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par

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Preface

Sometimes in life, we stumble into serendipitous circumstances which only reveal later in time what an impact they will have on us; at least that is how I would describe my journey with biological applications of non-thermal plasmas with my beginnings at ETH Zurich. With a formal background in microbiology, I was looking for a food science project to try out of curiosity and happened to choose a project from a long list; the inactivation of bacteria on sprout seeds using non-thermal plasmas. Unexpectedly over time, I became more curious about how the seeds and plants 'felt' after plasma exposure, especially during my Master thesis. What I did not expect was how strongly this interest would later captivate my mind. It suddenly hit me, late in the evening as I was writing my Master thesis, a feeling of satisfaction followed by sadness. Realizing how little was known about this new field called plasma agriculture and that my project was coming to an end, it was then that I considered working on this topic for a longer period of time, however, I still was not entirely sure if it would be possible. A few months after the Master thesis, in my tiny dorm room in Zurich before bed, I couldn't shake off a stream of ideas that suddenly came to me out of the blue and forced me to open my laptop and write them down. In that moment, I knew without a doubt that I now needed to find a way to work on this topic but where?

Surprisingly, it didn't take long to answer this question. After seeking the counsel of a respected individual, I decided to reach out with my proposal and ideas to my current supervisor without knowing that the Swiss Plasma Center had just received a generous infrastructure grant and that they were open to embracing a new research direction; impeccable timing to say the least. I knew I did not fully comprehend the challenges that would come from building a bioplasma lab and interdisciplinary project from the ground up. However, the vision I had in my mind and a very odd sense of certainty convinced me to commit and at times, was the only thing that made me persist through challenging times. Dr. Basil Duval had given me wise advice during my interview and I paraphrase his words here: you need a reason to do research because when, not if, you are thinking of giving up, you need to remind yourself why you started this in the first place. His words echoed in my mind several times throughout the four years and although I had feelings of doubt momentarily and a lack of results initially, I refused to give up.

Admittingly, there were moments where I thought things should have gone differently but as I look back now as I write this preface, I wouldn't change anything about the process; it all makes sense in hindsight. Intuition is a funny thing. Trusting the process is not easy. It likely will not go as you originally planned and you will likely be blindsided along the way, yet somehow, it all works out. Sometimes, even better than you imagined.

To the four who gave me life and the one who set me free

"Can it be done, Father? Can a man change the stars?"

"Yes, William. If he believes enough, a man can do anything."

- A Knight's Tale

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Acknowledgements

People come into your life for a moment, a day, a month, a year, or a lifetime. It matters not the time they spent with you but how they impacted your life in that moment. Whether it was perceived as a pleasant or challenging interaction or experience, all of it contributes to your growth and advancement.

I would like to thank my supervisor, Prof. Ivo Furno, for being open-minded and agreeing to begin this adventure with me. He was always optimistic and always did his best to acknowledge the difficulties of being an interdisciplinary student; in my case, a biologist surrounded by physicists and engineers. This understanding certainly was helpful as I was growing through the process. Because of this interdisciplinary project, working and discussing with others who are different from me enabled me to pick up on their analytical and problem-solving skills and it enriched my way of thinking. Thank you Ivo for the freedom and the trust in me to design and build a bioplasmas lab, as well as, my own project in plasma agriculture.

I realize that the final decision for beginning this new research direction and ensuring the generous funding remained with Prof. Ambrogio Fasoli. I still remember with great clarity, sitting across from him in his office and being asked whether I am a pioneer during my interview, to which I firmly said yes. Although no one could have known with certainty how things would work out, I want to thank you for taking this risk anyway. It gave me the opportunity to prove that I am a pioneer and it will provide others the opportunity to work on biological applications of plasmas in now one of the few labs in the world equipped with plasma sources, diagnostics, and biology laboratory equipment.

I would also like to thank Prof. Ambrogio Fasoli, Dr. Milan Simek, and Prof. Milvia De Miccolis who have agreed to be the examiners. I appreciate your willingness to take the time to evaluate this thesis. Likewise, thank you to Prof. Paolo De los Rios for being the president of my private exam and thank you to my candidacy committee, Dr. Stefano Alberti, Dr. Paolo Angelino, and Prof. Andy Oates, for your feedback during the exam and annual meetings.

I would like to thank Dr. Alan Howling for being unofficially like a second supervisor to me. He asked me questions which made me reflect, shared his observations, read through numerous manuscripts, often several times, and provided detailed feedback wherever possible. Not only did this help me grow scientifically, but it was also a pleasant experience. I could easily talk to him and always enjoyed his ability to insert song lyrics into conversations, especially from the Beatles. Even when I had my doubts, I could quickly bounce my ideas off of him, which would help me come to a decision. Discussing with him also gave me a sense of reassurance that I was progressing with my project and every interaction left me more clarity. I am very grateful that I had the opportunity to work with him right before his retirement. This journey undoubtedly would not have been the same without you, Alan.

I would like to thank the past and present group members of BPPA. Dr. Matthias Van Gompel was there at the beginning alongside me. He was always supportive and there to listen to me whether it was about building the lab, designing the DBD reactor, or giving me personal advice. Matthias, I have always felt that you had my back, then and now, and that is a wonderful gift to have from a coworker. Dr. Gennady Plyushchev helped me scientifically, technically, and personally. Gennady, you're incredibly gifted with hands-on tasks and were always willing to help me, whether it was to lift heavy objects, design or find DBDs, or help me take DBD measurements. We were able to vent our frustrations but not for too long before having a good laugh. I would like to thank Remy Jacquier for also being there for me. Although he was not directly involved in the bioplasmas lab, he would help out group members with different tasks, i.e. when the tables for the biolab arrived and needed to be assembled using tools or building the loadlock for the Sterilux ozone machine. Remy, you've been a great friend to me that has helped me see things from a different perspective at critical times in my life. I would like to thank Lorenzo Ibba, Rita Agus, Dr. Fabio Avino and now, Dr. Brayden Myers for hopping onboard this bioplasma adventure. I am happy that they are continuing to keep the lab and projects alive in their own way. Thank you Lorenzo and Max Leftley for your work with the power and FTIR measurements. Thank you Marion von Allmen for your work on the humidity and DBD topic. I want to thank all BPPA group members for the memories, like Dr. Marcelo Ruiz Baquero for the nice discussions on the metro in pre-COVID times, Dr. Riccardo Agnello for the electric field simulations and entertaining conversations during our short breaks between intense work, Dr. Christine Stollberg and Dr. Jia Han for the constructive inputs during my rehearsals, and Dr. Fabian Manke for his help with the assistantships.

