„ALEXANDRU IOAN CUZA" UNIVERSITY OF IAȘI
FACULTY OF BIOLOGY
DOCTORAL SCHOOL OF BIOLOGY

# The effects of atmospheric-pressure plasma treatments on germination and development of economically valuable plants

## PHD THESIS SUMMARY

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# CONTENT

# KEY WORDS

Plasma activated water, dielectric barrier discharge, *Phaseolus vulgaris*, *Zea mays*, *Cucumis sativus*, potassium nitrate, germination percentage, germination rate, germination parameters, assimilatory pigments, chlorophyll A, chlorophyll B, carotenoids, gene expression, Real-Time PCR, radicle, indirect treatment.

# THEORETICAL CONSIDERATIONS

## INTRODUCTION

According to data provided by the United Nations, the global population is continuously increasing, and it is estimated that by 2050 it will reach approximately 10 billion inhabitants (Department of Economic and Social Affairs, 2019). These data are correlated with findings published by the Food and Agriculture Organization regarding acute food insecurity and the depletion of food stocks (Food Agriculture Organization; World Health Organization, 2003). Considering all this information, the production of high-quality food capable of meeting the nutritional requirements of the population is of critical importance.

Plants are sessile organisms and are unable to escape unfavorable environments in order to seek alternative habitats that provide more stable conditions of temperature, moisture, or nutrient availability. The adaptive response of plants involves both alterations in gene expression, which strongly influence metabolic pathways, and pronounced phenotypic plasticity, aimed at modulating growth and developmental processes (Zhu, 2016).

Over time, there has been increased interest in identifying an interdisciplinary solution capable of ensuring both the decontamination of plant material and the stimulation of seed germination and subsequent plant development (Ji et al., 2016). Utilizarea plasmei la presiune atmosferică pare să satisfacă toate aceste condiții și nu implică folosirea de fertilizanți chimici care ar putea interfera cu sănătatea oamenilor. The use of atmospheric pressure plasma appears to meet these requirements and does not involve the application of chemical fertilizers that could interfere with human health. Moreover, worldwide research has investigated the effects of plasma on a wide range of substrates, including food products, seeds, and textile materials(Cho et al., 2009), wood (Altgen et al., 2016; Asandulesa et al., 2010; Talviste et al., 2020) and many others. Each individual treatment type may confer specific benefits to the analyzed material; therefore, a thorough evaluation of the induced effects is required to identify the optimal treatment method for each substrate. In addition, it is essential to characterize plasma properties prior to treatment, as well as those of the treated substrate, in order to identify the consequences for the chemical and physical components of samples exposed to this type of gas. The observed effects may vary in a dose-dependent manner, and experimental conditions can be adjusted according to the research objectives(Adhikari et al, 2020).

# OBJECTIVES OF THE STUDY

The aim of this research is to investigate the effects of atmospheric-pressure plasma treatments on germination and development processes in economically valuable plants, through morphological (radicle length, root system length, and shoot length), biochemical (quantification of photosynthetic pigments), and molecular evaluations (assessment of the expression of genes involved in plant development and abiotic stress responses). The specific objectives and activities were distributed over the four years of the doctoral program according to the Gantt chart (Figure 1) and are as follows:

**O.1. Morphological and physiological characterization of *Phaseolus vulgaris*, *Zea mays*, and *Cucumis sativus* samples;**

A.1.1. Selection of samples from the collection of the "Mihai Cristea" Plant Genetic Resources Bank, Suceava;

A.1.2. Treatments with plasma-treated water;

A.1.3. Measurement of radicle length and, subsequently, of belowground and aboveground plant parts in samples irrigated with plasma-treated water, in comparison with control samples;

A.1.4. Determination of chlorophyll a, chlorophyll b, and carotenoid contents using spectrophotometric methods in plasma-treated water samples, compared to the control group;

**O.2. Gene expression analysis in *Phaseolus vulgaris*, *Zea mays*, and *Cucumis sativus* samples;**

A.2.1. Evaluation of the expression of genes involved in plant development and abiotic stress responses.

**O.3. Dissemination of results through publication in peer-reviewed journals and participation in scientific conferences;**

**O.4. Preparation and defense of the doctoral thesis.**

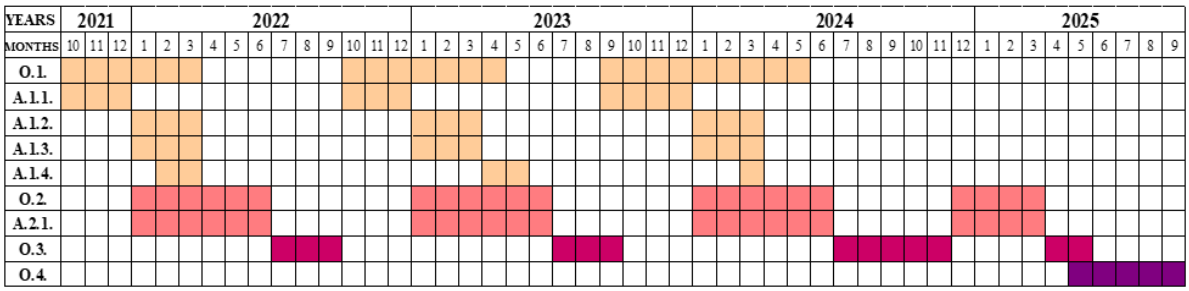


**Figura 1.** Gantt diagram

# MOTIVATION AND ORIGINALITY OF THE STUDY

This research was motivated by the hypothesis that a proportion of plant material exhibits germination-related problems, which negatively affect field productivity. In addition, stress factors pose a continuous threat to seed integrity and survival; these include both biotic factors, such as pathogens, and abiotic factors, among which extreme weather conditions and fluctuations in moisture availability are particularly relevant.

Furthermore, long-term seed storage represents an essential aspect of biodiversity conservation and food security, which constitutes the fundamental objective of Gene Banks. These institutions are specialized in the preservation of plant genetic material by maintaining seeds under optimal conditions, enabling their use after decades or even centuries. However, long-term storage under controlled conditions, including low temperature and humidity, may adversely affect the viability of certain seed stocks. Continuous testing of germination capacity and periodic regeneration of seed lots are core activities of Gene Banks, aimed at maintaining viable and healthy collections. For seed lots that are nevertheless affected by these storage conditions, it is necessary to identify a technique capable of revitalizing plant material without altering the species-specific morpho-physiological traits.

Within this context, scientific studies published in recent years have demonstrated that seed exposure to cold plasma or treatment with plasma-activated water can induce a range of positive effects on germination and subsequent plant development. The emergence of this non-invasive approach represents a major advancement in agricultural biotechnology, and its applicability across diverse plant species constitutes a rapidly expanding field of research.

In order to ensure a comprehensive approach, species belonging to the main groups of cultivated plants, namely cereals, legumes, and vegetables—were selected. From the cereal group, maize was chosen, as it is one of the most important cereal crops due to its high productivity and multiple uses, both for animal feed and human consumption. The legume representative is common bean, a plant of major importance owing to its high protein content, which makes it an essential food source, as well as to its positive impact on soil fertility through biological nitrogen fixation. Among vegetable crops, cucumber was selected, which, in addition to its high nutritional value, is associated with the accumulation of significant

amounts of nitrogen and phosphorus, thereby enhancing plant productivity. Consequently, the three selected species have a major impact on global agriculture, and plasma-based treatments may offer new perspectives for ensuring improved seed quality.

Following the analysis of 57 scientific articles published between 2008 and 2024 that investigated the effects of plasma treatments on seeds, the most frequently studied plant-related parameters were identified as follows: 41 studies focused on the modulation of the germination process, while 34 addressed subsequent plant development. In 25 articles, aspects related to the activity of enzymes involved in various metabolic pathways were examined, whereas 22 studies investigated parameters associated with the scarification of the seed coat. Gene expression–related aspects were identified in only 16 studies; among these, the majority focused on genes involved in stress responses or on genes controlling metabolic processes. Only two studies evaluated plant defense mechanisms against pathogen attack and the effects of plasma on genes involved in plant growth processes, respectively.

Within this context, the present study aims to contribute to filling existing gaps in knowledge regarding the effects of plasma application on the molecular and biochemical mechanisms of the treated species. Furthermore, the originality of this research also arises from the selection of one representative from three distinct plant groups, whereas the majority of existing studies focus on samples belonging to a single species.

**The limitations of the present research** may be multiple, given the novelty of the addressed field and the limited availability of specialized literature, as well as the fact that researchers have applied highly diverse treatment methods to a wide range of species, involving individuals at different developmental stages. Thus, although this study investigated three distinct species, it is possible that the selection of alternative representatives from the plant groups of interest could have resulted in a more pronounced response to plasma-activated water. In addition, the present research focused on *in vitro* analyses of the samples; however, the extrapolation of the experimental variants to *in vivo* conditions would likely have provided a more comprehensive understanding of the induced effects. Finally, the biochemical laboratory facilities available at the time the experiments were conducted did not allow for more complex analyses aimed at identifying potential variations among experimental groups, and some of the analyzed genes were either not influenced by the tested treatments or the selected primers were not sufficiently efficient, preventing their proper analysis.

# 1. General considerations on plasma

## *1.1. What is atmospheric-pressure plasma*

Plasma represents one of the four fundamental states of matter, and among the first researchers to describe plasma was Irving Langmuir at the beginning of the 20th century (Goldston & Rutherford, 1995). Plasma is quite similar to a gas; however, it consists of a complex mixture of electrons, ions, neutral atoms, radicals, and various molecules carrying electrical charges (Attri et al., 2021). It is estimated that more than 99% of the universe exists in the plasma state, except for cold celestial bodies and planetary systems (Dave et al., 2018). The sun, solar winds, stars, and the aurora borealis are examples of naturally occurring plasma phenomena found near humanity (Peratt, 2015).

Considering the temperature of its components, plasma can be classified into hot (thermal) plasma or cold (non-thermal) plasma. Cold plasma is characterized by a lack of thermodynamic equilibrium, with certain components exhibiting low temperatures, while electrons possess significantly higher temperatures, a feature associated with low levels of ionization (Dave et al., 2018). This particular type of plasma can be generated at ambient temperatures and can be applied in both biological and medical research (Talviste et al., 2020). The present study focuses on evaluating the effects of cold atmospheric-pressure plasma on seeds.

## *1.2. Methods of plasma generation*

### 1.2.1. Plasma generated by dielectric barrier discharge

The first researcher to develop and use dielectric barrier discharge was Siemens in 1857 (Kogelschatz et al., 1999). This technique involves the use of two metal electrodes, one acting as the high-voltage electrode and the other as the grounded electrode. A gas, also referred to as the carrier gas, is present between the two electrodes and becomes ionized during the electrical discharge, leading to plasma formation. For this purpose, an alternating high-voltage current is required, with operating frequencies typically in the kilohertz range. Moreover, although the basic operating principle remains the same, numerous variations in electrode configuration may exist; for example, the electrodes may be cylindrical or planar, and the dielectric material may cover only one electrode or both (Hoffmann et al., 2013).

### 1.2.2. Radiofrequency plasma jet

The development of radiofrequency cold plasma jet technology is attributed to the research group led by Koinuma in 1992. In the years that followed, numerous improvements were made to this type of equipment, and some researchers reported the creation of the so-called plasma needle, whose main advantages are related to its reduced dimensions, resulting in significantly increased experimental precision (Priatama et al., 2022).

## *1.3. Physicochemical characteristics of plasma-activated water*

The modulation of the properties of plasma-activated water (PAW) includes variations in redox potential and electrical conductivity, as well as the generation of reactive oxygen and nitrogen species (reactive oxygen and nitrogen species – RONS) (Thirumdas et al., 2018). The oxidation–reduction potential of water is correlated with its disinfectant capacity (Suslow, 2004) as PAW participates in the degradation of bacterial cell membranes due to its high oxidative potential (Ma et al., 2015). In addition, variations in hydrogen ion concentration occur because of the electrical discharge, leading to a decrease in pH values (Lee et al., 2020). The acidification of PAW is caused by the accumulation of chemical species such as nitric acid ($HNO_3$), which plays an important role in antimicrobial activity (Oehmigen et al., 2010).

Therefore, numerous studies reported in the literature indicate a decrease in water pH following plasma treatment due to the generation of reactive species that subsequently diffuse into the aqueous solution. Furthermore, long-term storage of PAW leads to further pH acidification. After 70 hours of storage, a decrease in pH from 2.07 to 1.5 was observed (Shainsky et al., 2010), while after 30 days the pH decreased from 6.8 to 2.3, regardless of storage temperature (Shen et al., 2016).

## *1.4. Chemical changes occurring in plant material following the use of non-thermal plasma*

Hydroxyl radicals (OH•) are highly reactive, short-lived species that are generated in the gas phase but rapidly recombine and are therefore difficult to detect. This limitation can be overcome by using optical emission spectroscopy, a technique that allows the identification of the spectral signatures of NO• and OH• radicals, as well as hydrogen and oxygen atoms (Dufour et al., 2021).

# 2. Effects of atmospheric-pressure plasma treatment in various fields of interest

In recent years, there has been a rapid increase in data generated by research groups regarding the testing of plasma treatments across various fields of interest. The wide variety of reactive oxygen and nitrogen species produced during plasma generation induces beneficial effects when applied to both biotic and abiotic surfaces. Based on the analysis of studies addressing plasma-related applications, the most common research areas include the medical, food, and agricultural sectors.

## *2.1. Effects on food products*

The effects of plasma have been investigated on both fruits and fruit juices. The results indicate that plasma can influence vitamin content (Rodríguez et al., 2017), ascorbic acid levels (Rodríguez et al., 2017), sugars (Rodríguez et al., 2017), starch (Okyere et al., 2022), and carotenoids (Ramazzina et al., 2015), aiming to enhance the nutritional properties of fruit juices. In addition, plasma treatment has also been applied to various types of meat, including poultry (Zhuang et al., 2019), pork (Kim et al., 2013) and beef (Sarangapani et al., 2017).

## *2.2. Effects in the medical field*

Due to its chemical and physical properties, cold plasma represents an innovative therapeutic approach that is currently applied in numerous fields of interest. Certain applications include the sterilization of surfaces (Mai-Prochnow et al., 2014) as well as of living tissues, such as skin (Gan et al., 2018), teeth (Stancampiano et al., 2019) or chronic wounds (Mirpour et al., 2020).

## *2.3. Effects on plant material*

The use of cold plasma in agriculture improves both germination and growth processes (Recek et al., 2021; Volkov et al., 2020), with effects on morphological properties, such as modifications of seed surface characteristics (Recek et al., 2021; Rüntzel et al., 2019), a

reduction in the contact angle (Bormashenko et al., 2012) and variations in gene expression (Holubova et al., 2021).

