphorous deficiency increases, transgenic tobacco leaves turn purple. When enough phosphorus is given to the plant, the leaves of tobacco returned to the green colour. Under P deficiency, several anthocyanin genes are expressed in large quantities compared to wild type. Therefore, although it is physiologically harmful to plants in general, the protein expression of some genes is dramatically enhanced in the absence of phosphorus.[58] Thus, by optimising phosphorus, it is possible to significantly increase the expression of the recombinant protein.

### Metal Ions

Metal ions play an important role in many biochemical and physiological processes. If the plant cannot obtain enough metal ions from the environment, many of its functions are impaired. Despite its large amount on Earth, iron (Fe) is a limiting resource due to challenges in its uptake by plants. Due to the low solubility of the oxidised ferric form in aerobic conditions, iron is the third most limiting nutrient for plant growth and metabolism.[59] Metal-ion transporters are responsible for the uptake of iron from soil. Iron plays an important role in respiration, photosynthesis, and the activation of catalase, peroxidase, and cytochrome oxidase in plants and ensures the catalysis of many chemical reactions. During the deficiency of this substance, the overall growth of the plant is reduced. The absorption of iron is highly dependent on the soil pH. For example, if the pH of the environment in which the plant is growing is above 6.5, iron will be converted into a form that the plant cannot absorb, resulting in iron deficiency.[60] The number of proteins expressed in transgenic tobacco roots was increased by Fe deficiency.[61–63] In another study, the relationship between iron mobilisation and phosphate was examined. Iron mobilisation is highly dependent on the amount of phosphate present in the environment. Phosphorus deficiency causes iron immobilisation in the root apical meristem.[64]

## EFFECTS OF LIGHT, TEMPERATURE, AND HUMIDITY ON RECOMBINANT PROTEIN EXPRESSION IN *N. benthamiana*

### Light

Light plays a crucial role in photosynthesis, which is the process by which plants and other photosynthetic organisms convert light energy into chemical energy to produce ATP and NADPH, which are required for protein synthesis. Some studies have reported that increasing light intensity can enhance the expression of certain proteins by stimulating gene expression and increasing the efficiency of protein folding and post-translational modifications (Table 1).[65-72] Light intensity and photoperiod (the length of time plants is exposed to light) are important environmental factors that affect plant growth.[73] The light conditions under which transient expression occurs in plants can significantly affect the accumulation of recombinant proteins. In fact, controlling light conditions is an important factor in optimising the expression of recombinant proteins in plants. Light plays a critical role in the regulation of protein expression in plants.[49,74] The intensity of light can affect various aspects of protein production, including gene expression, protein folding, and post-translational modifications.

In plant-based systems, such as tobacco and Arabidopsis, increasing light intensity during the post-inoculation phase can increase protein production. For example, increasing light intensity from 70 to 120 μmol.m$^{-2}$.s$^{-1}$ increased the expression of the human erythropoietin gene in tobacco plants. Light exposure is essential for recombinant protein production, particularly in photosynthetic hosts such as plants, algae, and cyanobacteria. As mentioned in the example, Cazzonelli and Velten (2006) found that the recombinant luciferase content in *N. tabacum* leaves treated in the dark was only 20% of that treated at a photosynthetic photon flux density (PPFD) of 80-100 μmol.m$^{-2}$.s$^{-1}$.[65] This indicates that light is necessary for optimal protein production in this system. Therefore, light intensity can influence protein production by affecting photosynthetic rates, energy production, and the efficiency of protein synthesis and post-translational modifications. However, the optimal light conditions for recombinant protein production can depend on various factors, such as the host gene expression, the specific protein being produced, and growth conditions. It is important to optimise the light conditions to achieve maximal protein yield without causing photodamage or other adverse effects on cell growth and viability. While light conditions can have a significant impact on the accumulation of recombinant proteins during transient expression in plants, some studies have shown that exposure to light may not always have a positive effect on protein accumulation.[68]

Overall, the effect of light intensity on recombinant protein content can vary depending on the specific expression system and protein being produced. It is important to optimise the light conditions to achieve maximal protein yield without causing photodamage or other adverse effects on cell growth and viability. Light quality, intensity, and duration affect the accumulation, folding, and stability of recombinant proteins in plant cells. For example, some studies have shown that blue light can enhance the accumulation of certain recombinant proteins in tobacco plants, whereas red light can reduce protein aggregation and improve their solubility.[75] Red and blue light have different effects on the expression of certain proteins involved in photosynthesis and other processes. Similarly, a high light intensity can increase the accumulation of some recombinant proteins, whereas a low light intensity may be more suitable for other proteins.

Plants have evolved sophisticated photoreceptors that enable them to sense and respond to changes in their environment, including changes in light intensity, quality, and duration.