I would like to thank all members of the Swiss Plasma Center for the nice working atmosphere and social events, despite the fact that I spent most of my time dwelling in the basement (by choice and by my own suggestion to have the lab in the basement sorry). Thank you to Edith Grueter, Roxane Tschanz, and Thushi Poopalasundram

for always handling my order invoices for the lab and papers since there were certainly many in 2018 and 2019. Thank you to all those who helped me with my seminar apero and to all those who helped me survive physics assistantships, especially in the beginning. You managed to keep me just below the critical stress threshold.

I would like to thank members of the Goloubinoff group and the DBMV at the University of Lausanne for their support wherever it was possible. Prof. Pierre Goloubinoff, thank you for your advice and for being a true scientist who is incredibly objective. You have shown me a standard to which all scientists should aim and work towards. I will always remember your captivating storytelling and of course, that the data is sovereign. Thank you Dr. Anthony Guihur for your bioinformatics skills for the RNA sequencing studies and for answering my plant biology questions throughout the years. Thank you Dr. Baptiste Bourgine for being the first to welcome me into the DBMV community and for teaching me the techniques used in the group. Thank you Dr. Elia Stahl for the great scientific discussions and help with the bioassays and qPCR. Your scientific curiousity and confidence made it absolutely brilliant to work with you even though it was regrettably limited due to my project constraints. Thank you to all of the other members, specifically Dr. Raphael Groux, Dr. Esteban Alfonso, Dr. Jules Deforges, Dr. Pauline Anne, and Dr. Moritz Graeff for the scientific advice as I was treading through this new world of plants. Thank you to the rest of the members in the DBMV for the nicely organized events.

I would like to thank Dr. Paolo Ambrico. Our interactions started in 2020 because of the extensive troubleshooting for my project. I was searching for answers in the literature and through trial-and-error, but discussions with you were exactly what I needed to overcome the hurdles in a timely manner. I am certain that the turning point for my project was through our interactions over Zoom and my visit to your lab for two weeks. I am very grateful to you, specifically for the chance to collaborate, design a parametric study, and publish together. Your infectious, positive attitude also gave me a renewed sense of hope when I most needed it.

Thank you to the people I have worked with in the facilities at UNIL and EPFL, especially Pierre Mettraux (rest in peace) for the XPS, Antonio Mucciolo for the SEM-EDX, Dr. Hannes Richter and Dr. Johann Weber for the RNA quality control and sequencing. Many of the results in this thesis would not have been here without your skills. Thank you to Jeremie Dubray, Steve Couturier, Damien Fasel, and Thierry Leresche for the power supply and electrical support, Robert Bertizzolo for your help with the reactor design and manufacturing, and Blaise Marletaz and Coralie Beuchat for handling our first generation SDBDs. Thank you Dr. Patrick Blanchard for the discussions which helped me develop the safety documents and procedure for the biolab. Thank you to Christian Moura for building parts of the lab infrastructure and Pierre Etienne for the IT support.

Thank you to my EDBB mentor. I resonated with his career journey and it turned out to be a great match. You have given me meaningful advice and I always left uplifted from our conversations. Although our interactions were not frequent, they undoubtedly had a positive impact on me, especially at trying moments.

Thank you to Dr. Denis Butscher, Dr. Markus Schuppler, Dr. Gina Oberbossel, and Prof. Philipp Rudolf von Rohr who I worked for or alongside during my Master in Zurich. I had not realized how much I learned during my time with you until I embarked on my own journey. Although I pursued a different direction for my thesis, I carried forward my knowledge and experiences with me from Zurich to Lausanne, which set my foundation and provided me with a sense of direction.

Thank you to all of the friends that I have made throughout these years and to those that were in my life before, especially from Zurich. Through being part of ADSV, BSNL, EDBB mini-symposia, casually PolyDoc, surprisingly an intensive French course, and through playing badminton and tennis, I have met proactive and kind people. I share great memories with you and you have brought smiles and laughs into my life.

Above all, thank you to my family. To my cat (rest in peace), you were in my life since I was a child, and were almost like a sibling to me. I would imagine equally moody as a human sibling except very small, furry, and bound by a limited vocabulary. I was hoping that you would still be here at the end of my thesis. Nevertheless, you never failed to increase my endorphin levels and make me smile. To my absolutely wonderful parents, I never imagined that I would find myself here but I know with certainty that I wouldn't have made it this far without you. As I have gone off on my own, first to Zurich and now Lausanne, I have been able to better appreciate all that you have done for me and yet you still continue to support me and believe in me. I do not take this for granted, especially since none of us ever knows what idea I am going to come up with next. Thank you for your continuous encouragement, love, and for giving me the freedom to explore this world and find my way and place in it.

Lausanne, August 8, 2022

From little seeds grow mighty trees.

A seed neither fears light nor darkness, but uses both to grow.

Abstract

The motivation driving plasma-seed treatment research is the renewed importance of sustainable, eco-friendly agriculture. There is a constant interest in finding alternatives to minimize resource use and environmental degradation, while ensuring healthy seed and plant development. Plasma agriculture is being intensively investigated since many laboratories and industries see the potential of plasma-seed treatments. However, the lack of understanding of the mechanisms and lack of standardized protocols has created a situation where interdisciplinary groups work with their own customized setup. Often times, the setup is developed through trial-and-error, and consequently, the process is not shared publicly. This thesis, therefore, addresses both the mechanisms and the design of plasma-seed treatments.

For identifying the mechanisms, a short study was first designed to better understand the effect of plasma and its separate components, heat and ozone. This revealed that plasma is unique in its ability to effectively change the seed surface within a short time frame, which was not observed with heat or ozone alone. Next, the plasma conditions were then established for accelerated germination in *Arabidopsis thaliana* seeds, a plant model organism. These plasma treatment conditions were then analyzed using *in situ* FTIR to determine the plasma gas chemistry and differences between parameter changes. The results suggest that it is likely short-lived species, such as NO, which are responsible for the effect, although more studies need to be conducted. To understand how the plasma affects the seed and its subsequent development, RNA sequencing was used and it revealed upregulated plant stress and defense responses. Depending on the plasma treatment time and extraction time point, genes were differentially expressed in the phenylpropanoid or glucosinolate pathways. From this information, a tentative hypothesis was proposed; plasma exposure could be interpreted as a wounding and oxidative stress.