According to data published by the Statista Research Center in 2024, the global amount of pesticides used has doubled, from approximately 1.7 million tonnes in 1990 to about 3.54 million metric tonnes reported in 2020 (https://www.statista.com/statistics/1263077/global-pesticide-agricultural-use/, accessed on 10.08.2024). This fact highlights the importance of obtaining high yield crops capable of surviving under biotic and abiotic stress conditions, without considering the potential secondary effects associated with the use of these chemical compounds.

### 2.3.1. Decontamination of plant material

Decontamination is a consequence of the generation of UV radiation and free radicals, such as hydrogen peroxide and reactive nitrogen species (Varilla et al., 2020). Plasma exhibits a strong capacity to inactivate a wide range of microorganisms, including spores (Liao et al., 2019), biofilms (Mai-Prochnow et al., 2016) and viruses (Filipić et al., 2020).

### 2.3.2. Changes of the seed coat

Plasma treatment affects both the chemical structure and the rigidity of the treated surface (Lommatzsch et al., 2007). Although numerous studies have demonstrated that plasma treatment can enhance water uptake and, consequently, germination, the exact mechanism underlying surface permeabilization remains unclear. A possible explanation may involve the oxidation of the outer seed layers and the removal of carbon atoms through volatile molecules. Among the effects induced by plasma treatment are the weakening, abrasion, or even rupture of the original structures (Stolárik et al., 2015). All these mechanisms lead to changes in the hydrophobic layer, promoting its transition to a hydrophilic state (Kim et al., 2006).

### 2.3.3. Decrease in the contact angle

The contact angle can be measured at the interface where a liquid meets air on a solid surface and represents a means of quantifying the degree of wettability. Contact angles lower than 90° are associated with increased water absorption, whereas angles greater than 90°

correspond to low wettability (Bracco & Holst, 2013). A decrease in the contact angle is associated with the hydrophilization of the seed coat, a condition that facilitates water penetration into the interior of the seed.

### 2.3.4. Seed germination and plant development

Numerous techniques have been tested to improve plant growth processes and protective mechanisms. The use of cold plasma may represent an alternative to conventional treatments, including physical scarification as well as thermal or chemical treatments (Dhayal et al., 2006).

### 2.3.5. Molecular changes induced by plasma treatment in seeds

Cold plasma and plasma-activated water (PAW) treatments have been extensively studied in recent years due to their potential to modulate seed germination, plant development, and molecular responses to both abiotic and biotic stress. Guo et al. (Guo et al., 2017) investigated the effects of dielectric barrier discharge (DBD) plasma on wheat seeds by analyzing the expression of the LEA1, SnRK2, and P5CS genes, which are involved in drought stress responses. The results showed a stimulation of gene expression under plasma treatment alone as well as under combined plasma and drought conditions, suggesting the activation of abscisic acid–dependent pathways and proline accumulation. In contrast, LEA1 expression rapidly decreased under combined plasma and drought conditions, indicating that plasma treatment does not influence all protective mechanisms associated with abiotic stress.

Iranbakhsh et al. (Iranbakhsh et al., 2018) highlighted the importance of the analyzed tissue, reporting an increase in HSFA4A gene expression in roots three hours after a short-duration treatment, compared to green tissue. Panngom et al. (Panngom et al., 2014) reported rapid activation of pathogen resistance (PR) genes in tomato seeds infected with *Fusarium oxysporum*, attributing this response to the reactive oxygen and nitrogen species generated by plasma. Similar results were obtained by Adhikari et al. (Adhikari et al., 2019) who demonstrated that plasma-activated water induces the expression of defense-related genes in a manner dependent on both treatment duration and tissue type.

The epigenetic dimension of plasma treatment is supported by several studies. Adhikari et al. (Adhikari et al., 2020) highlighted the activation of genes involved in redox homeostasis,

pathogen resistance, and epigenetic modifications, particularly under longer exposure conditions. Zhang et al. (Zhang et al., 2017) demonstrated the demethylation of genes involved in metabolism and growth in *Glycine max*, confirming the role of plasma as an epigenetic modulator.

In conclusion, cold plasma treatment represents a promising technology; however, its effects depend on the species, the type of plasma, the duration of exposure, and the timing of analysis.

# 3. General considerations on the species of interest

## *3.1. Phaseolus vulgaris*

### 3.1.1. General aspects

Common bean (*Phaseolus vulgaris* L.) is an important plant species cultivated worldwide for its seeds. With respect to human consumption, common bean is the most widely consumed legume and represents a major source of proteins, vitamins, minerals, and dietary fiber for populations around the world (Sathe et al., 1984)..

### 3.1.2. Effects of non-thermal plasma treatment on *Phaseolus vulgaris*

Various diseases that may affect seeds hinder healthy plant development and reduce productivity, and dry common bean seeds can be naturally contaminated by various fungi. The most commonly used methods to control infections caused by pathogenic agents involve the application of chemical substances such as pesticides, which prevent the occurrence of diseases (Gupta & Singh, 2020).

#### *3.1.2.1. Erosion of the external seed surface*

The induced modifications can be observed both at the macroscopic level, through the wrinkling of the external seed coat, and by electron microscopy studies, which reveal a transition from a smooth surface in untreated seeds to a surface characterized by numerous depressions and abrasions of the outer seed layer (Rüntzel et al., 2019).

#### *3.1.2.2. Variation in the contact angle*

The effects on the seed coat can be observed in the scientific study published by Recek et al. (Recek et al., 2021). The treatment method involved direct exposure of seeds to the plasma generation source for 3 seconds at a pressure of 10 Pa. Following plasma treatment, the contact angle decreased to approximately 10° and became almost undetectable after 3 seconds of exposure to the plasma source. This result indicates a highly hydrophilic seed surface that absorbs the water droplet almost completely.

#### *3.1.2.3. Stimulation of germination and developmental processes*

Fan et al. (Fan et al., 2020) investigated the effects of plasma-activated water treatment for 15, 30, 60, and 90 minutes on *Phaseolus vulgaris* seeds. The results indicated stimulation of the germination process for almost all tested experimental variants, apart from the 90-minute incubation. Regarding germination, similar results were also reported by the research group led by Bormashenko (Bormashenko et al., 2012), which demonstrated an increase in germination speed following direct plasma treatment, as well as by Volkov et al. (Volkov et al., 2020) who reported both stimulation of radicle development under plasma treatment and enhanced overall plant growth at later developmental stages.

## *3.2. Zea mays*

### 3.2.1. General aspects

Maize is the most widely cultivated cereal worldwide (Qi et al., 2012). Due to its extensive distribution and affordable price, maize has numerous applications. For example, it is important for human nutrition, industrial food processing, animal feed, and non-food industries, such as the production of starch, acids, and alcohols.

### 3.2.2. Effects of non-thermal plasma treatment on *Zea mays*

The effects of plasma treatment on *Zea mays* seeds have been investigated mainly from morphological and physiological perspectives. For example, stimulation of the germination process and subsequent plant development were reported in the studies by Šerá et al. (Šerá et al., 2021) and Sohan (Sohan et al., 2022). Aspects related to modifications of the external seed

coat that allow increased water uptake were identified by Karmakar et al. (Karmakar et al., 2021). In addition, all these studies reported a quantitative increase in enzymes such as catalase, superoxide dismutase, and ascorbate peroxidase.

#### *3.2.2.1. Seed decontamination*

Naturally, plant material serves as a habitat for numerous microorganisms, with normal concentrations considered to range between $10^2$ and $10^6$ colony-forming units per gram of seeds (Tancinová et al., 2001). In the study conducted by Zahoranová et al. (Zahoranová et al., 2018), the decontamination effect of plasma treatment on maize seeds was evaluated for both bacteria and fungi, and the optimal treatment was found to differ depending on the group of microorganisms analyzed.

#### *3.2.2.2. External seed surface*

The effects of plasma treatment on the external seed coat have frequently been investigated using electron microscopy, revealing the development of surface depressions and microcracks that enhance water absorption. Similar results were also reported in the study published by Karmakar et al. (Karmakar et al., 2021).

#### *3.2.2.3. Water contact angle*

In the study published by Zahoranová et al. (Zahoranová et al., 2018) the water contact angle of untreated samples was reported to be 107.9 ± 4.1, indicating a hydrophobic seed surface. In contrast, direct plasma treatment led to an almost twofold reduction in this value (55.9 ± 3.9), corresponding to hydrophilization of the external seed coat. This effect was associated with enhanced water uptake and the initiation of the germination process.

#### *3.2.2.4. Seed germination and plant development*

The study conducted by Zahoranová et al. (Zahoranová et al., 2018) indicates that the effects of cold plasma treatment are dependent on the duration of seed exposure to the plasma generation source. The research group led by Henselová reported a series of beneficial effects of plasma treatment on maize seed germination within an exposure range of 40 to 80 seconds, with an optimal treatment duration of 60 seconds (Henselová et al., 2012).

In the study published by Zahoranová et al. (Zahoranová et al., 2018) a 60-second treatment was shown to stimulate shoot development by 12% and root development by 35% compared to the untreated control.

## *3.3. Cucumis sativus*

### 3.3.1. General aspects

Cucumber (*Cucumis sativus* L.) belongs to the family Cucurbitaceae, which comprises 118 genera and 825 plant species (http://www.faostat.fao.org/ faostat/). It is considered to have originated in India approximately 3,000 years ago and was subsequently introduced to Europe by the Greeks and Romans. Approximately 95% of the cucumber's composition consists of water, making it particularly important in the prevention of dehydration; however, it also contains a diverse range of vitamins, minerals, and essential compounds that contribute to proper physiological functioning.

### 3.3.2. Effects of non-thermal plasma treatment on *Cucumis sativus*

In the specialized literature, research on the effects of plasma and plasma-activated water treatments on cucumber seeds remains very limited, with greater attention being given to other vegetable species, particularly tomatoes. Among the available studies, the research published in 2023 by a group of scientists from Nepal provides one of the most comprehensive investigations and offers valuable insights into the behavior of seeds following exposure to plasma (Guragain et al., 2023).

#### *3.3.2.1. Decontamination of plant material*

In a study published in 2015, the authors investigated the use of atmospheric-pressure plasma to treat whole pieces of apples, tomatoes, cucumbers, and carrots for different exposure durations, with a maximum treatment time of 10 minutes (Baier et al., 2015). The results varied among the tested species; for example, cucumber and carrot exhibit rougher surface characteristics, which favor the accumulation of pathogenic microorganisms.

In the study published by Yarabbi et al. (Yarabbi et al., 2023) a positive effect of argon plasma treatment on cucumber samples was reported, as evidenced by a reduction in microbial load.

#### *3.3.2.2. External seed surface*

The results reported in the specialized literature generally indicate that direct exposure of seeds to the plasma generation source induces changes in the surface morphology of the seed coat, including the occurrence of microcracks that facilitate increased water uptake. Stepanova et al. (Štěpánová et al., 2018) exposed cucumber seeds to the plasma generation source for 20 seconds and, based on electron microscopy analysis, reported the absence of detectable alterations in the external seed coat. However, a more detailed analysis using X-ray Photoelectron Spectroscopy revealed the presence of variations in the chemical composition of the seed coat, which could not be identified by microscopy-based techniques.

#### *3.3.2.3. Water contact angle*

A study published in 2023 (Guragain et al., 2023) analyzed *Cucumis sativus* seeds using the sessile drop method with the aid of DROP software. The initial water contact angle was 72.05 ± 1.05° and following treatment it decreased to 30.46 ± 7.16°.

#### *3.3.2.4. Seed germination and plant development*

The beneficial effects of argon plasma treatment on seed germination and plant development were reported by Guragain et al. (Guragain et al., 2023). Thus, on the 16th day after planting, the total germination percentage was assessed, and a 1-minute plasma exposure resulted in a 13% increase in germination rate, while a longer exposure time (7 minutes) led to a more pronounced stimulation, reaching 20%.

Štěpánová et al. (Štěpánová et al., 2018) investigated the effects of direct plasma treatment on cucumber seeds using three different exposure durations to the plasma generation source, namely 10, 20, and 30 seconds. An exposure time of 20 seconds resulted in the highest increase in germination, reaching 96%, whereas the 10-second treatment led to a 2% increase and the 30-second treatment to a 1% increase in germination percentage.

# 4. Genes involved in plant development and resistance to biotic and abiotic stress factors

Within this study, various genes involved in plant development and in plant responses to stress factors were analyzed.

**a) ABA 8'- hydroxylase**

Abscisic acid is a plant hormone that plays an essential role in the normal regulation of developmental processes and in the modulation of adaptive responses to various stress factors, with effects that vary depending on the plant growth stage (Mizutani & Todoroki, 2006). The catabolic pathway of abscisic acid occurs predominantly through hydroxylation at the C-8′ position, a reaction catalyzed by ABA 8′-hydroxylase.

**b) CCD1 (carotenoid cleavage dioxygenase)**

The CCD gene family is composed of two subfamilies, namely CCD and NCED (9-cis-epoxycarotenoid dioxygenase) (Cheng et al., 2022). Carotenoids are C40 isoprenoid pigments that absorb light in the ultraviolet and blue regions of the spectrum.

**c) DREB family genes**

The DREB (Dehydration-Responsive Element-Binding) gene family encodes transcription factors that play a crucial role in regulating the expression of numerous genes involved in abiotic stress tolerance. Proteins encoded by the DREB1, DREB2, and DREB6 genes function in distinct signaling pathways associated with responses to abiotic stress exposure and also participate in the regulation of ERF (Ethylene-Responsive Factor) gene expression (Ethylene Responsive Factor) (Wu et al., 2022).

**d) ERF (Ethylene Responsive Factor)**

ERF (Ethylene-Responsive Factor) transcription factors play an essential role in a wide range of processes associated with plant development, hormonal regulation, immunity, and responses to various stress factors (Hong et al., 2022; Lee et al., 2016; Tiwari et al., 2012).

**e) LEA (Late Embryogenesis Abundant)**

The information accumulated to date regarding LEA gene expression indicates that it encodes a multifunctional stress protein involved in maintaining metabolic processes within normal limits under various conditions (Magwanga et al., 2018). LEA gene expression is induced by abscisic acid (ABA) (Hundertmark & Hincha, 2008).

**f) MYB (myeloblastosis)**

MYB transcription factors belong to a large gene family with diverse functions in key plant processes, including plant development, primary and secondary metabolic pathways, and responses to abiotic stress factors (Cao et al., 2016; Cao et al., 2013; Du et al., 2012).

**g) MYC (myelocytomatosis)**

The roles of MYC genes in plants are diverse, as they are involved in numerous regulatory processes governing plant growth and development. In addition, MYC genes participate in jasmonic acid–mediated plant development, contribute to the regulation of the plant circadian rhythm, and influence a wide range of phytohormones (Song et al., 2022).

**h) POR (Protochlorophyllide oxidoreductase)**

POR1 and POR2 proteins (protochlorophyllide oxidoreductase) share approximately 75% sequence identity; however, they exhibit distinct regulatory patterns with respect to plant development (Masuda et al., 2002). POR1 and POR2 enzymes catalyze one of the late steps in chlorophyll biosynthesis, namely the light-dependent reduction of protochlorophyllide to chlorophyllide in the presence of NADPH (Garrone et al., 2015).