**Table 1.** Studies on the effects of environmental factors on *Agrobacterium tumefaciens*-mediated transient expression of recombinant proteins in *N. benthamiana.*

| Environment Factor | Recombinant Protein | Agroinfection method | Effects of environmental factors |
|---|---|---|---|
| Temperature | Luciferase [65] | Syringe | The optimal temperature for efficient recombinant protein expression is 15-25°C. Dark treatment has a negative effect on protein production. |
| | Hemagglutinin [66] | Vacuum | |
| | Brome mosaic virus coat [67] | Syringe | |
| | Endoglucanase [68] | Vacuum | |
| | GFP [69] | Syringe | |
| Light | Luciferase [65] | Syringe | Vital for recombinant protein expression. No positive effect on accumulation No significant effect on protein content. |
| | Hemagglutinin [66] | Vacuum | |
| | Endoglucanase [68] | Vacuum | |
| | GUS [70] | Co-Culture | |
| | GFP [69] | Syringe | |
| Humidity | Brome mosaic virus coat [67] | Syringe | Recombinant protein production increased with low relative humidity in detached leaf inoculation and with high relative humidity in whole plant inoculation. |
| | Endoglucanase [68] | Vacuum | |
| | Alpha-1-antitrypsin [71] | Vacuum | |
| | Hemagglutinin [72] | Vacuum | |

One key mechanism through which light affects protein expression in plants is the regulation of gene expression. Photoreceptors such as phytochromes and cryptochromes can directly interact with DNA and modulate the activity of transcription factors, which in turn regulate the expression of target genes.[76] This can lead to changes in the levels of specific proteins, including enzymes involved in photosynthesis and other metabolic pathways. In addition to regulating gene expression, light can influence post-transcriptional and post-translational processes that affect protein expression.[77] It has been shown that light can modulate the activities of ribosomes and other components of the protein synthesis machinery, leading to changes in the rate of protein synthesis.[78–80] Light can also activate protein kinases and other enzymes that modify existing proteins, altering their stability, activity, and localisation within the cell. The specific effects of light on protein expression in plants depend on a variety of factors, including the wavelength and intensity of the light, the duration of exposure, and the developmental stage of the plant.

The regulation of protein expression by light is a complex and dynamic process essential for plant growth, development, and adaptation to changing environmental conditions. Although light intensity can affect plant growth and development, it is not always the primary determinant of protein production. In some cases, the effect of light intensity on protein expression may be relatively small, especially if other environmental factors, such as temperature, humidity, and nutrient availability, are optimal.[81] However, the specific effect of light intensity on protein production in plants may vary depending on the type of plant and the particular protein being produced. Studies have shown that low light intensity can decrease the expression of certain photosynthetic proteins, whereas high light intensity can increase the expression of stress response proteins.[82,83] It is also worth noting that the effect of light intensity on protein expression may depend on the duration of exposure because short-term changes in light intensity may have a different effect on protein expression than long-term changes. In addition, the effects of light intensity on protein expression may differ at different stages of plant development, including germination, vegetative growth, and reproduction.[81] Photoperiod is another important factor that can affect the timing of flowering and other developmental processes in plants.[49,81,84]

Overall, although changes in light intensity may not always have a significant effect on protein production in plants, it is important to consider this factor along with other environmental variables when studying the regulation of protein expression in plants. The intensity, duration, and quality of light affect the vital activities of plants. The intensity of light changes with seasons and affects leaf colour, stem length, and flowering. The duration of light refers to the time the plant is exposed to light and regulates photosynthesis by dividing it into day and night.[85] Without natural or artificial light, the plant would not be able to grow, reproduce, or find enough oxygen to support life. Sunlight is extremely important for the growth and flowering of plants, and it includes various colour, and plants absorb some colours and reflect others to perform photosynthesis.[49] While natural light is the most important source of plant growth, blue light is particularly important in the early stages of plant growth and red light during the blooming stage. Artificial light sources such as fluorescent, high-pressure sodium, and LEDs also work similarly to sunlight to support plant growth. Artificial light sources make it possible to control plant growth and produce plants faster.

In recombinant protein production using *Agrobacterium*, it was possible to increase the amount of recombinant protein produced by changing the light intensity. In a study using *N. benthamiana* as a bioreactor and changing the ambient light intensity, Zhang et al.[81] used green fluorescent protein (GFP) as a reporter to examine the effects of different light conditions (grown at different light: dark regime) on pre- and post-agroinfiltration. The highest level of transient expression was observed in plants grown under light conditions for 5 weeks compared with those grown for 4–8 weeks. The level of GFP expression was also decreased by shortening the photoperiod or the increase in light intensity. Changing the light intensity before and after inoculating the plant with *Agrobacterium* affected the amount of recombinant protein GFP.[81] It was shown that moderate light intensity post-agroinfiltration was needed for a higher level of transient expression efficiency. A moderate light intensity post-agroinfiltration showed that when light intensity increases during pre-agroinfiltration, protein production decreases. However, protein production increases to a certain level during post-agroinfiltration and then decreases. In addition to this study, a review of the literature revealed conflicting results regarding the impact of light on recombinant protein production in *N. benthamiana*. Some studies reported no effect of light exposure on protein production[68,70], whereas others demonstrated that increasing light intensity and the use of LED enhanced recombinant protein expression.[55,84] In addition, some studies did not observe any influence of light on protein production.[66,69]