In terms of plasma-seed treatment design, each variable concerning the biological and physical aspects of this interdisciplinary field is mentioned to raise awareness and explain the relevance. This information was compiled into guidelines for the entire community in hopes that data collection can be done systematically and lead to a meta-analysis in the future. Additionally, specific diagnostics were explored and recommended based on their relative merits. For example, plasma-treated seed surfaces were analyzed and compared using SEM, EDX, XPS, AFM, and ATR-FTIR. A similar comparison was done for the plasma gas chemistry, which revealed that FTIR is mainly useful for long lifetime species and other diagnostics, such as LIF, are more suitable for short lifetime species and are therefore complementary. The importance of analyzing multiple variables through a parametric study was discovered and recommended as an approach for others to follow suit in order to disentangle the complex number of variables in these treatments and minimize the risk of false interpretations and conclusions.

Additional studies are needed to understand how to design experiments with reproducible and consistent results from plasma-seed treatments using a systematic approach. My hope is that this work will be the foundation for future studies.

Keywords: Non-thermal plasma, seeds, *Arabidopsis thaliana*, germination, surface analysis, mechanisms, protocol, FTIR, RNA sequencing

Résumé

La motivation qui anime la recherche sur le traitement des semences par plasma est l'importance renouvelée d'une agriculture durable et respectueuse de l'environnement. Il y a un intérêt constant à trouver des alternatives pour minimiser l'utilisation des ressources et la dégradation de l'environnement, tout en assurant un développement sain des semences et des plantes. L'agriculture au plasma fait l'objet d'études intensives car de nombreux laboratoires et industries voient le potentiel des traitements des semences au plasma, cependant, le manque de compréhension des mécanismes et le manque de protocoles standardisés ont créé une situation où des groupes interdisciplinaires travaillent avec leur propre configuration personnalisée. Cette thèse aborde donc les mécanismes et la conception des traitements plasma-semences.

Pour identifier les mécanismes, une courte étude a d'abord été conçue pour mieux comprendre l'effet du plasma et de ses composants séparés, la chaleur et l'ozone, qui a révélé que le plasma est unique dans sa capacité à modifier efficacement la surface de la graine dans un court laps de temps, ce qui n'était pas le cas observé avec la chaleur ou l'ozone seul. Ensuite, les conditions du plasma ont ensuite été établies pour une germination accélérée des graines d'*Arabidopsis thaliana*, un organisme modèle végétal. Ces conditions de plasma ont ensuite été analysées à l'aide de FTIR in situ pour déterminer la chimie du gaz plasma et les différences entre les changements de paramètres. Les résultats suggèrent que ce sont probablement des espèces à courte durée de vie, telles que le NO, qui sont responsables de l'effet, bien que d'autres études doivent être menées. Pour comprendre comment le plasma affecte la plante, le séquençage de l'ARN a été utilisé et a révélé que le stress et les réponses de défense des plantes sont régulés positivement; gènes spécifiquement dans les voies des phénylpropanoïdes et des glucosinolates en fonction du temps. Une hypothèse provisoire est proposée; l'exposition au plasma pourrait être interprétée comme une blessure en plus d'un stress oxydatif.

En termes de conception de traitement de semences plasma, chaque variable concernant les aspects biologiques et physiques dans ce domaine interdisciplinaire est mentionnée à la fois pour sensibiliser et expliquer la pertinence. Ces informations ont été compilées dans des lignes directrices pour l'ensemble de la communauté dans l'espoir que la collecte de données puisse être effectuée de manière systémique et conduire à une méta-analyse à l'avenir. De plus, des diagnostics spécifiques ont été explorés et recommandés en fonction de leurs mérites. Par exemple, les surfaces de semences traitées au plasma ont été analysées et comparées. Une comparaison similaire a été effectuée pour la chimie du gaz plasma, qui a révélé que le FTIR est principalement utile pour les espèces à longue durée de vie et que d'autres diagnostics tels que le LIF sont plus adaptés aux espèces à courte durée de vie et sont donc complémentaires. L'importance d'analyser plusieurs variables par le biais d'une étude paramétrique a été découverte et recommandée comme approche à suivre par d'autres afin de démêler le nombre complexe de variables dans ces traitements et de minimiser le risque de fausses interprétations et conclusions.

Des études supplémentaires sont nécessaires pour comprendre comment concevoir des résultats reproductibles et cohérents à partir de traitements plasma-graines en utilisant une approche systématique utilisant simultanément le plasma et les paramètres des graines.

Mots-clés: Plasma non-thermique, graines, *Arabidopsis thaliana*, germination, analyse de surface, mécanismes, protocole, FTIR, séquençage d'ARN

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Chapter 1

Biological applications of non-thermal plasma

1.1 Aims of the thesis

The personal motivation for this research was to contribute towards the development of a plasma treatment for agriculture, specifically a seed pre-treatment which improves plant health, in order to minimize pesticide use. To facilitate its transfer into industry, the underlying mechanisms of how plants respond to non-thermal plasma treatments first need to be better understood.

Currently, molecular studies of plasma-treated seeds are largely lacking and the interpretation of the results is further complicated by the plethora of variables in the plasma-seed treatment. This is because different plasma sources are used with varying treatment styles, geometries, electrical characteristics, or operating parameters, such as power supply, gas type, and treatment time to name a few. This, in turn, makes it challenging to compare studies and calls for the use of standardized conditions and protocols. To a degree, this standardization is required to address the ambiguity surrounding plasma-seed mechanisms.

Therefore, the overarching goal of this thesis was to better understand the mechanisms of plasma-seed treatments using a systematic approach. This entailed the design and establishment of a bioplasmas lab, as well as, the infrastructure and operating parameters of a plasma-seed treatment which affects a macroscopic property of *Arabidopsis thaliana* seeds.

After the infrastructure for the bioplasmas lab was established, the following aims were investigated:

The **first aim** was to construct a plasma device, which ignited at sufficiently low temperatures for biological substrates, and design a plasma-seed treatment which changes one or more plant properties, such as germination rate.

The **second aim** was to better understand the influence of each plasma component and plasma-seed treatment parameter, in order to identify which parameters are most determinant (power supply type, power, gas type etc.). In other words, the goal was to better comprehend the requirements necessary for a successful plasma-seed treatment and develop a structured approach for designing plasma-seed treatments.

The **third aim** was to identify the key molecular event(s) after plasma treatment by analyzing differentially expressed genes in seedlings with accelerated germination. The motivation here was to contribute towards the very limited number of transcriptomic studies using plasma-treated seeds. Therefore, the goal was to develop insights into how plants interpret plasma exposure and investigate the possibility of a molecular marker or gene signature specific to plasma.