**i) PP2C (protein-phosphatase 2C)**

The PP2C gene is strongly involved in plant responses to various stress factors by influencing numerous metabolic processes, participating in hormonal level regulation, and contributing to the production of growth-related factors (Wu et al., 2023).

**j) P5CS (1-pyrroline-5-carboxylate synthetase)**

Proline represents a key component in the regulation of protein synthesis in plants (Kishor et al., 2015). Proline accumulation is dependent on the activation of the P5CS gene, which induces the synthesis of $\Delta^1$-pyrroline-5-carboxylate synthetase, an enzyme that catalyzes the first step in the proline biosynthetic pathway (Kishor et al., 2015).

**k) WRKY**

The WRKY transcription factor family is involved in plant adaptation processes to a wide range of stress factors (Bai et al., 2018; Guo et al., 2022; Jiang et al., 2017; Ma & Hu, 2024). These transcription factors also participate in the modulation of metabolic pathways related to the biosynthesis of abscisic acid, jasmonic acid, and salicylic acid, as well as in the regulation of reactive oxygen and nitrogen species scavenging, thereby protecting cellular homeostasis (Guo et al., 2022).

# EXPERIMENTAL PART

## 5. Materials and methods

### *5.1. Biological material*

The biological material originated from the collection of the *"Mihai Cristea" Plant Genetic Resources Bank* in Suceava. The selection criterion was the germination capacity of the samples, as recorded by the Viability Laboratory. In this context, two samples exhibiting different germination percentages (70% and 100%, respectively) were selected. The aim was to assess whether plasma treatment could induce an increase in the germination percentage and/or accelerate the germination rate. In addition, observations were conducted during subsequent stages of plant development in order to determine whether any beneficial effects of plasma treatment are maintained over the long term.

Accordingly, two samples were selected for each species, one reported as having a germination percentage of 70% and the other 100%. The plant material belonging to the species *Phaseolus vulgaris*, registered in the internal database of the Bank with a germination percentage of 70%, was abbreviated as Pv70, while samples recorded with 100% germination were designated Pv100. A similar approach was applied to samples belonging to *Zea mays* (Zm70 and Zm100) and *Cucumis sativus* (Cs70 and Cs100). These values do not represent the germination percentages obtained in the present study, but rather those previously reported by the Viability Laboratory of the institution.

### *5.2. Plasma-Activated Water Production*

Plasma-activated water was obtained at the *Advanced Rsearch Center in Plasma Physics* in Iași, within the Faculty of Physics, „Alexandru Ioan Cuza" University of Iași. The method used for plasma generation at atmospheric pressure was based on the dielectric barrier discharge (DBD) principle, which involves two metallic electrodes (a pin-type metal electrode and a metal foil electrode) separated by a glass dielectric containing the volum of water to be treated (Figure 2). The discharge gap between the pin-type electrode and the water surface was approximately 5 mm, and the voltage selected to achieve electrical breakdown of the air in the dischage space was 19 kV (peak-to-peak).

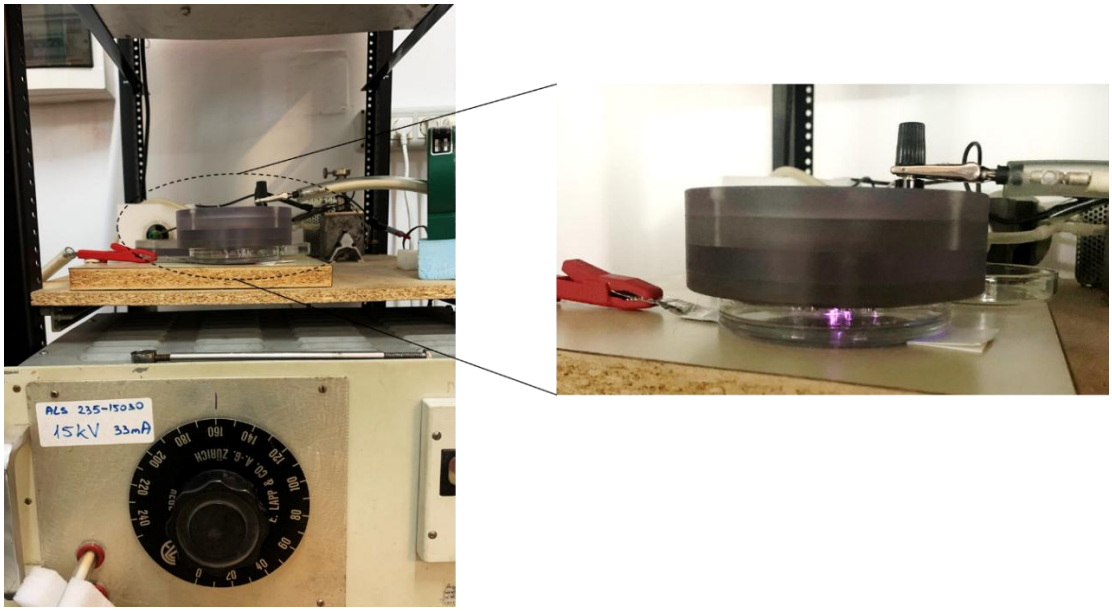


**Figure 2.** Water exposure to the plasma generating source at 19 kV power and 15 kV amplitude at the Advanced Research Center in Plasma Physics, Faculty of Physics, ”Alexandru Ioan Cuza” University of Iași

### *5.3. Seed Germination of the Samples*

For each of the three species, four experimental variants were established (25 seeds per variant), and the experimental design is schematically presented in Figure 3:

- **UW** – exclusive use of untreated water (abbreviated UW);
- **$KNO_3$+UW** – use of a 0.2% $KNO_3$ solution, applied to the seeds prior to germination, followed by the use of untreated water;
- **PAW+UW** – use of PAW until the first evaluation of the germination (day 5 for *Phaseolus* vulgaris, day 4 for *Zea mays,* day 3 for *Cucumis sativus),* after which the seeds were exposed only to UW;
- **PAW** – exclusive use of PAW.

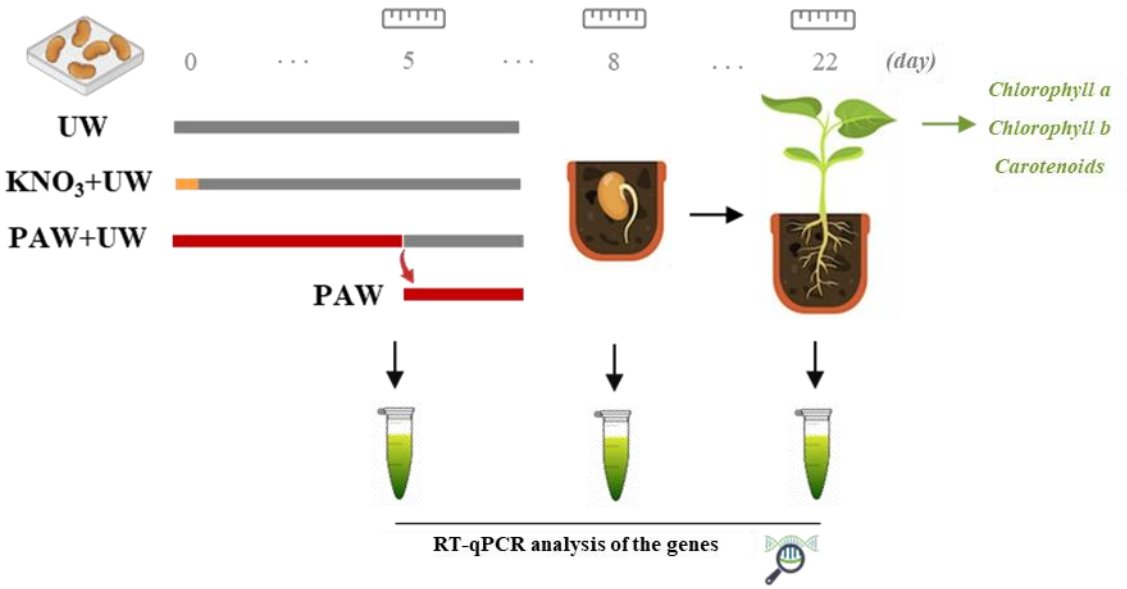


**Figure 3.** Schematic representation of the experimental design applied to seeds of *Phaseolus vulgaris* and the analyses performed: determination of sample length and evaluation of the expression of genes of interest on days 5, 8 and 22, as well as analysis of photosynthetic pigments on day 22

At the end of the germination period (day 8 for *Phaseolus vulgaris* and day 7 for *Zea mays* and *Cucumis sativus*), five germinated seeds from each experimental variant were planted in soil and allowed to continue their development for an additional 14 days. Germination was carried out in a Binder growth chamber at 75% humidity, under alternating temperature conditions of 20 °C for 8 hours and 30 °C for 16 hours for all three analyzed species.

### *5.4. Assessment of seed germination*

To characterize the seeds in terms of germination, the following germination parameters were calculated: **germination percentage**, **relative germination percentage**, **germination index**, **mean germination time**, and **the time required for the germination of a specific percentage of the tested seeds**.

### *5.5. Soil transfer of the samples*

On the 8th day of germination, five germinated seeds from each experimental variant were transferred to soil to continue their development. Plastic pots measuring 6 × 6 cm were used, each filled with approximately 30 g of soil. The samples were subsequently placed in a

climate-controlled chamber and watered daily with 2 mL of untreated water or plasma-treated water, depending on the experimental variant, for a period of 14 days.

### *5.6. RNA isolation*

For RNA isolation, five individuals were collected from each experimental variant. Sampling was performed in 1.5 mL tubes containing RNA Save solution (RNA Save, Biological Industries). The samples were stored at −80 °C in the solution until RNA isolation experiments were conducted. RNA isolation was performed using the Promega kit (SV Total RNA Isolation System).

### *5.7.RNA cuantification*

The qualitative and quantitative assessment of the isolated RNA was performed using a NanoDrop™ One Microvolume UV-Vis Spectrophotometer.

### *5.8. Reverse transcription step*

The reverse transcription reaction was performed using the GoScript™ Reverse Transcription System kit (Promega).

### *5.9. Real Time quantitative PCR (RT-qPCR) step*

In the present study, the GoTaq qPCR Master Mix kit (Promega) was used. The genes of interest, depending on the analyzed species, were as follows: *Phaseolus vulgaris*: ABA8'H, DREB1, DREB2A, DREB6B, DOX, ERF, LEA3, NCED3, POR1, POR2, PP2C, P5CS10, WRKY53; *Zea mays*: ABA8'H, CCD1, DREB1, LEA, MYB, MYC, PP2C, POR, WRKY53; *Cucumis sativus*: ABA8'H, ERF110, LEA2, MYB, P5CS, PP2C, POR, WRKY2.

RT-qPCR analysis was performed using the relative quantification method ($2^{-\Delta\Delta CT}$). The values obtained for the tested samples were statistically analyzed using GraphPad software, and the analytical method applied was one-way ANOVA.

### *5.10. Morphological analysis of the samples*

Morphological analyses initially involved measuring five individuals from each of the four experimental variants. An electronic caliper was used to determine radicle length on days 5 and 8 of germination for *Phaseolus vulgaris*, days 4 and 7 for *Zea mays*, and days 3 and 7 for *Cucumis sativus*.

### *5.11. Determination of photosynthetic pigment content*

For the determination of chlorophyll a, chlorophyll b, and carotenoids in the samples of interest, a protocol adapted from Sumanta et al., 2014 was used.

### *5.12. Assessment of Plasma-activated water pH*

After three months of storage of PAW at −80 °C, the pH was determined using a portable pH meter. Prior to pH measurement of the samples of interest, a calibration curve was performed using standard values of 4.01, 7.00, and 10.01.

# 6. Results and discussion

## *6.1. Analysis of the pH of untreated water compared to plasma activated water*

The pH value obtained for untreated water was 5.06, compared to plasma-treated water, which exhibited a pH of 3.25. Thus, acidification of PAW was observed, a result consistent with literature reports indicating that the accumulation of reactive oxygen and nitrogen species, such as nitric acid, promotes a decrease in pH values (Adhikari et al., 2019; Ma et al., 2016).

## *6.2. Phaseolus vulgaris*

### 6.2.1. Evaluation of the germination process

The results obtained for the Pv70 sample highlight a positive influence of the applied treatments on seed germination compared to the control variant (UW). The lowest germination percentage was recorded for the UW variant (60%), while all other treatments induced significant increases, with the maximum value observed for the PAW+UW combination (84%). These trends are also supported by the germination index and the relative germination percentage. Moreover, analysis of the mean germination time indicates the lowest value for the PAW+UW variant (4.95 days), corresponding to a higher germination rate, whereas the $KNO_3$+UW treatment exhibited the longest duration of the process, despite a high germination percentage.

Evaluation of the T10, T25, T50, T75, and T90 indices shows similar values among variants during the initial stages of germination; however, at higher germination percentages (50–90%), the lowest values were recorded for PAW treatments, confirming both an increase in germination percentage and an acceleration of the process compared to the control.

In the case of the Pv100 sample, which exhibits maximum germination potential, PAW treatment led to a reduction in mean germination time, with the lowest values obtained for PAW+UW and PAW compared to the control variant. These differences become more pronounced at higher germination percentages, where PAW treatment significantly shortened the duration of the process, highlighting the beneficial role of plasma-treated water in enhancing germination speed.

### 6.2.2. Evaluation of plant growth parameters

For the Pv70 sample, analysis of radicle length indicated the absence of significant differences among the experimental variants on both day 5 and day 8 of germination. These results suggest that, although plasma-treated water (PAW) stimulated the germination percentage, it did not accelerate early seedling development. Likewise, potassium nitrate treatment did not result in relevant improvements in radicle growth. Observations performed on day 14 of development in soil confirmed these findings, as neither PAW nor $KNO_3$ led to increases in belowground, aboveground, or total plant length compared to the control variant.

In the case of the Pv100 sample, growth differences were more pronounced. Although variations were minimal on day 5, a trend toward stimulation of radicle elongation under the influence of PAW was observed. On day 8, PAW treatments induced significant increases in radicle length compared to the control, with a more pronounced effect than that observed for potassium nitrate. Results obtained on day 14 showed values up to 1.5-fold higher for total plant length in PAW-treated plants compared to UW, for both temporary and continuous application. $KNO_3$ treatment yielded values similar to those obtained with PAW+UW, without significant differences compared to PAW, indicating a moderate beneficial effect on plant development (Figure 4).