## Temperature

Temperature is another important environmental factor that can affect plant growth, as plants have specific temperature requirements for germination, growth, and reproduction.[86,87] Temperature has a significant effect on plant distribution, growth, development, and metabolism of plants.[88–90] Optimum temperatures facilitate rapid growth, development, and efficient photosynthesis. However, exposure to extreme temperatures can induce stress, impede growth, damage the photosynthetic machinery, and diminish overall metabolic efficiency. Temperature affects the metabolic processes of plants. For example, photosynthesis and respiration rates are temperature dependent.[91–93] Optimal temperatures promote efficient photosynthesis, whereas extremes in temperature can damage the photosynthetic machinery and reduce overall metabolic efficiency.[92] Recent studies have demonstrated that air temperature significantly influences the accumulation of target proteins. Exposing a whole plant, a detached leaf, or calli to a constant temperature within a few days after infiltration with *A. tumefaciens*, it was shown that the optimal temperature for recombinant protein accumulation depends on the specific protein being produced.[74,94] Several studies examining the effect of temperature on recombinant protein accumulation in leaves or callus using *Agrobacterium*-mediated transient expression suggest that the optimal temperature range for recombinant protein accumulation is typically between 15°C and 25°C (Table 1).[65,67,74,95,96] This temperature range effectively facilitates the transfer of T-DNA during *Agrobacterium*-mediated gene transfer and induces temperature-dependent plant stress hormone responses.[97,98] At elevated temperatures, plant stress hormones reduce protein production and necrosis. Note that the accumulation of human monoclonal anti-HIV immunoglobulin G antibody at 21°C was 5-10 times higher than that at 30°C. The accumulation of red fluorescent protein expressed in *N. tabacum* was significantly higher at 25°C than at 15°C or 30°C.[99] The studies mentioned above have shown that moderate temperatures rather than extreme (high or low) temperatures are optimal for the accumulation of recombinant protein in leaves. However, as previously mentioned, the optimal temperature may vary depending on the specific protein being produced, and the roles of other environmental factors are important to optimise the conditions for each individual target protein of interest to maximise their expression levels.

## Humidity

Humidity is another factor that can affect plant growth, as plants require a certain amount of moisture in the air to support their physiological processes.[100] High humidity levels can promote the growth of fungal and bacterial pathogens, whereas low humidity can cause stress and reduce plant growth. Inside the plant, water and nutrients move in tubes called xylems with pulling forces that allow the water to evaporate when the stomata are open, and the pressure rises in the tense leaf cells to deliver water and nutrients to all parts of the plant. Therefore, transpiration is considered a major factor in normal growth and development. Transplant density depends on humidity, and a high amount of water vapour in the air weakens the transpiration process, whereas a small amount accelerates it.[72] When the humidity level is too low, the plant evaporates water intensively, which can quickly lead to water loss and plant destruction. In this case, the plant creates a special stress condition and closes its stomata to prevent water loss.[71,72]

Currently, there is limited information available about the effects of humidity on transient protein production in plants.[49,71,72] High humidity can promote the growth of fungi and bacteria, which can be detrimental to the plant and affect protein expression. Conversely, low humidity can cause dehydration and stress in plants, leading to reduced protein expression. Therefore, it is important to maintain optimal humidity levels for specific plant species and the recombinant protein being produced. It was shown that ambient humidity level is important for leaf-based transient expression in *N. benthamiana* (Table 1).[67,71,72] The findings of these studies indicate that elevated humidity levels enhance the production of homologous proteins when optimal environmental conditions, such as temperature, are maintained. Thus, pre-determining optimal humidity conditions is important for achieving high levels of recombinant protein expression in plants, which can be useful for developing plant-based expression systems for biopharmaceutical production.

## CONCLUSION

Before producing recombinant proteins using *N. benthamiana* as a bioreactor, environmental factors such as humidity, light intensity, temperature, pH, and the availability of nutrients can all impact the efficiency and quality of the production process. Optimising these environmental conditions is critical for achieving high recombinant protein expression, stability, and yield in plants. There is a paucity of studies examining the optimisation of environmental conditions for enhancing protein production during transient expression in *N. benthamiana*. By focusing on optimising the environmental conditions, the preference for *N. benthamiana*, which is currently one of the most preferred heterologous expression systems, will increase.


| | |
|---|---|
| Ethics Committee Approval | Ethics committee approval was not required for the study. |
| Peer Review | Externally peer-reviewed. |
| Author Contributions | Conception/Design of Study- T.M.; Data Acquisition- T.M., D.Y., G.Y.; Drafting Manuscript- T.M., D.Y., G.Y., A.S.H.; Critical Revision of Manuscript-T.M.; Final Approval and Accountability- T.M., D.Y., G.Y., A.S.H. |
| Conflict of Interest | Authors declared no conflict of interest. |
| Financial Disclosure | Authors declared no financial support. |



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