1.2 Structure of the thesis

Plasma applications for smart and sustainable agriculture include: low-temperature plasma treatment on seeds, plants, or food items, as well as the use of plasma-treated water (PTW) on the same types of substrates as growth medium, fertilizer, or disinfectant. The focus of this thesis is on low-temperature plasma-seed treatments and the main contributions are first, the development of guidelines for designing treatments and second, the molecular investigation of seedlings grown from plasma-treated seeds.

In **Chapter 1**, the aims, structure of the thesis, and the construction of a bioplasmas lab is presented.

In **Chapter 2**, the concept of plasma agriculture is introduced, first with seed development, and then continues with what is known about plasma and its components, and ends with how each component affects the seed and its subsequent development. The state-of-the-art for plasma-seed mechanisms is presented in order to demonstrate the limited number of molecular biology studies and highlight the gaps in our understanding of which plasma component is responsible for the plant effect.

In **Chapter 3**, a broad overview of plasma sources is first given with a focus on dielectric barrier discharges (DBDs) since they are used for the plasma-seed treatments in this thesis. This is followed by explanations of the DBD ignition mechanisms, DBD characterization, such as power measurements using the Lissajous figures, and differences between power supplies to better understand the experimental design in the following chapters.

The results of this chapter are the evolution of the plasma source and the plasma-seed treatment, which includes temperature, humidity, ozone, and power measurements. The plasma-seed treatment, which accelerated the germination rate, is an alumina surface DBD with printed high voltage electrodes, powered by an alternating current (AC) power supply using a duty cycle with the following standard parameters: 10 kHz, 8 kVpp, 60 s plasma treatment time, 3.7 mm distance between seeds and plasma, and 2 L/min of dry synthetic air (80:20 N_2:O_2).

In **Chapter 4**, a brief descriptive summary of the surface analysis methods, which are most often used on plasma-treated seeds, is presented and covers the following: scanning electron microscopy (SEM), contact angle goniometry, energy-dispersive X-ray spectroscopy (EDX), X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR).

The results of this chapter are a comparison of the above listed methods and their relative merits. It also includes the results of an experiment designed in such a way to produce separate controls which mimic the plasma components in an attempt to disentangle the multiple components of plasma. SEM and XPS were the most fruitful diagnostics. The seed surface was slightly eroded at longer treatment times and was oxidized after direct air plasma treatment, yet only plasma, not heat or ozone, appeared to have this unique effect under the given experimental conditions.

In **Chapter 5**, a parametric study was performed to optimize the plasma-seed treatment and determine which conditions yield a change in a macroscopic property, specifically germination rate. This was done using different combinations of power supplies, DBDs, voltages, frequencies, plasma-seed gap distances, times, and gas flow rates.

The results of this chapter show that seeds treated using a modulated AC power supply and a printed striped SDBD yielded the most promising results. These conditions were scanned using *in situ* Fourier transform infrared spectroscopy (FTIR) to better understand how each parameter influences the plasma gas composition and find out which component is responsible for the altered germination rate. The plasma was operating in ozone mode and with increased time or voltage, an increase in ozone concentration was observed, whereas increased gas flow rate decreased ozone concentration. Although the active agent behind the accelerated germination remains inconclusive, nitric oxide stands as a likely candidate.

In **Chapter 6**, a background about methods used to study molecular biology, such as quantitative polymerase chain reaction (qPCR) and RNA sequencing, is given. The plasma-treated seeds with an accelerated germination rate were analyzed using RNA sequencing to determine which genes are differentially expressed, and then this information was used to better understand how plants interpret plasma treatment.

The results of this chapter, specifically of the transcriptomic studies, are the increases in plant primary and secondary metabolism 6 days after plasma-seed treatment or in other words, 4 days after accelerated germination. A phenylpropanoid or glucosinolate response was elicited depending on the plasma treatment time. The results from another set of experiments with a delayed extraction time of 24 hours and similar plasma treatment showed increased expression of nitriles, a catabolic breakdown product of glucosinolates. A tentative hypothesis is proposed that plants not only perceive plasma as an oxidative stress but also possibly as a wounding. In parallel, a first attempt was made to determine whether a marker gene for plasma treatment could be identified. Therefore, the relevance of reactive oxygen and nitrogen species is shortly presented and followed by experiments exploring potential gene candidates responding to plasma components, such as ozone and hydrogen peroxide. However, it remains difficult to pinpoint a set of genes distinctly responding to plasma.

In **Chapter 7**, each variable for the plasma-seed treatment design is described and relevant examples from the thesis work are shared, such as DBD aging with repetitive use, exposure to high humidity, or nanoparticle deposition to name a few. These examples demonstrate the importance of reproducibility and standardization of protocols which is currently lacking in plasma agriculture.

The result of this chapter is the establishment of guidelines for plasma-seed treatments to first, encourage the recording of all relevant details and second, help identify the necessary parameters which lead to a plant effect.

In **Chapter 8**, the achievements of this thesis are outlined and a future outlook is provided. It is followed by examples for future experiments and ends with advice on potentially relevant applications and steps which should be taken prior to transferring this technology into real-world applications.

1.3 Construction of a bioplasmas laboratory

In 2018, a new research direction exploring biological applications of low-temperature non-thermal plasmas at the Swiss Plasma Centre was officially decided. This required building the infrastructure of a bioplasmas lab and developing the project from the ground up. The room for the bioplasmas lab was selected on the basis of limited human traffic in order to minimize contamination and ensure stable temperature, light and humidity conditions. Previous activities were performed in this room so this required the removal of existing infrastructure and tools to then introduce the necessary equipment for the bioplasmas lab.

Figure 1.1. The state of the lab prior to the construction of the bioplasmas lab.

The design concept was to have the plasma sources, plasma diagnostics, and biology laboratory equipment easily accessible. Therefore, all elements were housed in one room and were organized according to the expected workflow. This was inspired by the personal experience of traveling between different labs and buildings for experiments, which is typical for an interdisciplinary field like plasma decontamination and plasma agriculture. In the first half of the room, the lab is equipped with a bench dedicated to DBD prototyping, which is neighboring well-ventilated workstations for the plasma treatments. With greater spatial separation, the biology portion of the lab is stationed in the other half of the room to prevent the spread of contamination. Inevitably, a small office space was established to provide a space to record, analyze, and discuss experimental results.