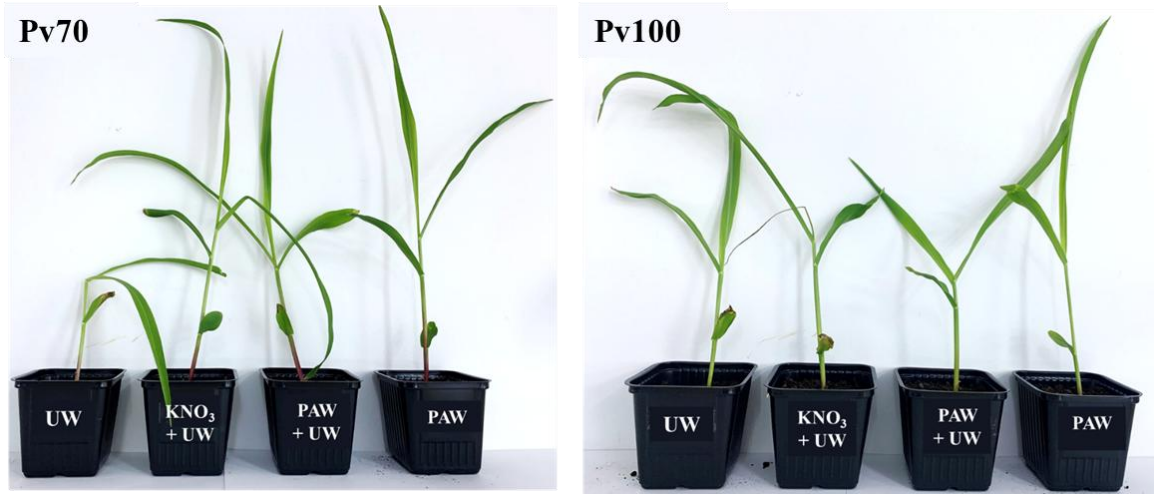


**Figure 4.** Selection of one individual from each of the four experimental variants for *Phaseolus vulgaris*, for comparative observation of plant development (day 14 of growth in soil) (UW – untreated water, $KNO_3$ – potassium nitrate solution, PAW – plasma activated water)

### 6.2.3. Evaluation of chlorophyll a, chlorophyll b, and carotenoid content

The results obtained from the biochemical analyses showed no significant differences among the experimental variants of the Pv70 sample, with even a decrease in the recorded values being observed. In contrast, for the Pv100 sample, a slight increase in the content of chlorophyll a, chlorophyll b, and carotenoids were observed across all treatment variants. This result supports the enhanced morphological development of plants.

### 6.2.4. Evaluation of the expression of genes involved in the response to plasma activated water in *Phaseolus vulgaris*

Analysis of the expression of genes involved in germination, stress response, and plant development highlights a complex, time-dependent effect of the applied treatments, particularly plasma-treated water (PAW), on the Pv70 and Pv100 samples. In the case of the **ABA8'H** gene, which is involved in abscisic acid catabolism, the results support the physiological data regarding the acceleration of the germination process. Inhibition of the expression of this gene is associated with a reduction in ABA levels and, consequently, with stimulation of germination and early development. For the Pv70 sample, a statistically significant decrease in expression was observed on day 8 for the PAW variant compared to the UW control, whereas for the Pv100 sample, similar values on days 5 and 8 reflect the comparable germination percentages. On day 22, reduced ABA8'H expression under PAW conditions, significant for Pv100, correlates with intensified metabolic processes, normal chloroplast development, and stimulation of radicle growth, confirming the beneficial role of PAW during later stages of development.

Expression of the **P5CS10** gene, associated with proline biosynthesis and stress tolerance, showed a marked increase on day 5 in all treated variants, both with $KNO_3$ and PAW, for both samples. The differences compared to the control group were statistically significant, indicating early activation of cellular adaptation and protection mechanisms. In subsequent days, expression levels tended to converge among variants, suggesting that the initial treatment-induced stimulation allows rapid attainment of an optimal functional level, compared to the control, in which expression remained consistently lower.

In the case of the **LEA3** gene, associated with desiccation tolerance, PAW treatments did not induce significant changes in expression, except for an isolated increase observed in the Pv100 sample on day 8 under temporary treatment conditions. This result suggests that

PAW does not induce severe water stress but rather acts through optimization of metabolic processes.

The response of the **PP2C** gene, an important component of the ABA signaling pathway, indicated a twofold increase in expression on day 5 under the influence of PAW for both samples, with statistical significance in the case of Pv100. This early response suggests rapid adaptation to treatment-induced conditions; however, the lack of significant differences on days 8 and 22 indicates stabilization of physiological processes as development progresses.

Genes belonging to the **DREB** family (DREB1, DREB2, and DREB6), involved in abiotic stress responses, exhibited a similar pattern, characterized by strong stimulation on day 5 under both $KNO_3$ and PAW treatments. Particularly in the case of PAW, this stimulation was maintained on day 8 for certain genes and samples, highlighting a sustained adaptive response. By day 22, differences among variants disappeared, suggesting that the effects of the treatments are predominant during the early stages of development.

Expression of the **ERF** gene, associated with growth regulation and stress responses, was significantly increased on day 5, especially under PAW treatment, for both samples. At the final stage of the experiment, enhanced expression was mainly associated with $KNO_3$ treatment, indicating differential effects of the treatments depending on the timing of application.

For the **MYB07** gene, involved in the transcriptional regulation of developmental processes, a significant decrease in expression was observed on day 5 following $KNO_3$ and PAW treatments, suggesting a temporary deregulation of control mechanisms in favor of accelerated germination. Subsequently, expression tended to normalize, and on day 22, $KNO_3$ treatment induced a significant increase for the Pv70 sample.

The **POR1** and **POR2** genes, involved in chlorophyll biosynthesis, showed a significant increase in expression, particularly under the influence of PAW, on day 5 for both samples. The results obtained on day 22 for POR2, especially in the Pv100 sample, support the biochemical data regarding increased chlorophyll a and carotenoid content, confirming the role of PAW in stimulating developmental and photosynthetic processes.

In the case of the **WRKY53** gene, expression was low during the early stages, followed by a transient stimulation on day 8, particularly in the PAW and PAW+UW treatment variants. At the final stage, expression levels became similar across all variants, indicating a temporary effect of the treatments on this regulatory pathway.

Some of the results regarding the modulation of gene expression of interest are illustrated in Figure 5.

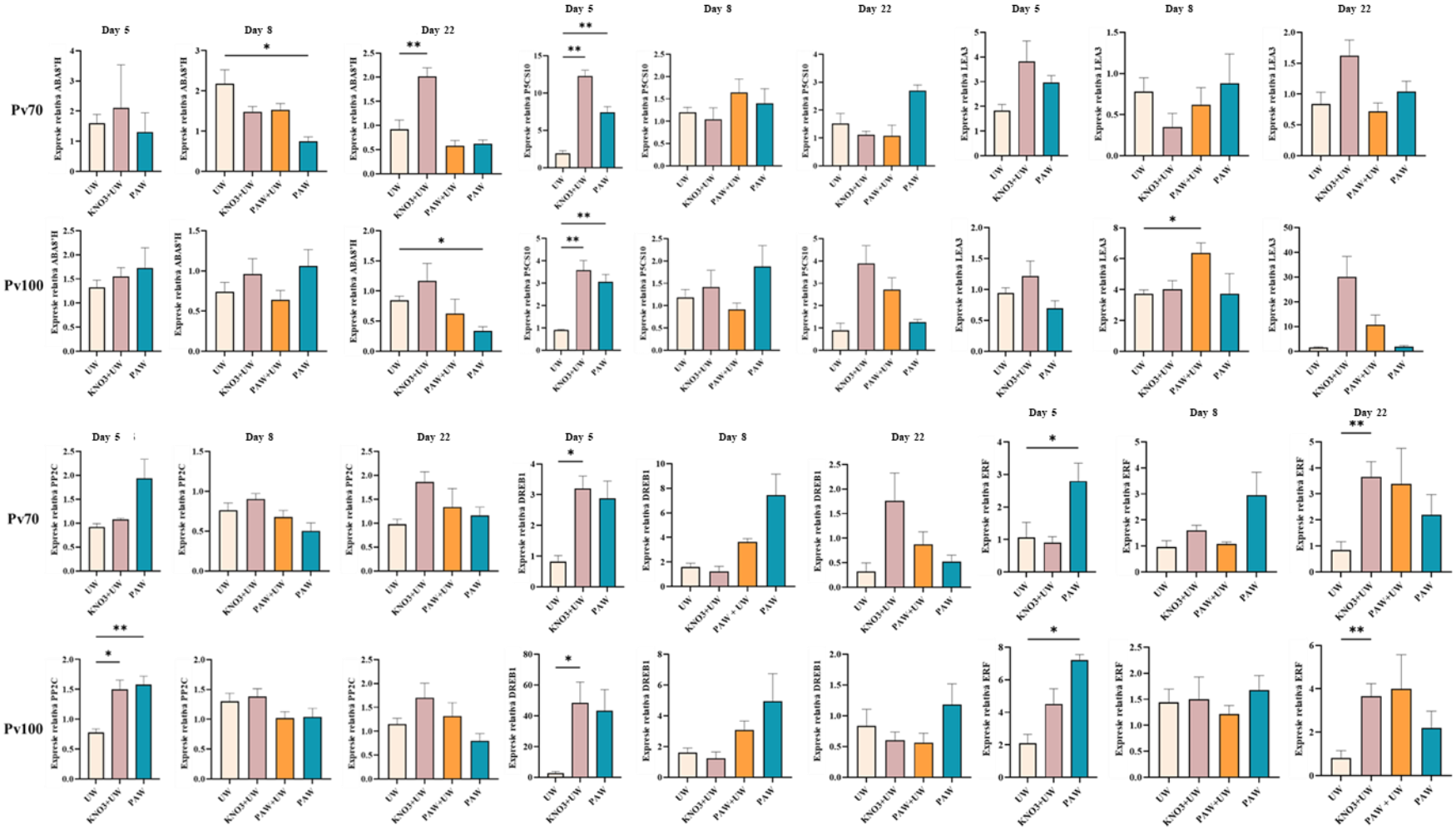


**Figure 5.** Emphasizing gene expression in sample belonging to the species *Phaseolus vulgaris* for a subset of the studied genes: ABA8'H, P5CS10, LEA3, PP2C, DREB1 și ERF

(UW – untreated water, $KNO_3$ – potassium nitrate solution, PAW – plasma activated water)

### *6.3. Zea mays*

#### 6.3.1. Evaluation of the germination process

Similar to the germination results obtained for Phaseolus vulgaris in sample Zm70 (recorded with a germination rate of 70%), in Zea mays the PAW treatment also increased the germination percentage from 60% in the UW variant to 84%. In fact, an increase in the number of germinated seeds was observed in all three experimental groups, reaching the same final germination percentage ($KNO_3$+UW = PAW+UW = PAW = 84%). This finding supports the potential replacement of potassium nitrate solutions with PAW, which offers lower costs and multiple advantages for plant growth-related processes.

The increase in the number of germinated seeds is also associated with a higher germination rate, as indicated by the values obtained for the T10, T25, T50, T75, and T90 indices. The largest difference was recorded for T90, with a value of 5.3 days in the UW variant, 3.6 days for $KNO_3$, and 3.8 days for both PAW+UW and PAW. This represents another example of a situation in which no differences are observed between temporary and permanent treatment variants, highlighting the importance of PAW application at the early stages of plant material development.

For the Zm100 sample, the results regarding the germination indices do not reveal differences among the treatment groups analyzed in this study. Both the number of germinated seeds and the time required for radicle protrusion show similar values under untreated water irrigation as well as under stimulation with potassium nitrate or plasma-treated water.

#### 6.3.2. Evaluation of plant growth parameters

Regarding the evolution of radicle length during the first and second days of germination assessment, an acceleration of seed development induced by plasma-treated water was observed in the Zm70 sample. During the first four days, a pronounced increase in radicle growth was recorded, followed by a slower growth rate thereafter.

In the Zm100 sample, an increase in radicle length was observed at the first assessment of the germination process (UW = 9.9 mm; PAW = 14.1 mm). By the second day of germination evaluation, a homogenization of the values obtained for all tested experimental variants was noted.

The aspects illustrated in Figure 6 support the previously mentioned results, according to which, in maize samples with a 70% germination rate (Zm70), plasma-treated water increased the number of germinated seeds, whereas in the Zm100 sample (100%), an acceleration of seed development occurred, as evidenced by greater radicle lengths.

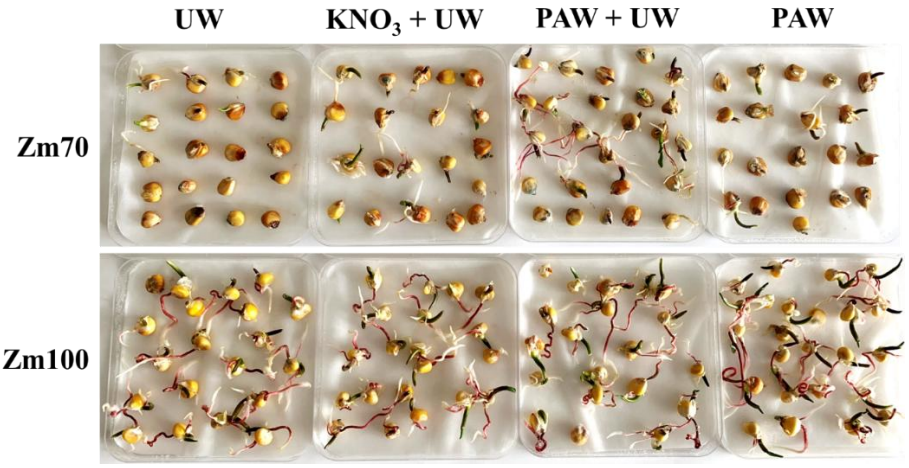


**Figure 6.** Overview of the growth plates in which *Zea mays* seeds were subjected to the germination process under four experimental variants (day 7).

(UW – untreated water, $KNO_3$ – potassium nitrate solution, PAW – plasma activated water)

Additionally, the different effects exerted by the four experimental variants on the analyzed samples can be observed in Figure 7. Thus, by selecting one individual from each sample (Zm70 and Zm100, respectively) and placing them side by side, the stimulatory effect on growth processes in the Zm70 sample becomes evident, while a relatively similar pattern of development is observed in the Zm100 sample.