Therefore, throughout 2018, the lab was equipped with the basic necessary equipment to be able to grow living organisms, such as bacteria and plants, and store biological materials or chemical reagents. The infrastructure includes: a laminar flow bench, milliQ water purification system, sink, incubators, phytotron, 4°C refrigerator, -20°C and -80°C freezers, spectrophotometer, centrifuges, autoclave, pH meter, hot plates, pipettes, and consumables. The molecular work station was established in 2019 and includes: a PCR machine, a qPCR machine, nanodrop, and tissue lyser.

Figure 1.2. The process of establishing the biology and bioplasma work stations in 2018.

Throughout 2019 to 2021, more plasma treatment work stations were added, a chemical fume hood and safety cabinet were installed, and an ozone machine by Sterilux was purchased as a control to compare to plasma treatments. More plasma diagnostics were purchased and introduced into the lab: a picosecond laser for LIF and FTIR for plasma gas chemistry analysis, as well as, an EPR and microscope for plasma-treated water experiments. The completed lab can be viewed **here**.

Figure 1.3. The fully equipped and established bioplasma lab in 2022 (courtesy of Humberto Torreblanca).

Chapter 2

Introduction to plasma agriculture

Abstract

The purpose of this chapter is to introduce the mechanisms underlying the effects of plasma-seed treatments, which is the framework of this thesis work. This will be done by explaining the seed structure and germination process and then, introducing the definition of non-thermal plasma and outlining its individual components. The remainder of the chapter elaborates on how each plasma component can affect the plant and its performance on a physical, chemical, biochemical, and molecular level.

This chapter is based on the review paper cited below. I conceptualized the review and figures, performed the literature search, illustrated the figures, wrote the original manuscript, selected the journal, and handled the submission process.

Frontiers in Physics

Mechanisms of plasma-seed treatments as a potential seed processing technology

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Keywords: plasma, seeds, processing, mechanisms, germination, surface modification, stress or disease resistance

Plasma treatments are currently being assessed as a seed processing technology for agricultural purposes where seeds are typically subjected to pre-sowing treatments to improve the likelihood of timely and uniform germination. The main motivation driving plasma-seed treatment research is the importance of food. The world population is projected to increase to 10 billion by 2050 and even without increasing the food supply, it is necessary to maintain the current food production and quality (FAO, 2017). Here, an introduction to plasma, seeds and plasma-seed treatment mechanisms as well as the physical and biological variables will be presented.

2.1 Seed structure and development

Although very diverse, all seeds have generally evolved to contain all their needs to develop into plantlets once the environmental conditions are perceived as appropriate. The living tissues of seeds are protected by the seed coat (testa), which can vary between species and cultivars. Inside the dry mature seed, the embryo is in a partially desiccated, quiescent state, poised to germinate upon the addition of water or, in other words, imbibition. It is then provided with stored foods through the endosperm during germination, a process which is the transition from an inactive to active seed. It grows, ruptures the seed coat, and develops from a seedling into a plantlet, which is generally still frail and particularly sensitive to external stresses, to then a more stress-resistant autotrophic plant (Fig. 2.1).

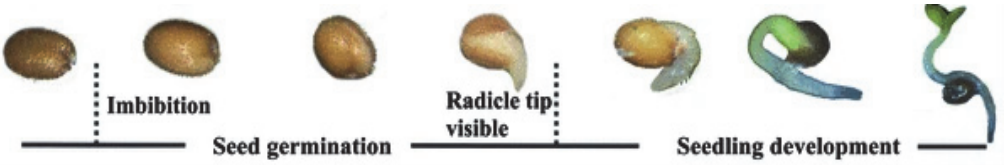

Figure 2.1. Stages of seed development in *Arabidopsis thaliana* seeds (Silva et al., 2017).

The trigger for germination requires a number of external parameters to be met, which will vary from seed to seed, but are generally a combination of water, temperature and light. Additionally, each seed has different requirements due to structural differences, particularly in the seed coat. Imbibition is the first step where the seed undergoes three stages with initially rapid, then slow and finally rapid water uptake. Water reactivates the enzymes that can repair DNA and membrane damages by using pre-existing RNA transcripts produced during seed maturation, and activates enzymes involved in beta-oxidation and amylases to break down stored oil and starch into sugars for energy and cell wall production, respectively. Proteases break down storage proteins into amino acids for protein synthesis.

After the third water uptake stage, the seed is swollen since the tissues have expanded and the embryo grows. The first visible sign of germination is the protrusion of the radicle, which later becomes the root. From there, the hypocotyl, which connects the root and shoot, hooks out and brings out the shoot with the cotyledon/s, then the true leaves. Once photosynthesis starts, the plantlet can grow independently from prior storages of organic matter and will only take nutrients and water from the soil and the surrounding media.

Prior to germination, there are hormones and inhibitors that prevent the process, to ensure the right environment and maximize the probability of the germinated seed to survive and thrive. The hormone abscisic acid (ABA) is known for its role in maintaining dormancy and for inhibiting germination. When this is removed by a lengthy water imbibition, another hormone gibberellic acid (GA) is produced and germination begins. As

the embryo grows, both auxin and cytokinin are involved in cell expansion and cell division, respectively, whereas later during the stressful life of the plant, hormones such as salicylic acid, jasmonic acid and ethylene play a role in plant defense to protect it against abiotic stresses such as cold, heat, dehydration, and biotic stresses, such as herbivores, viral, fungal, and bacterial pathogens (Fig. 2.2). More information about plant defense can be found in Andersen et al. (2018).

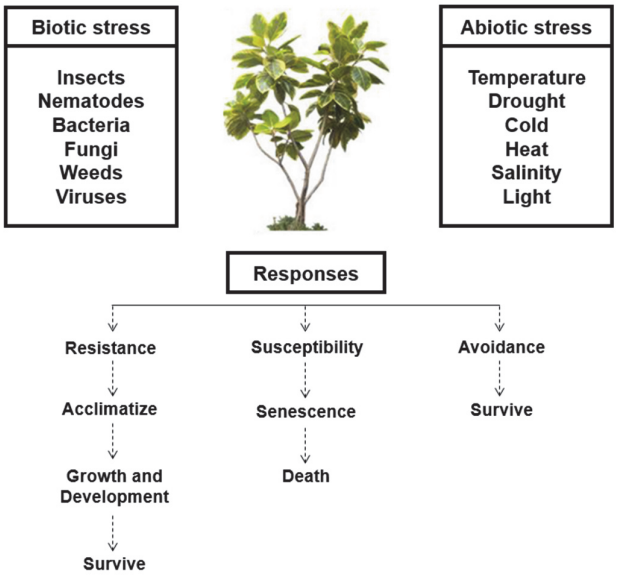

Figure 2.2. Examples of plant stresses and their corresponding responses (modified from Giri and Sharma, 2020).

2.2 Seed performance

The best chance for survival is to provide the seed with an opportunity to germinate from the very beginning, assuming they are not dead. For food production, there are additional criteria beyond successful germination which are germination uniformity and rate for a single harvest when done on an industrial scale. For this reason, many centuries ago, a method called priming was developed to ensure a more uniform and faster germination.

Seed priming is a method which can increase plant growth parameters, such as germination rate and uniformity and contribute to higher yields and greater plant resistance. As reviewed by Lutts et al. (2016) and Pawar and Laware (2018), the concept of priming is to provide water and activate the metabolism of the seed to repair damage before continuing the embryo development and root emergence.

Priming is frequently applied using water (hydro-priming), which requires soaking the seeds for a given timeframe. This water treatment can be modified to ameliorate germination rate, efficiency and uniformity with the addition of salts (halo-priming), solutes to change the osmotic pressure (osmo-priming), micronutrients like boron and iron (nutri-priming), hormones like gibberellic acid (hormonal priming) or beneficial microorganisms, such as *Pseudomonas* species (bio-priming), and metallic nanoparticles like iron and silver (nano-priming). Priming can also be done without water by using a solid and non-soluble material, such as sand or clay, called matrix priming (Pawar and Laware, 2018).

Depending on the seed and its structure, it can be primed with wet treatments by soaking in cold, warm, boiling water or dry treatments using dry heat or microwaves. Seeds can also be primed using acid

scarification and physical scarification. Seeds, specifically their seed coats, can also be modified with compounds such as selenium or salicylic acid (Wang et al., 2016) or agents that are protectants, nutrients, symbionts, soil adjuvants (hydrogels), and colorants (Pedrini et al., 2017). Protectants, such as pesticides, and colorants make up the bulk of coatings and are applied mostly to crops and vegetables to mainly deter insects (44%), weeds, and fungi (Aktar et al., 2009).

Pesticides in the seed coatings can transfer into the soil through rain and enter the groundwater and wastewater treatment plants. Since these compounds are highly toxic, persist for a long time, and become more toxic with time when held in storage, remediation methods can be used to remove toxic chemical compounds. This can be done using microorganisms, clay, polymeric materials, UV-H_2O_2 and UV-ozone, hydroxyl radicals, and at times, can require additional water resources (Marican and Duran-Lara, 2018).

Although protective coatings are important to preserve plant health and occupy a multi-billion-dollar market, the amount of investment in remediation techniques highlights that it would be beneficial to consider alternatives to minimize pesticide use. For this reason, alternatives have been considered, such as biocontrol using fungi or bacteria, biopesticides derived from natural compounds such as grapefruit seed extract (Choi, 2017), physical methods such as ultrasound (Tito, 2017; Guo et al., 2018), as well as genetic engineering (Paoletti and Pimentel, 2000). It would also be ideal to find a method with minimal energy consumption in view of energy savings considering that energy input is five to ten times greater than the output in the form of food in North America (Lockeretz, 2012).

2.3 Non-thermal plasma (NTP) as a seed processing technology

Ideally, more effective solutions for seed treatment to ensure rapid and uniform germination should not include toxic residues, consume little energy, have low penetration depth to avoid injuring cells, and favour long storage time (Hussain et al., 2015), while subsequently supporting optimal seed development. These criteria can possibly be met with non-thermal plasma (NTP) treatments.

Plasma is a fourth state of matter, which is partially or fully ionized gas. It can be subdivided into thermal plasma, which exists at temperatures as high as 100 million degrees Celsius, or non-thermal plasma, which exists at gas temperatures as low as room temperature. The properties of a non-thermal plasma are mostly determined by the temperature of the electrons, which are responsible for the plasma chemistry. Gas temperature is a measure of the kinetic energy of particles, not their internal energy. The electrons are accelerated by electric fields and the transfer of the electron thermal energy to the heavy particles is weak and remains mostly unchanged with elastic collisions since the electrons have a small mass. If the electrons have sufficient energy, they can transfer almost all their thermal energy through inelastic collisions to change the internal energy of the heavy particles. At high pressures (more than 10 or 100 mbar), the elastic collisions begin to dominate and the plasma tends to thermodynamic equilibrium where $T_e = T_i = T_{gas,}$ where *e* is for electrons and *i* is for ions (Fig. 2.3). When in thermodynamic equilibrium, both the electrons and heavy particles are at the same high temperature as in thermal plasmas. When not in equilibrium, the electrons have a high temperature of several eV (where 1 eV is 11600 K) but the heavy particles remain at a low temperature because gas has the same temperature as the walls, and ions have similar temperature to the gas (the kinetic energy exchange in elastic collisions is very efficient between equal-mass particles) and therefore, the overall gas temperature is low in non-thermal plasmas.

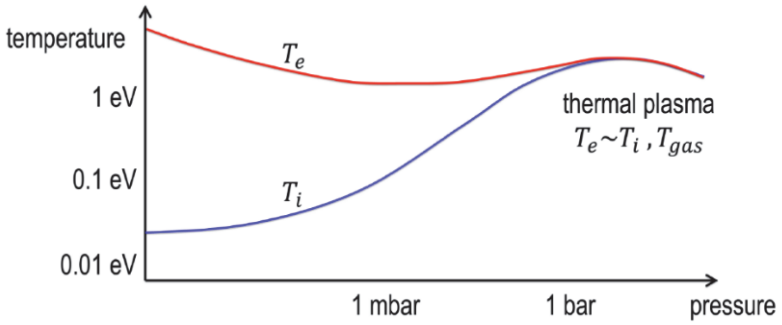

Figure 2.3. Temperature of ions and electrons are not the same in non-thermal plasmas, but are the same in thermal plasmas which often happen at or above atmospheric pressure (courtesy of Dr. Alan Howling).

The main advantage in using non-thermal plasmas is the high temperature chemistry that is available at a low gas temperature. Plasma can be ignited at both low or atmospheric pressure. Low pressure is easier to ignite and maintain at a low temperature due to less frequent collisions but requires vacuum, which would require working in batches in vacuum chambers. In contrast, atmospheric pressure is more difficult to ignite, increases in temperature easily (Fig. 2.3), but is easier to continuously work with provided that a DBD is used since the dielectric barrier prevents arcing. Therefore, this option is more applicable for industrial processes.