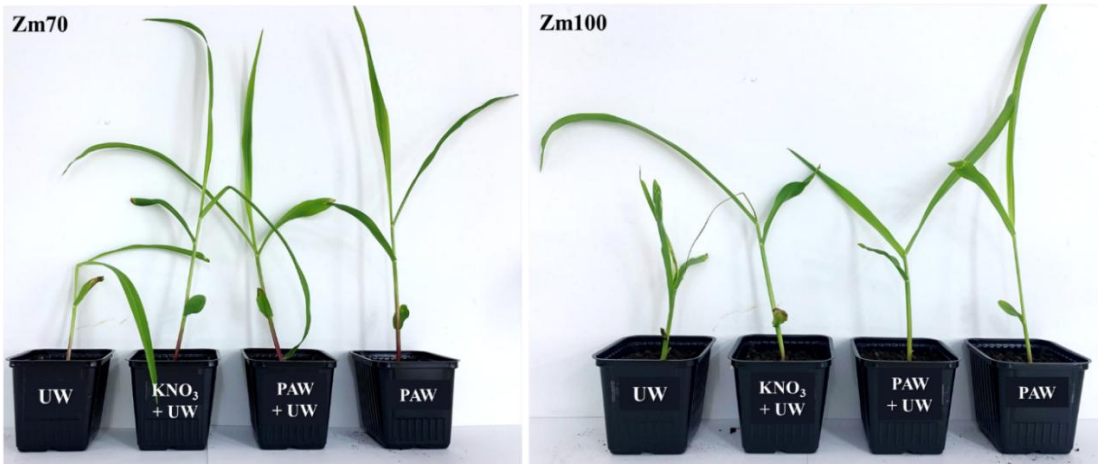


**Figure 7.** Selection of one individual from each of the four experimental variants in *Zea mays* to observe the different developmental stages on day 21 of the experiment.

By analyzing root length, shoot length, and total plant length of maize plants obtained 14 days after planting in soil, the values recorded for the Zm70 sample indicate a beneficial effect of PAW stimulation on plant development. Both root and shoot lengths in PAW-treated plants were markedly higher than those of the control group, with values approximately four times greater (e.g., shoot length: UW = 7.1 mm, PAW = 28.5 mm).

Treatment with $KNO_3$ yielded results that fall between those obtained for UW and PAW but were closer to the PAW values. This suggests that, despite the positive effects associated with $KNO_3$ treatment, the use of PAW is more efficient, with more pronounced effects.

The differences between temporary and permanent treatments are minimal, suggesting the importance of PAW application during the initial days of seed development, when the chemical compounds present in the treated water meet the seed surface.

Regarding the development of plants belonging to the Zm100 sample, the results are similar across the analyzed variants, and no stimulatory effects of potassium nitrate or plasma-treated water were observed. This can also be explained by the fact that the sample exhibited good germination capacity from the outset, making additional treatments unnecessary.

### 6.3.3. Evaluation of chlorophyll a, chlorophyll b, and carotenoid content

The results obtained from the biochemical analyses for the Zm70 sample do not show statistically significant differences. However, in the case of the Zm100 sample, statistically significant differences were observed for chlorophyll a and carotenoid content. For chlorophyll a, comparison of the value obtained under permanent PAW treatment with the control group yielded $p = 0.0486$ (*), while for carotenoid analysis, the difference between the same experimental variants was $p = 0.0376$ (*).

### 6.3.4. Evaluation of gene expression involved in the respose to plasma activated water in *Zea mays*

Analysis of gene expression in *Zea mays* reveals a moderate, time-dependent response to the applied treatments, with differentiated effects depending on the sample and developmental stage. For the **ABA8'H** gene, no statistically significant differences were identified in most analyses, except for day 21 in the Zm100 sample, when all treatment variants induced a significant decrease in expression levels. This reduction is associated with decreased

degradation of the ABA hormone and suggests the activation of adaptive and protective mechanisms, even in the absence of evident morphological differences compared to the control group.

The expression of the **CCD1** gene was very low in the Zm70 sample, with no significant variations among treatments. In contrast, in the Zm100 sample, a significant decrease in expression was observed on day 21 following all applied treatments, indicating a late regulation of the metabolic processes associated with this gene.

For the **DREB1** gene, which is involved in the response to abiotic stress, an early increase in expression was observed on day 4 of germination in both samples, being more pronounced under plasma-treated water. This stimulation was statistically significant only for the Zm100 sample. Subsequently, expression levels became uniform by day 7 and decreased markedly by day 21, suggesting a transient response specific to the early stages of development.

The expression of the **LEA** gene was not influenced by any treatment on day 4; however, on day 7, moderate stimulation was observed in the Zm70 sample for all treated variants. For the Zm100 sample, the increase in expression was induced only by potassium nitrate, while PAW treatments showed values similar to those of the control group. By day 21, the differences among variants disappeared.

The **MYB** and **MYC** genes, involved in transcriptional regulation and stress responses, were not significantly affected by the applied treatments, except for an isolated response of the MYC gene on day 21 in the Zm100 sample subjected to permanent PAW treatment. These results suggest a limited impact of the treatments on these regulatory pathways.

For the **POR** gene, which is associated with chlorophyll biosynthesis, permanent PAW treatment induced an early increase in expression on day 4 in both samples, which was statistically significant only for Zm70. At later stages, gene expression decreased, and no differences were observed among the analyzed variants.

The expression of the **PP2C** gene did not show statistically significant variations; however, different trends were observed among samples and treatments; without being consistently maintained throughout the experiment. In the case of WRKY53 gene, plasma activated water induced an early stimulation of expression, which was significant for the Zm70 sample on day 4, but this effect did not persist. For the Zm100 sample, expression levels increased on days 4 and 21 under PAW treatment, whereas on day 7 potassium nitrate induced the highest stimulation (Figure 8).

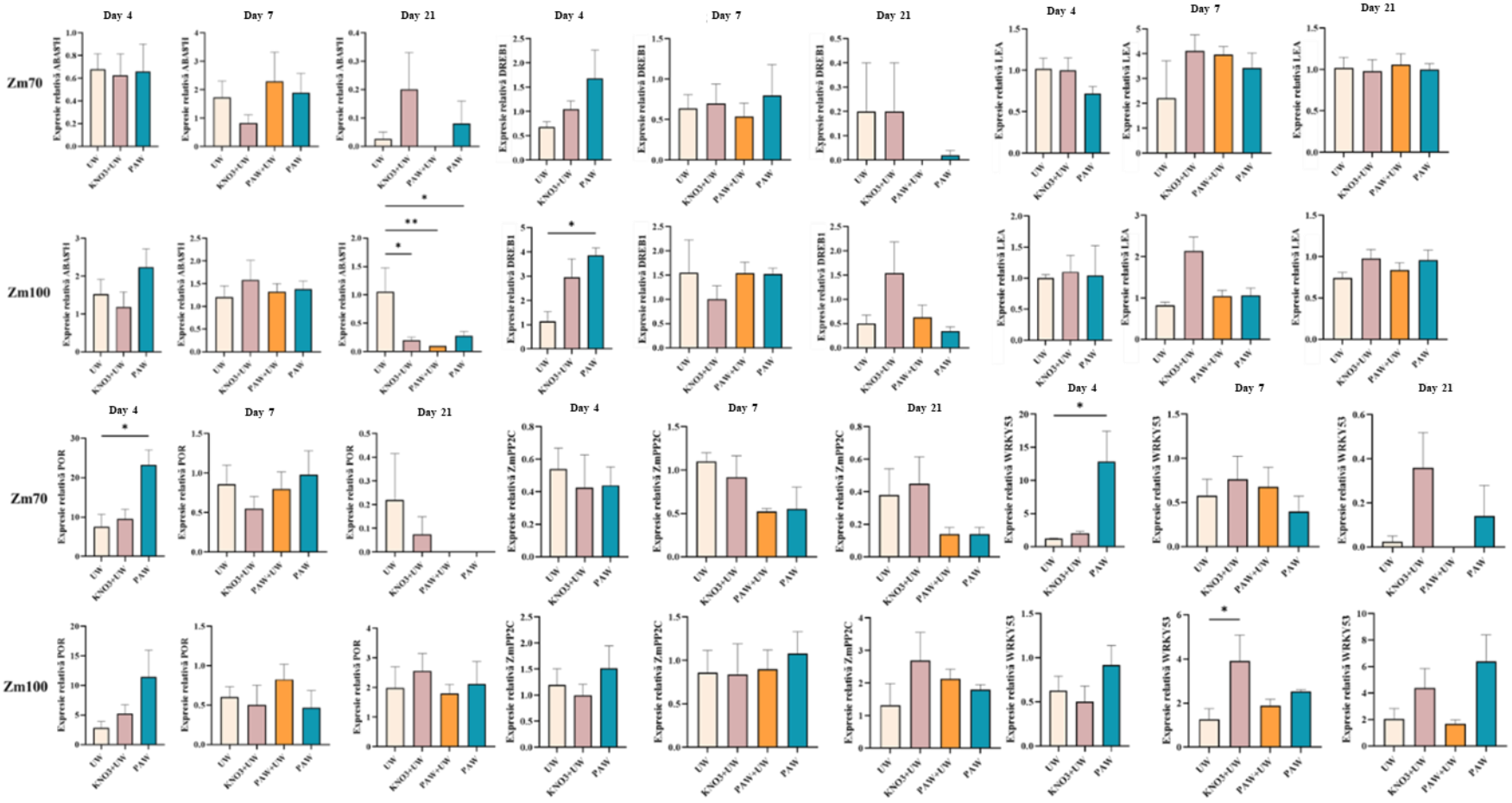


**Figure 8.** Emphasizing gene expression in sample belonging to the species *Zea mays* for a subset of the studied genes: ABA8'H, DREB1, LEA, POR, PP2C and WRKY53

(UW – untreated water, $KNO_3$ – potassium nitrate solution, PAW – plasma activated water)

### *6.4. Cucumis sativus*

#### 6.4.1. Evaluation of the germination process

Regarding the germination process of the cucumber samples, the results do not show significant differences. The differences between untreated samples and those treated with $KNO_3$ or PAW are minimal, as the untreated seeds already exhibited a high germination capacity and the applied treatments did not induce notable changes. In the Cs70 sample, the lowest values were recorded for the PAW+UW variant (3.44), whereas in the Cs100 sample, potassium nitrate treatment led to a decrease in the recorded values. This germination index was also calculated for the 10%, 25%, and 50% thresholds; however, due to the rapid germination rate of the samples, the results obtained were not considered relevant.

The dynamics of the germination process were assessed by monitoring the number of newly germinated seeds during each of the seven days of germination. For the Cs100 sample, the experimental variants accelerated developmental processes, as the seeds germinated from the first day of the experiment. Under PAW treatments, the recorded values were 1.52 and 1.56 days, respectively, whereas for the untreated seed variant the value was slightly higher, at 2 days. The lowest value was recorded for seeds treated with $KNO_3$, namely 1.44 days. In the Cs70 sample, the mean germination time was slightly reduced following temporary PAW treatment (2.92 days, compared with UW = 3.2 days); however, treatment with $KNO_3$ or permanent PAW resulted in values similar to those of the untreated variant.

#### 6.4.2. Evaluation of plant growth parameters

In the Cs70 sample, PAW treatment stimulated plant material development from the early days, with higher values recorded even in comparison with the $KNO_3$ treatment ($KNO_3$+UW = 28 ± 10.3 mm; PAW = 34.1 ± 10.8 mm; UW = 19.5 ± 7.4 mm). This trend was maintained in later stages of plant development, as on day 7 of germination the mean radicle length under PAW treatment was 16 mm higher than that recorded in the control group (PAW = 86.6 ± 35.1 mm; UW = 60.6 ± 24.6 mm). Moreover, both temporary plasma treatment and $KNO_3$ treatment resulted in plant development exceeding that of the control group (PAW+UW = 80 ± 21.4 mm; $KNO_3$+UW = 76.2 ± 22.2 mm).

The Cs100 sample exhibited uniform radicle development values on the third day of germination, with a slight increase observed under plasma-treated water (PAW = 19.3 ± 6.3

mm). On the seventh day of germination, $KNO_3$ and PAW treatments did not promote enhanced plant material development, with radicle length being even shorter compared to the control samples.

Figure 9 indicates that, in cucumber samples with an initial germination rate of 70% (Cs70), plasma-treated water increased radicle length, whereas in the Cs100 sample (100%), the most developed plants belonged to the control group.

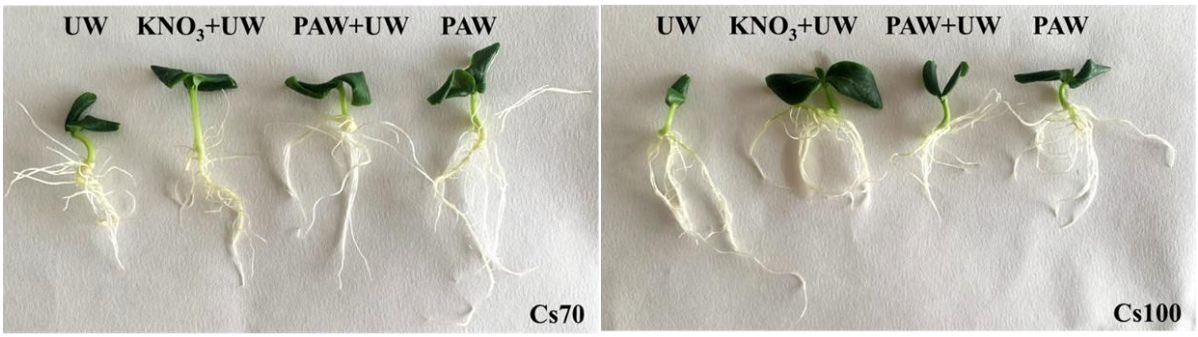


**Figure 9.** Different developmental stages of *Cucumis sativus* plants on the 7th day of germination, corresponding to the four experimental variants

(UW – untreated water, $KNO_3$+UW – potassium nitrate solution, PAW – plasma activated water)

Additionally, the different effects exerted by the experimental variants on the analyzed samples can be observed in Figure 10. Thus, by selecting one individual from each sample (Cs70 and Cs100, respectively) and placing them side by side, a stimulatory effect on growth processes is evident for the potassium nitrate treatment in both analyzed samples. For individuals belonging to the Cs70 sample, enhanced development is observed under both PAW treatments compared with the individual control. In contrast, for the Cs100 sample, PAW-treated individuals exhibit slower development compared with those from the control and potassium nitrate–stimulated variants.

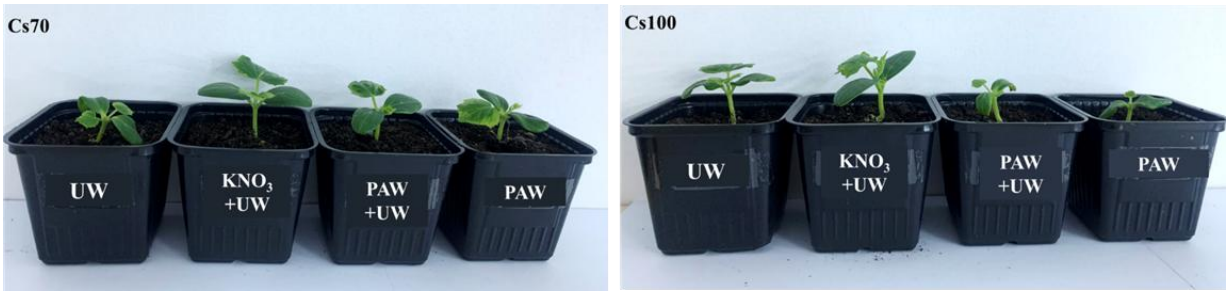


**Figure 10.** Developmental stage of *Cucumis sativus* plants on day 21 of the experiment

(UW – untreated water, $KNO_3$ – potassium nitrate solution, PAW – plasma activated water)

The results obtained on the 14th day of growth in soil (day 21 of the experiment) regarding root length, shoot length, and total plant length in the Cs70 sample indicate certain differences compared with the results recorded for radicle length on days 3 and 7. Thus, although on the final day of radicle evaluation the highest values were obtained for plants subjected to permanent PAW treatment, two weeks after planting a more pronounced shoot development was observed in plants treated with potassium nitrate (5.2 cm), followed by those subjected to temporary PAW treatment (4.4 cm), whereas permanent PAW treatment resulted in development similar to that of the control group (mean value of 3.3 cm). Within this study, the development of *Cucumis sativus* plants was not favored under in vitro conditions, which may explain why both shoot and root lengths were considerably smaller compared with the results obtained for bean and maize plants.