The plasma composition depends on the operating parameters such as voltage, frequency, humidity, flow rate, and gas mixture. Gases such as argon, oxygen, nitrogen, helium, and/or air can be ionized by electric fields to form electrons, ions, UV, thermal radiation, and reactive species. Specifically, air plasmas contain reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), and ozone (O_3), and reactive nitrogen species (RNS), such as nitric oxide ($NO^{\bullet}$), peroxynitrite ($ONOO^{\bullet}$), and nitrogen dioxide radical ($NO_2^{\bullet}$) (Li et al., 2016). More details about the mechanisms and plasma device configurations are given in Chapter 3.

Plasma treatment may enhance seed survival without lingering toxic residues since almost all of the constituents in plasmas can be found in nature and recombine shortly afterwards. Moreover, components in plasma have approximately 10 nm deep penetration, limiting it to surface functionalization (Guo et al., 2018). Importantly, plasma treatments are considered to be low maintenance with low energy costs (Randeniya and de Groot, 2015). Therefore, many studies have been performed involving plasma treatments of agronomic interest, such as quinoa, basil, tomato, wheat, radish, soybean, mung bean, rice, Ajwain, Umbu, and seeds deemed important for the landscape, like Norway spruce (Pauzaite et al., 2018).

Randeniya de Groot (2015) and Puac et al. (2018) reviewed the observed effects on germination and subsequent plant growth. The extensive list of these effects in this review has been summarized in Figure 2.4. It can increase germination probability and biomass (Măgureanu et al., 2018) or increase disease resistance or stress resistance (Jinkui et al., 2018; Wu et al., 2007). Additionally, it can accelerate or delay germination and subsequent development (Volin et al., 2000), decrease water consumption (Bormashenko et al., 2012), decrease levels of microbial pathogens or insects (Shintani et al., 2007; Shintani et al., 2010), without detectable toxic residues (Sivachandiran et al., 2017). Molina et al. (2018) proposed to make a hydro-absorbant polymer coating with short plasma treatments on specific seed types, and Kopacki et al. (2017) suggested to use plasma for seed coatings against fungal pathogens.

Other than treating the seed or plant directly, plasma treatment can also be used on the plant's surroundings by degrading volatile organic compounds in the soil to improve soil health (Stryczewska et al., 2005; Chen et al., 2009). It may also have potential as an alternative to fungicides (Pérez-Pizá et al., 2019) and be implemented in industry as field studies have been documented (Li et al., 2016; Zhang et al., 2018).

Table S1 shows the legend describing the categories of Table S2, which is a compilation of papers described in four categories: seed coat modification, growth parameters, metabolism and disease or stress resistance and further divided by the scale of information i.e. macroscopic, microscopic or molecular properties (see Appendix, supplemental section, Chapter 2). Decontamination of seeds was intentionally left aside and can instead be found in more detail in the food processing field (Butscher et al., 2020).

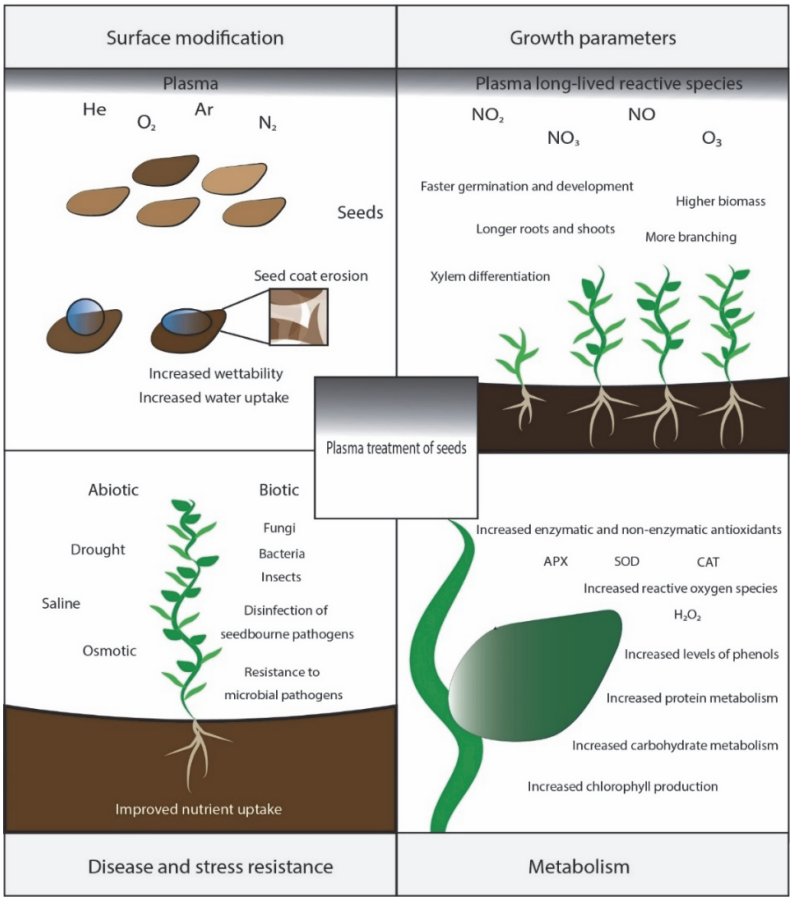

Figure 2.4. A summary of plasma-seed treatment effects which includes surface modifications, changing growth parameters, and modulating disease and stress resistance through metabolism.

2.4 Mechanisms of how non-thermal plasma affects seeds and their subsequent development

Quite consistently, most reports refer to optimized plasma setups that can significantly change germination and plant growth parameters. For example, germination rate may be accelerated, shoot and root lengths may be longer (Jiang et al., 2018) and more branching of the roots (Măgureanu et al., 2018) or stronger root system (Mildažienė et al., 2017) were reported. However, it is difficult to know the causative agent behind these effects due to varying plasma-seed treatment methodologies.

Not all observe the same effects and instead, plant growth can be improved without changing germination rate (Sidik et al., 2019) or scientists have strong variation in their experiments (Hosseini et al., 2018; Mildažienė et al., 2019). This could mean that further optimization is required, which is time-consuming considering the number of variables in the experimental design. It is not yet clear by which mechanism(s) these effects arise but understanding this will simplify the experimental design and potential future scale up so that the results are reproducible. Here, we take a closer look at the physical, chemical, and biochemical factors derived from plasma treatments to have a more detailed understanding of what is happening to the seed. A summary of the plasma-seed interactions at the seed surface and at a molecular level are given in the following sections.