In the Cs100 sample, the most developed shoot and root were observed in the untreated water variant (shoot length 4.1 cm, root length 5.2 cm). Potassium nitrate treatment resulted in more pronounced development compared with both PAW treatment variants.

### 6.4.3. Evaluation of chlorophyll a, chlopohyll b and carotenoid

The results obtained from the biochemical analyses do not show significant differences among the experimental variants of the Cs70 sample, with the recorded values being similar. In contrast, for the Cs100 sample, a slight increase in chlorophyll a content was observed under potassium nitrate treatment, a result that supports the observations made on day 14 of growth in soil.

### 6.4.4. Evaluation of the expression of genes involved in the response to plasma activated water in *Cucumis sativus*

Analysis of gene expression in the Cs70 and Cs100 samples highlights a complex, development stage–dependent response to potassium nitrate and plasma-treated water (PAW) treatments. For the **ABA8'H** gene, which is associated with abscisic acid metabolism, a decrease in expression was observed on day 7 in the Cs70 sample under the influence of both treatments, followed by an increase on day 21 in the $KNO_3$+UW and PAW variants. In the Cs100 sample, PAW induced an early increase in expression on day 3, followed by a homogenization of values on day 7, while at the final stage the lowest expression level was

associated with exclusive PAW treatment, suggesting a differential regulation of hormonal pathways depending on germination potential.

The **ERF110** gene exhibited one of the most pronounced responses to the applied treatments, with significant stimulation of expression in both Cs70 and Cs100 samples. In the Cs70 sample, permanent PAW treatment induced a significant increase in expression, which correlated with reduced plant length on day 21, suggesting the activation of stress adaptation mechanisms at the expense of growth processes.

The results obtained for the **LEA2** gene indicate stimulation of expression primarily under the influence of PAW treatment, an effect that was evident on day 7 for both samples and on day 3 for the Cs100 sample. At the final stage, temporary PAW treatment was associated with the highest expression levels in Cs100, whereas in Cs70 the effect was more pronounced under permanent application, suggesting the involvement of this gene in the adaptive response to water stress.

The expression of the **MYB07** gene shows sample- and time-dependent variations, without a clearly defined general pattern. PAW treatment induces both decreases and increases in expression, particularly in the Cs100 sample, indicating a finely tuned, context-dependent regulation of transcriptional processes.

For the **POR** gene, the response differed between samples. In Cs70, the treatments did not induce stimulation of expression, with the lowest value being associated with PAW during the early stages. In contrast, in Cs100, significant increases in expression were observed on day 3 under the influence of both treatments, after which PAW continued to induce slight stimulation, particularly under temporary treatment on day 21, suggesting involvement in the regulation of photosynthetic processes.

The response of the **PP2C** gene is characterized by limited variation; however, in the Cs70 sample, an early decrease in expression under PAW treatment was observed, followed by a significant increase on day 21, indicating a late activation of stress resistance mechanisms.

The **P5CS1** gene, involved in proline biosynthesis, showed significant increases in expression particularly at the final stage of the experiment, predominantly in PAW-treated variants, in both Cs70 and Cs100 samples.

For the **WRKY2** gene, no significant variations were observed in the Cs70 sample; however, in the Cs100 sample, a progressive stimulation of expression was recorded, culminating on day 21 in a significant increase induced by permanent PAW treatment. This finding indicates a more pronounced adaptive response in plants with high germination potential (Figure 11).

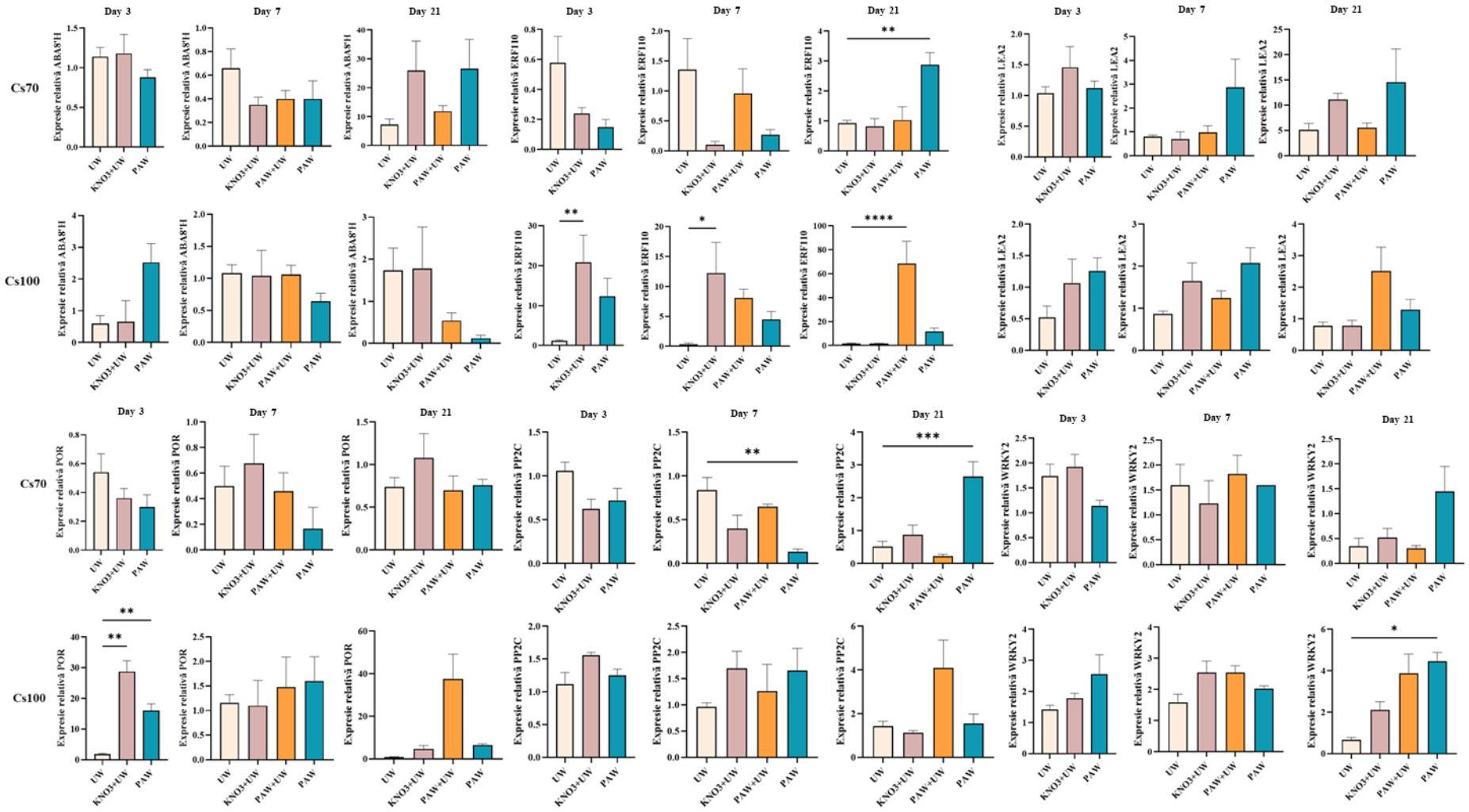


**Figure 8.** Emphasizing gene expression in sample belonging to the species *Cucumis sativus* for a subset of the studied genes: ABA8'H, ERF110, LEA2, POR, PP2C and WRKY2

(UW – untreated water, $KNO_3$ – potassium nitrate solution, PAW – plasma activated water)

## *6.5. Comparative analysis of the effects of potassium nitrate and plasma activated water on the three studied species*

### 6.5.1. Assessment of the germination process

#### *6.5.1.1. Assessment of the germination percentage*

The observed results are complex and require further studies to identify the molecular basis of the underlying processes; however, the following conclusions can be drawn. With respect to the germination percentage of seeds recorded in the internal Bank system as having a 70% germination rate (Pv70, Zm70, Cs70), both temporary and permanent plasma-treated water (PAW) treatments stimulated the number of germinated seeds. PAW treatments significantly increased the germination percentage in bean and maize compared with the control group, with effects that were superior to or comparable with those obtained using potassium nitrate. In cucumber, the germination percentage remained constant at 100%, regardless of the applied treatment.

Seeds originating from samples reported with a 100% germination rate (Pv100, Zm100, Cs100) exhibited slightly different behaviors among the three analyzed species. While in cucumber all experimental variants maintained a germination percentage of 100%, maize seeds subjected to temporary or permanent PAW treatments showed a slight decrease (96%) compared with the UW variant (100%). In the case of bean seeds, the highest germination percentage was recorded in the UW variant (92%), whereas potassium nitrate and PAW treatments induced a reduction in this value (80% and 76%, respectively).

Table 1 schematically illustrates how the experimental variants induced variations in germination percentage relative to the control group.

**Table 1.** Schematic representation of the variation in seed germination percentage for the three analyzed species following $KNO_3$ and PAW treatments, relative to the control group.

↑ = increase compared to UW, ↓ = decrease compared to UW, ~ = similar to UW

| | *Phaseolus vulgaris* | | *Zea mays* | | *Cucumis sativus* | |
|---|---|---|---|---|---|---|
| | **Pv70** | **Pv100** | **Zm70** | **Zm100** | **Cs70** | **Cs100** |
| **$KNO_3$+UW** | ↑ | ↓ | ↑ | ↑ | ~ | ~ |
| **PAW+UW** | ↑ | ↓ | ↑ | ~ | ~ | ~ |
| **PAW** | ↑ | ↓ | ↑ | ~ | ~ | ~ |

### *6.5.1.2. Assessment of the germination speed*

Evaluation of germination speed for samples with an initial germination rate of 70% (Pv70, Zm70, Cs70) highlights an overall favorable effect of plasma-treated water and potassium nitrate treatments, both of which reduced the time required for radicle protrusion. In the Pv70 sample, both temporary and permanent PAW treatments accelerated germination during the early phases of the process, as reflected by lower T10 and T50 index values. As germination progressed, the differences among variants diminished, and potassium nitrate treatment resulted in slower progression compared with the other experimental variants.

For the Zm70 sample, PAW treatments induced a clear acceleration of germination from the early stages, with the effect becoming more pronounced toward the end of the process, when T90 values were significantly lower compared with the control group. Potassium nitrate treatment also increased germination speed, yielding values even lower than those obtained under PAW treatments. In cucumber seeds, germination was rapid and efficient across all variants; however, temporary PAW treatment resulted in a slight acceleration of the process, particularly evident for the T75 and T90 indices.

For samples with an initial germination rate of 100% (Pv100, Zm100, Cs100), the treatments primarily influenced germination speed rather than germination percentage. In the Pv100 sample, all treatments reduced the mean germination time compared with the control group, with differences becoming more evident at higher percentages of germinated seeds. Similar results were observed for Zm100, where PAW treatment accelerated germination during the later stages. In the Cs100 sample, both potassium nitrate and PAW induced early acceleration of germination, an effect that diminished toward the final stages of the process (Table 2).

**Table 2.** Schematic representation of the variation in germination speed of seeds belonging to the three analyzed species following $KNO_3$ and PAW treatments, relative to the control group.

↑ = increase compared to UW, ↓ = decrease compared to UW, ~ = similar to UW

| | ***Phaseolus vulgaris*** | | ***Zea mays*** | | ***Cucumis sativus*** | |
|---|---|---|---|---|---|---|
| | **Pv70** | **Pv100** | **Zm70** | **Zm100** | **Cs70** | **Cs100** |
| **$KNO_3$+UW** | ↓ | ↑ | ↑ | ↓ | ↓ | ↑ |
| **PAW+UW** | ↑ | ↑ | ↑ | ~ | ↑ | ↑ |
| **PAW** | ↑ | ↑ | ↑ | ↑ | ~ | ↑ |

### 6.5.2. Assessment of growth parameters

#### *6.5.2.1. Assessment of radicle length*

For the Pv70 sample, radicle development did not show significant differences among the experimental variants, indicating that although PAW treatments stimulated germination percentage and speed, they did not influence radicle growth, and neither the mode of PAW application nor potassium nitrate exerted long-term effects (Table 3).

In the Zm70 sample, PAW and $KNO_3$ treatments induced significant stimulation of radicle elongation during the early stages, with the effect being more pronounced under temporary PAW treatment, which resulted in the highest radicle length values. Subsequently, the differences between temporary and permanent treatments diminished, indicating that early stimulation is decisive for initial development. In Cs70, PAW application led to a marked increase in radicle length from the first assessment, and by the end of the germination period the best results were obtained under exclusive PAW treatment, followed by PAW+UW and $KNO_3$.

For samples with an initial germination rate of 100% (Pv100, Zm100, Cs100), the effects of treatments were less pronounced. In Pv100, temporary PAW treatment resulted in the greatest radicle length values at the end of the germination period, suggesting the importance of PAW application during early stages. In Zm100 and Cs100, the initial stimulation induced by PAW diminished over time, and by the end the differences among variants were minimal.

**Table 3.** Schematic representation of the variation in radicle length for samples belonging to the three analyzed species following $KNO_3$ and PAW treatments, relative to the control group.

↑ = increase compared to UW, ↓ = decrease compared to UW, ~ = similar to UW

| **Specia** | ***Phaseolus vulgaris*** | | ***Zea mays*** | | ***Cucumis sativus*** | |
|---|---|---|---|---|---|---|
| | **Pv70** | **Pv100** | **Zm70** | **Zm100** | **Cs70** | **Cs100** |
| | Ziua 5 | | Ziua 4 | | Ziua 3 | |
| **$KNO_3$+UW** | ↑ | ~ | ↑ | ↑ | ↑ | ↓ |
| **PAW** | ↓ | ↑ | ↑ | ↑ | ↑ | ↑ |
| | Ziua 8 | | Ziua 7 | | Ziua 7 | |
| **$KNO_3$+UW** | ~ | ↑ | ↑ | ↓ | ↑ | ↓ |
| **PAW+UW** | ↓ | ↑ | ↑ | ~ | ↑ | ↓ |
| **PAW** | ↓ | ↑ | ↑ | ↓ | ↑ | ↓ |

### *6.5.2.2. Assessment of belowground and aboveground component lengths*

The development of the analyzed seedlings largely reflects the trends previously observed at the radicular level, with differential responses depending on species and germination potential. In the Pv70 sample, none of the applied treatment methods led to significant improvements in seedling growth, confirming that stimulation of germination is not automatically accompanied by accelerated vegetative development (Table 4).

For the Zm70 sample, PAW treatments had a pronounced effect on growth, inducing considerable elongation of both the aboveground parts and the root system, with the effect being more evident under temporary PAW application. $KNO_3$ and PAW+UW treatments also produced significant increases compared with the control group, supporting the beneficial role of these stimulations on the early development of maize plants.

In the Cs70 cucumber samples, the positive effects initially observed at the radicle level diminished after two weeks of growth in soil, with only moderate increases in total length being recorded, particularly under potassium nitrate treatment. For samples with an initial germination rate of 100%, the response was variable: in Pv100, PAW treatments—especially temporary application—resulted in the highest values of both belowground and aboveground components, whereas in Zm100 stimulation was evident only at the radicular level, without an effect on total plant length.

**Table 4.** Schematic representation of the variation in belowground parts, aboveground parts, and total plant length for the three analyzed species following $KNO_3$ and PAW treatments, relative to the control group.

↑ = increase compared to UW, ↓ = decrease compared to UW, ~ = similar to UW

| Species | *Phaseolus vulgaris* | | *Zea mays* | | *Cucumis sativus* | |
|---|---|---|---|---|---|---|
| | Pv70 | Pv100 | Zm70 | Zm100 | Cs70 | Cs100 |
| | | | Aboveground part | | | |
| $KNO_3$+UW | ~ | ↑ | ↑ | ↑ | ↑ | ↓ |
| PAW+UW | ↓ | ↑ | ↑ | ↑ | ↑ | ↓ |
| PAW | ~ | ↑ | ↑ | ↑ | ~ | ↓ |
| | | | Belowground part | | | |
| $KNO_3$+UW | ↑ | ↑ | ↑ | ↑ | ↑ | ↓ |
| PAW+UW | ↑ | ↑ | ↑ | ↓ | ↑ | ↓ |
| PAW | ↓ | ↑ | ↑ | ↓ | ↓ | ↓ |
| | | | Total length | | | |
| $KNO_3$+UW | ↑ | ↑ | ↑ | ~ | ↑ | ↓ |
| PAW+UW | ↓ | ↑ | ↑ | ↓ | ↑ | ↓ |
| PAW | ~ | ↑ | ↑ | ↓ | ~ | ↓ |

### 6.5.3. Assessment of chlorophyll a, chlorophyll b and carotenoid content

The applied treatments had a limited impact on the accumulation of photosynthetic pigments, with effects depending on species and germination potential. In the Pv70 sample, no increases in pigment content were observed, a result consistent with the uniform morphological development of the plants. In the Zm70 sample, $KNO_3$ and PAW treatments led to increases in chlorophylls and carotenoids, in agreement with the enhanced development of both belowground and aboveground systems. In cucumber (Cs70), pigment content remained relatively constant, with slight increases observed under temporary PAW treatment.

For samples with an initial germination rate of 100%, the treatments induced moderate variations in pigment content. In Pv100, all treated variants showed higher values than the control, with a significant increase in chlorophyll a under PAW+UW treatment. Similar results were obtained for Zm100, where permanent PAW treatment induced significant increases in chlorophyll a and carotenoids. In Cs100, pigment content was low and relatively uniform across treatments, reflecting the absence of morphological differences. Overall, although the treatments influenced germination and vegetative growth, their effects on the accumulation of photosynthetic pigments were limited (Table 5).

**Table 5.** Schematic representation of the variation in chlorophyll a, chlorophyll b, and carotenoid content in the three analyzed species following $KNO_3$ and PAW treatments, relative to the control group. Statistical data interpretation was performed using GraphPad software and one-way ANOVA analysis, with the asterisk (*) indicating a statistically significant difference compared with the control group (*$p < 0.05$).

↑ = increase compared to UW, ↓ = decrease compared to UW, ~ = similar to UW

| Species | *Phaseolus vulgaris* | | *Zea mays* | | *Cucumis sativus* | |
|---|---|---|---|---|---|---|
| | Pv70 | Pv100 | Zm70 | Zm100 | Cs70 | Cs100 |
| | | | Chlorophyll a | | | |
| $KNO_3$+UW | ↓ | ↑ | ↑ | ↑ | ↑ | ~ |
| PAW+UW | ↓ | ↑ | ~ | ↑ | ↓ | ↓ |
| PAW | ↓ | ↑ | ↑ | ↑* | ↓ | ↓ |
| | | | Chlorophyll b | | | |
| $KNO_3$+UW | ↓ | ↑ | ↑ | ↑ | ↑ | ↓ |
| PAW+UW | ↓ | ↑ | ↓ | ↑ | ↓ | ↓ |
| PAW | ↓ | ↑ | ↑ | ↑ | ↓ | ↓ |
| | | | Carotenoids | | | |
| $KNO_3$+UW | ↓ | ↑ | ↑ | ↑ | ↓ | ↓ |
| PAW+UW | ↓ | ↑ | ↑ | ↑ | ↓ | ↓ |
| PAW | ↓ | ↑ | ↑ | ↑* | ~ | ↓ |

### 6.5.4. Evaluation of the expression of genes involved in plant development and the response to oxidative stress induced by atmospheric pressure plasma activated water

The ability of plants to adapt to variable environmental conditions is closely linked to their genetic makeup, which enables both species perpetuation and the fine regulation of growth and developmental processes. Seed treatments can influence these mechanisms through changes in gene expression, generated by complex interactions with hormonal and metabolic regulatory pathways. In this context, plasma-treated water (PAW), through the reactive compounds formed during its generation, can interfere with the chemical balance of the seed and induce variations in molecular responses. In the present study, gene expression was analyzed at early stages of germination and after seedling establishment in soil, for three plant species and two sample categories with initial germination rates of 70% and 100%.

Analysis of the expression of the **ABA8'H** gene, which is involved in abscisic acid (ABA) metabolism, indicates a response that is dependent on species, sample type, and timing of evaluation. During the early days of germination, expression levels were generally similar among experimental groups, suggesting the absence of an immediate differentiated response. Subsequently, particularly in samples with an initial germination rate of 70%, significant variations were observed, associated with either increases or decreases in transcript levels, depending on the treatment applied. The decreases in expression observed at later stages, especially under permanent PAW treatments, indicate cellular accumulation of ABA and activation of adaptive mechanisms oriented toward growth deceleration and enhanced stress resistance. In samples with an initial germination rate of 100%, PAW initially induced an increase in expression, suggesting accelerated ABA degradation and facilitation of germination processes, followed by a homogenization of expression levels among experimental variants.

The **PP2C** gene, which is directly involved in ABA signaling, exhibited more subtle yet physiologically relevant changes in expression. Early increases in expression, particularly under PAW and $KNO_3$ treatments, may be associated with inhibition of ABA-dependent pathways and stimulation of growth. In contrast, the decreases observed in certain PAW-treated samples suggest the possible activation of stress-adaptive responses. At the final stage of the experiment, potassium nitrate treatment was generally associated with higher expression levels, whereas PAW induced lower or intermediate values, indicating differential effects of the two treatments on hormonal regulation.

The expression of the **LEA** gene, an important marker of the abiotic stress response, was predominantly stimulated during the early stages of the experiment, especially in the $KNO_3$- and PAW-treated variants. Subsequently, the expression pattern became heterogeneous, with differences observed both among species and among samples within the same species, indicating regulation that is specific to the physiological context and environmental conditions.

The **POR** gene, which is involved in chlorophyll biosynthesis, exhibited early stimulation of expression, particularly under the influence of PAW, suggesting an acceleration of photosynthetic differentiation processes. At later stages, the effects of the treatments were more selective; however, the results indicate that PAW can match or even surpass the effects of potassium nitrate in stimulating this process, which is essential for plant survival.

In the case of the **WRKY** gene, which is associated with stress responses and developmental regulation, expression varied considerably among species and samples, with no general pattern being identified. This variability highlights the complexity of the WRKY gene family and the need to extend analyses to a larger number of gene representatives.

Overall, the results demonstrate that plasma-treated water induces dynamic transcriptional changes that depend on the developmental stage and the germination potential of the seeds. PAW emerges as a viable alternative to potassium nitrate, capable of modulating the expression of genes involved in germination, photosynthesis, and stress responses; however, the complexity of the underlying mechanisms justifies the need for further molecular studies (Table 6).

**Table 6.** Schematic representation of how the application of $KNO_3$ or PAW resulted in increased, decreased, or unchanged expression levels of the genes of interest, relative to the control group, in the three analyzed species. Statistical interpretation of the data was performed using GraphPad software with one-way ANOVA, and the asterisk (*) indicates a statistically significant difference compared with the control group

(* $p < 0,05$, ** $p < 0,01$, *** $p<0,001$ ****, $p < 0,0001$)

↑ = increase compared to UW, ↓ = decrease compared to UW, ~ = similar to UW

/ = on day 5 for bean, day 4 for maize, and day 3 for cucumber, the PAW+UW experimental variant was not included.

| **Species** | ***Phaseolus vulgaris*** | | | | | | ***Zea mays*** | | | | | | ***Cucumis sativus*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Pv70** | | | **Pv100** | | | **Zm70** | | | **Zm100** | | | **Cs70** | | | **Cs100** | | |
| | **Day 5** | **Day 8** | **Day 22** | **Day 5** | **Day 8** | **Day 22** | **Day 4** | **Day 7** | **Day 21** | **Day 4** | **Day 7** | **Day 21** | **Day 3** | **Day 7** | **Day 21** | **Day 3** | **Day 7** | **Day 21** |
| **ABA8'H** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | ↑ | ↓ | ↑ | ↑ | ↑ | ↑ | ~ | ↓ | ↑ | ↓ | ↑ | ↓* | ↑ | ↓ | ↑ | ~ | ~ | ~ |
| PAW+UW | / | ↓ | ↓ | / | ↓ | ↓ | / | ↑ | ↓ | / | ↑ | ↓** | / | ↓ | ↑ | / | ~ | ↓ |
| PAW | ↓ | ↓* | ↓ | ↑ | ↑ | ↓* | ~ | ↑ | ↑ | ↑ | ↑ | ↓* | ↓ | ↓ | ↑ | ↑ | ↓ | ↓ |
| **CCD1** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | | | | | | | ↑ | ↑ | ~ | ↓ | ↓ | ↓** | | | | | | |
| PAW+UW | | | | | | | / | ↑ | ↓ | / | ~ | ↓** | | | | | | |
| PAW | | | | | | | ↑ | ~ | ↓ | ↓ | ~ | ↓** | | | | | | |
| **DREB1** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | ↑* | ↓ | ↑ | ↑* | ↓ | ↓ | ↑ | ↑ | ~ | ↑ | ↓ | ↑ | | | | | | |
| PAW+UW | / | ↑ | ↑ | / | ↑ | ↓ | / | ↓ | ↓ | / | ~ | ↑ | | | | | | |
| PAW | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↓ | ↑* | ~ | ↓ | | | | | | |

| Species | *Phaseolus vulgaris* | | | | | | *Zea mays* | | | | | | *Cucumis sativus* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pv70 | | | Pv100 | | | Zm70 | | | Zm100 | | | Cs70 | | | Cs100 | | |
| | Day 5 | Day 8 | Day 22 | Day 5 | Day 8 | Day 22 | Day 4 | Day 7 | Day 21 | Day 4 | Day 7 | Day 21 | Day 3 | Day 7 | Day 21 | Day 3 | Day 7 | Day 21 |
| **DREB2A** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | ↑*** | ~ | ↑ | ↑** | ↑ | ↑ | | | | | | | | | | | | |
| PAW+UW | / | ↑ | ↑ | / | ~ | ↑ | | | | | | | | | | | | |
| PAW | ↑* | ↑ | ↑ | ↑** | ↑ | ↑ | | | | | | | | | | | | |
| **DREB6B** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | ↑** | ↑ | ↑ | ↑** | ↓ | ↑ | | | | | | | | | | | | |
| PAW+UW | / | ↑ | ↓ | / | ↓* | ↑ | | | | | | | | | | | | |
| PAW | ↑** | ↑* | ↑ | ↑** | ↓* | ↓* | | | | | | | | | | | | |
| **ERF** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | ↓ | ↑ | ↑** | ↑ | ↑ | ↑** | | | | | | | ↓ | ↓ | ~ | ↑** | ↑* | ~ |
| PAW+UW | / | ~ | ↑ | / | ~ | ↑ | | | | | | | / | ↓ | ~ | / | ↑ | ↑**** |
| PAW | ↑* | ↑ | ↑ | ↑* | ↑ | ↑ | | | | | | | ↓ | ↓ | ↑** | ↑ | ↑ | ↑ |
| **LEA** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | ↑ | ↓ | ↑ | ↑ | ↑ | ↑ | ~ | ↑ | ~ | ~ | ↑ | ↑ | ↑ | ~ | ↑ | ↑ | ↑ | ~ |
| PAW+UW | / | ↓ | ↓ | / | ↑* | ↑ | / | ↑ | ~ | / | ~ | ↑ | / | ↑ | ~ | / | ↑ | ↑ |
| PAW | ↑ | ↑ | ↑ | ↓ | ~ | ~ | ↓ | ↑ | ~ | ~ | ~ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| **MYB** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | ↓** | ↑ | ↑* | ↓* | ↑ | ~ | | | | | | | ↓ | ↓ | ↑* | ↑ | ↑ | ↓ |
| PAW+UW | / | ↓ | ↑ | / | ~ | ↓ | | | | | | | / | ↓ | ~ | / | ↑* | ~ |

| Species | *Phaseolus vulgaris* | | | | | | *Zea mays* | | | | | | *Cucumis sativus* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pv70 | | | Pv100 | | | Zm70 | | | Zm100 | | | Cs70 | | | Cs100 | | |
| | Day 5 | Day 8 | Day 22 | Day 5 | Day 8 | Day 22 | Day 4 | Day 7 | Day 21 | Day 4 | Day 7 | Day 21 | Day 3 | Day 7 | Day 21 | Day 3 | Day 7 | Day 21 |
| PAW | ↓*** | ↓* | ↑ | ↓** | ↑ | ↑ | | | | | | | ↓* | ↓ | ↑ | ↑* | ↑** | ↓ |
| **MYC** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | | | | | | | ↓ | ↑ | ↑ | ↓ | ↓ | ↓ | | | | | | |
| PAW+UW | | | | | | | / | ↓ | ↓ | / | ↓ | ↓* | | | | | | |
| PAW | | | | | | | ↓ | ↓ | ↓ | ↑ | ↓ | ↓ | | | | | | |
| **POR1** | | | | | | | | | | | | | **POR** | | | | | |
| $KNO_3$+UW | ↑ | ↓ | ↑ | ↑** | ↑ | ↑ | ↑ | ↓ | ↓ | ↑ | ↓ | ↑ | ↓ | ↑ | ↑ | ↑** | ~ | ↑ |
| PAW+UW | / | ~ | ↑ | / | ↑ | ↑ | / | ↑ | ↓ | / | ↑ | ↓ | | ~ | ~ | | ↑ | ↑ |
| PAW | ↑* | ~ | ~ | ↑** | ↓* | ↑ | ↑* | ↑ | ↓ | ↑ | ↓ | ~ | ↓ | ↓ | ~ | ↑** | ↑ | ↑ |
| **POR2** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | ↑ | ↑* | ↑ | ↑** | ↑ | ↑ | | | | | | | | | | | | |
| PAW+UW | / | ↑ | ↓ | / | ↑ | ↑ | | | | | | | | | | | | |
| PAW | ↑* | ↑* | ↓ | ↑** | ~ | ↑* | | | | | | | | | | | | |
| **P5CS10** | | | | | | | | | | | | | **P5CS1** | | | | | |
| $KNO_3$+UW | ↑** | ↓ | ↓ | ↑** | ↑ | ↑ | | | | | | | ~ | ~ | ↑ | ↓ | ↓ | ~ |
| PAW+UW | / | ↑ | ↓ | / | ↓ | ↑ | | | | | | | / | ↑ | ↑* | / | ↓ | ↑ |
| PAW | ↑** | ↑ | ↑ | ↑** | ↑ | ↑ | | | | | | | ~ | ↑ | ↑*** | ↑* | ↓ | ↑* |

| Species | *Phaseolus vulgaris* | | | | | | *Zea mays* | | | | | | *Cucumis sativus* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pv70 | | | Pv100 | | | Zm70 | | | Zm100 | | | Cs70 | | | Cs100 | | |
| | Day 5 | Day 8 | Day 22 | Day 5 | Day 8 | Day 22 | Day 4 | Day 7 | Day 21 | Day 4 | Day 7 | Day 21 | Day 3 | Day 7 | Day 21 | Day 3 | Day 7 | Day 21 |
| **PP2C** | | | | | | | | | | | | | | | | | | |
| $KNO_3$+UW | ↑* | ↑ | ↑ | ↑ | ↑ | ↑ | ↓ | ↓ | ↑ | ↓ | ~ | ↑ | ↓ | ↓ | ↑ | ↑ | ↑ | |
| PAW+UW | / | ↓ | ↑ | / | ↓ | ↑ | / | ↓ | ↓ | / | ~ | ↑ | / | ↓ | ↓ | / | ↑ | ↑ |
| PAW | ↑** | ↓ | ↑ | ↑ | ↓ | ↓ | ↓ | ↓ | ↓ | ↑ | ↑ | ↑ | ↓ | ↓** | ↑*** | ↑ | ↑ | ↑ |
| **WRKY53** | | | | | | | **WRKY53** | | | | | | **WRKY2** | | | | | |
| $KNO_3$+UW | ↓ | ↑ | ↑ | ↓ | ↑ | ↑ | ↑ | ↑ | ↑ | ↓ | ↑ | ↑ | ↑ | ↓ | ↑ | ↑ | ↑ | ↑ |
| PAW+UW | / | ↑ | ↓ | / | ↑ | ↑ | / | ↑ | ↓ | / | ↑ | ~ | / | ↑ | ~ | / | ↑ | ↑ |
| PAW | ↓ | ~ | ↓ | ↓ | ↑ | ↑ | ↑* | ↓ | ↑ | ↑ | ↑ | ↑ | ↓ | ~ | ↑ | ↑ | ↑ | ↑* |

# CONCLUSIONS

Based on the analysis of **literature studies** regarding the effects of plasma on the species of interest, the following conclusions can be established:

- ***Phaseolus vulgaris***
  - ✓ Modification of the seed coat, both at the macroscopic level, through wrinkling of the outer layer, and at the microscopic level, through the appearance of erosion areas that facilitate increased water uptake;
  - ✓ Hydrophilization of the seed coat, as demonstrated by a decrease in the water contact angle on the seed surface;
  - ✓ Stimulation of germination and plant development processes, reflected by increases in germination percentage as well as in radicle, shoot, and root length.

- ***Zea mays***
  - ✓ Seed decontamination, with effects against various fungal and bacterial species;
  - ✓ Scarification of the seed coat, as evidenced by scanning electron microscopy analyses;
  - ✓ Reduction of the water contact angle, demonstrating increased water absorption;
  - ✓ Stimulation of germination and development processes, through increased germination percentage and greater plant length;
  - ✓ Stimulation of the expression of genes involved in the synthesis of heat shock proteins.

- ***Cucumis sativus***
  - ✓ Decontamination of plant material, through a reduction in total microbial load for various yeast and mold species;
  - ✓ Alteration of functional chemical groups on the seed surface;
  - ✓ Decrease in the water contact angle, indicating enhanced water absorption;
  - ✓ Stimulation of germination and plant development processes, reflected by increased germination percentage, germination speed, and plant length.

**The experiments and analyses conducted within this thesis** lead to the following conclusions:

- ***Phaseolus vulgaris,* Pv70**
  - ✓ $KNO_3$ and PAW treatments increased the germination percentage, by up to 20 percentage points in the PAW experimental variant, and a slight increase in germination speed was observed under PAW+UW and PAW treatments;
  - ✓ No changes were observed with respect to radicle length or the length of belowground and aboveground plant components;
  - ✓ No modifications were reported in chlorophyll a, chlorophyll b, or carotenoid content;
  - ✓ At the molecular level, the most relevant differences induced by the proposed treatments were as follows: on day 5, statistically significant variations compared with the UW control were recorded, including a decrease in MYB gene expression ($KNO_3$+UW** and PAW***), and increased expression of the genes DREB1 ($KNO_3$+UW*), DREB2A ($KNO_3$+UW***, PAW*), DREB6B ($KNO_3$+UW**, PAW**), ERF (PAW*), POR1 (PAW*), POR2 (PAW*), P5CS10 ($KNO_3$+UW**, PAW**), and PP2C ($KNO_3$+UW*, PAW**). On day 8, statistically significant decreases were observed for the ABA8'H (PAW*) and MYB (PAW*) genes, while increased expression levels were recorded for DREB6B (PAW*) and POR2 ($KNO_3$+UW*, PAW*). On day 22, no significant decreases in gene expression were detected; only increases were observed for the ERF ($KNO_3$+UW**) and MYB ($KNO_3$+UW*) genes

- ***Phaseolus vulgaris,* Pv100**
  - ✓ PAW treatment markedly increased seed germination speed;
  - ✓ Radicle length on day 8 of germination was greater by 8.9 mm under $KNO_3$ treatment, by 17 mm under PAW+UW, and by 12 mm under PAW, compared with the control plants;
  - ✓ Total plant length after 14 days of growth in soil was approximately 1.5-fold higher under PAW compared with UW (total length: PAW = 28.7 mm; UW = 17.8 mm). In addition, $KNO_3$ (total length = 27.8 mm) and PAW+UW (total length = 30.2 mm) treatments also stimulated plant development;
  - ✓ Chlorophyll a content was significantly increased in the PAW+UW variant compared with the UW control, whereas chlorophyll b and carotenoid contents showed slight increases following $KNO_3$ and PAW treatments, without statistical significance;

- ✓ At the molecular level, numerous statistically significant variations relative to the UW control were recorded, as follows: on day 5, a decrease in expression was observed only for the MYB gene ($KNO_3$+UW*, PAW**), while increased expression was recorded for DREB1 ($KNO_3$+UW*), DREB2A ($KNO_3$+UW**, PAW**), DREB6B ($KNO_3$+UW**, PAW**), ERF (PAW*), POR1 ($KNO_3$+UW**, PAW**), POR2 ($KNO_3$+UW**, PAW**), and P5CS10 ($KNO_3$+UW**, PAW**); on day 8, statistically significant decreases were recorded for DREB6B (PAW+UW*, PAW*) and POR1 (PAW*), while a significant increase was observed for the LEA gene (PAW+UW*); on day 22, the ABA8'H (PAW*) and DREB6B (PAW*) genes showed significant decreases relative to the UW control, whereas increased expression levels were observed for ERF ($KNO_3$+UW**) and POR2 (PAW).

- ***Zea mays,* Zm70**
  - ✓ The experimental variants $KNO_3$+UW, PAW+UW, and PAW increased the germination percentage by 14% and reduced the time required for seed germination;
  - ✓ The applied treatments enhanced radicle length. On day 4, the highest value was recorded for $KNO_3$+UW (8.5 mm, compared with UW = 5 mm), while on day 7 the maximum value was observed for PAW+UW (23 mm), representing an increase of 15 mm relative to the control group (UW = 7.16 mm)
  - ✓ Stimulation of developmental processes in $KNO_3$/PAW-treated samples persisted at more advanced stages of plant development. The results indicate a difference of 34.3 cm in total plant length between PAW+UW and UW (PAW+UW = 44.5 cm; UW = 10.2 cm), 19.2 cm between $KNO_3$+UW and UW ($KNO_3$+UW = 29.4 cm), and 28.7 cm between PAW and UW (PAW = 38.9 cm)
  - ✓ No significant differences were recorded with respect to the content of photosynthetic pigments;
  - ✓ Gene expression results indicate, on day 4, statistically significant increases for the POR (PAW*) and WRKY53 (PAW*) genes, and a statistically significant decrease on day 21 for the MYC gene (PAW+UW*), compared with the UW control.

- ***Zea mays,* Zm100**
  - ✓ A slight reduction in the time required for germination was observed for the exclusive PAW treatment variant;

- ✓ Regarding radicle length, an increase was recorded on day 4 under PAW treatment; however, by day 7 the values became uniform across experimental variants, a trend that was also observed on day 21 from the onset of the experiment;
- ✓ Chlorophyll a and carotenoid contents were significantly increased in the PAW experimental variant compared with the UW control, suggesting more pronounced development of these samples;
- ✓ At the genetic level, statistically significant variations were recorded on day 4 of the experiment, manifested as increased expression of the DREB1 gene (PAW*), and on day 21, as decreased expression of the ABA8'H ($KNO_3$+UW*, PAW+UW**, PAW*), CCD1 ($KNO_3$+UW**, PAW+UW**, PAW**), and MYC (PAW+UW*) genes.

- ***Cucumis sativus,* Cs70**
  - ✓ The germination percentage was 100% for all experimental variants, and germination speed was not substantially influenced by the applied treatments;
  - ✓ Permanent PAW treatment induced the greatest increase in radicle length on both day 3 and day 7 of evaluation. Temporary PAW and $KNO_3$ treatments also resulted in increased radicle length compared with the control group;
  - ✓ Fourteen days after planting in soil, a reduction in the effect of PAW on radicle development was observed. Total plant length was highest under $KNO_3$ treatment, followed by PAW+UW, PAW, and finally the UW control;
  - ✓ No significant differences were recorded in the content of photosynthetic pigment;
  - ✓ At the molecular level, the following variations in gene expression relative to the UW control were observed: on day 3 of the experiment, a decrease in MYB gene expression (PAW*); on day 7, a reduction in PP2C expression (PAW**); and on day 21, increased expression of the ERF (PAW**), MYB ($KNO_3$+UW*), P5CS10 (PAW+UW*, PAW***), and PP2C (PAW***) genes.

- ***Cucumis sativus*, Cs100**
  - ✓ The germination percentage was 100% for all experimental variants, and germination speed was slightly increased by $KNO_3$ and PAW treatments;
  - ✓ Although PAW treatment induced an increase in radicle length on day 3 of evaluation, this effect was not maintained over the long term;

- ✓ The applied treatments did not result in increases in plant length beyond the values recorded for the control group;
- ✓ The applied treatments did not result in increases in plant length beyond the values recorded for the control group;
- ✓ At the molecular level, no statistically significant decreases in gene expression were observed in $KNO_3$- or PAW-treated samples compared with the UW control; instead, only increases in expression were detected, as follows: on day 3 for the ERF ($KNO_3$+UW**), MYB (PAW*), POR ($KNO_3$+UW**, PAW**), and P5CS1 (PAW) genes; on day 7 for the ERF ($KNO_3$+UW*) and MYB (PAW+UW*, PAW**) genes; and on day 21 for the ERF (PAW+UW****), P5CS1 (PAW*), and WRKY2 (PAW*) genes.

Thus, the obtained data support the hypothesis that plasma-treated water can represent an effective method for stimulating physiological processes associated with germination and subsequent plant growth. The morphological observations are largely supported by variations in the expression of the genes of interest, and further studies will allow the identification of more specific mechanisms through which the chemical compounds in PAW exert their beneficial effects at different stages of plant development.

**Publications related to the doctoral thesis:**

1. Leti, L.-I.; Gerber, I.C.; Mihaila, I.; Galan, P.-M.; Strajeru, S.; Petrescu, D.-E.; Cimpeanu, M.-M.; Topala, I.; Gorgan, D.-L. The Modulatory Effects of Non-Thermal Plasma on Seed's Morphology, Germination and Genetics—A Review. Plants 2022, 11, 2181. https://doi.org/10.3390/plants11162181;
2. Galan, P.-M.; Strajeru, S.; Murariu, D.; Enea, C.-I.; Petrescu, D.-E.; Tanasa, A.-C.; Blaga, D.-D.; Leti, L.-I. The Effect of Plasma-Activated Water on Zea mays L. Landraces Under Abiotic Stress. Agriculture 2025, 15, 2037. https://doi.org/10.3390/agriculture15192037

**National scientific conferences:**

1. Scientific Conference of the Faculty of Biology, Poster: „Study on the Effects of Atmospheric-Pressure Non-Thermal Plasma-Activated Water Treatment on Germination and Development Processes in *Phaseolus vulgaris* L.", Authors: Leți Livia-Ioana, Galan Paula-Maria, Gorgan Dragoș-Lucian, 27-28.10.2023;

2. Scientific Communications Session BRGV 2023, Oral presentation: „Study on the Effects of Plasma-Activated Water Treatment on Germination and Development Processes in *Phaseolus vulgaris* L.”, Autori: Leți Livia-Ioana, Galan Paula-Maria, Gorgan Dragoș-Lucian;
3. Sesiunea de Comunicări Științifice BRGV 2024, Oral presentation: „Study on the Effects of Atmospheric-Pressure Plasma Treatments on Germination and Development Processes in Plants of Economic Interest”, Authors: Leți Livia-Ioana, Galan Paula-Maria, Gorgan Dragoș-Lucian.

**Member of National and International Research Projects:**

1. 07/2023 – 08/2025, Project Director, ADER 1.3.4.: „Study on the Effects of Plasma-Activated Water Treatments on Germination at Low and Standard Temperatures and on the Development of *Zea mays*, Based on Morphological, Biochemical, and Gene Expression Assessments”. National Project
2. 09/2020 – 08/2025, Project Member „INCREASE – Intelligent Collections of Food Legumes Genetic Resources for European Agrofood Systems”. European Project

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