2.5 Physical factors

The physical factors such as heat, ultraviolet, and electromagnetic fields and mechanical scarification are the first contact point with the seed coat and may then trigger downstream consequences from this initial interaction. In the following section, each physical factor and its effect will be presented individually (Fig. 2.5).

2.5.1 Heat

The temperature of plasma treatments can range from room temperature up to 90°C, so in principle it is possible to have an effect depending on the temperature and treatment time although not many authors think that increased temperature is responsible for the changes in plants. Temperature is monitored in a few studies by measuring the electrode temperature, calculating the gas temperature from spectra or measuring the temperature of the seed directly using an infrared camera or thermocouple (Lotfy, 2017; Kobayashi et al., 2020) but it has not yet been done on a molecular level by assessing the seed or plant response. Kitazaki et al. (2014) compared plasma and heat treatments by heating the seeds on a hot plate but did not see the same effect on the plant development.

Others have tried to look into heat shock proteins (HSPs), which are induced with temperatures ranging from 31 to 37°C, but HSPs are often not exclusively induced by heat. They may also accumulate under oxidative stress, high intensity irradiation, and desiccation (Al-Whaibi, 2011). Iranbakhsh et al. (2018) treated wheat seedlings with plasma and observed increased expression in heat shock factor A4A in both the root and shoot system in correlation with increased growth parameters and mitigated the negative effect of salinity stress. They only reached a maximum of 29°C in a 2-minute plasma treatment, suggesting that heat alone is not responsible for the observed effects in this particular study. Moreover, HSPs did not change in a study by Mildažienė et al. (2019). Therefore, the role of HSPs during plasma treatment remains unclear.

2.5.2 Ultraviolet light (UV)

The role of high-energy photons in plasma-seed treatments has been controversial since UV has had a negligible effect so far. However, the effects of UV on seeds and plants could theoretically contribute to the wettability or growth enhancement effects indirectly; for example, by producing radicals or reactive oxygen species (ROS), such as ozone (Lotfy et al., 2019). Gao et al. (2019) checked the effects of UV separately from plasma and observed that UV had only a minor contribution towards seed wettability. Sarinont et al. (2016) also reported that there was no effect from UV when they saw the lack of growth enhancement effect after they blocked UV (UV-B and UV-C) with a quartz glass plate.

It is known that just UV-B can accelerate germination of safflower seeds but then negatively affects growth (Farokh et al., 2010). Noble (2002) made the same conclusions with kale, cabbage, radish, and agave seeds. Likewise, Sadeghianfar et al. (2019) showed accelerated germination of maize and sugar with UV-C treatment but instead, saw an increase in plant growth parameters and suspected this may be due to the breakdown of the seed coat and increased temperature.

There may be differences in effects depending on the wavelength since it has been shown that UV-A had a more pronounced effect than UV-C but both were able to accelerate the germination rate and improve growth parameters (Ibrahim et al., 2012). On the one hand, UV is often associated with inducing DNA damage. Prakrajang et al. (2020) compared gamma radiation with plasma and saw only with gamma irradiation that the plants did not grow well and thus suspected that it induced DNA damage. On the other hand, plants treated with UV were able to better cope with drought stress, possibly by activating DNA repair mechanisms (Caldwell et al., 1998). This may be due to an increase in phenolic compounds, which often have a role in disease or stress resistance and this response can be triggered by intense UV light which is accompanied by high temperature and photo-oxidative damage in nature (Veberic, 2016).

Babajani et al. (2019) and Iranbakhsh et al. (2017) both considered that plasma-derived UV may be detected by photoreceptors, which then might affect secondary metabolism and trigger stress responses, if the treatment is done briefly. UV is also linked with photomorphogenesis, and cell elongation, division and differentiation (Huché-Thélier et al., 2016). Iqbal et al. (2019) compared laser and plasma treatment separately on seeds and saw similar types of effects: damage to the seed coat, an increase in water uptake and protein content.

2.5.3 Electromagnetic fields (EMF)

Pauzaite et al. (2018) and Mildažienė et al. (2019) also attributed the changes in growth parameters to radiation since Mildažienė et al. (2019) saw similar protein expression profiles when comparing seeds treated with plasma or electromagnetic fields (EMF). This is not entirely surprising since pulsed electric fields also affect seed germination (Dymek et al., 2012; Su et al., 2015). It needs to be kept in mind though that electric field treatment is also accompanied by oxidative stress and ozone (Teissie et al., 2005), where ozone has been suggested by Patwardhan et al. (2013) to be the main effective parameter in the treatment.

Static or alternating magnetic fields can also change germination probability, growth rate, increase root and shoot length, change redox status of plants possibly by increasing hydrogen peroxide (H_2O_2), alter photosynthesis, alleviate drought stress, or increase mineral content (Bilalis et al., 2012; Maffei et al., 2014). Arguably, UV may have a role but a minor contribution in terms of direct effect. Considering that the above studies used several hours long UV treatments to have an effect, most plasma treatment are in the seconds or minutes range. Nevertheless, photons and electromagnetic fields can still possibly contribute indirectly through the production of RONS.

2.5.4 Mechanical scarification and erosion

In terms of mechanical effects, it has been suggested that altering the seed coat may play a role in modifying germination rate. Typically, a seed has four layers: the cuticle, epidermis, hypodermis, and parenchyma. The differences in the microstructures and chemistry of these layers determine differences

between species and even cultivars. The seed coat role is like a water modulator; it controls the entry of water so it can be absorbed slowly by the cotyledons to minimize or avoid imbibition damage (Souza and Marcos Filho, 2001).

It has been suggested that the removal of the lipid layer allows for better access to water, a requirement for triggering germination (Sehrawat et al., 2017). Bafoil et al. (2019) showed the importance of the seed coat by using mutants of *Arabidopsis* plant model L*er* and Col-0 ecotypes and showed the rearrangement of lipid components, changes in lignin, and seed coat erosion. Many, although not all authors have observed with scanning electron microscopy (SEM) that seed surfaces treated with plasma have an eroded appearance (Stolárik et al., 2015; Junior et al., 2016; da Silva et al., 2017; Li et al., 2017; Wang et al., 2017; Gao et al., 2019). In contrast, Mildažienė et al. (2016) only saw etching on the seed surface facing plasma treatment and others did not see any changes (Sera et al., 2010; Bormashenko et al., 2012; Kitazaki et al., 2014).

A majority of these authors have been able to correlate these changes with increased water uptake. For example, Pawlat et al. (2018a) observed that plasma treatment changed the seed structure by removing the upper cuticle layers covered with wax, a polymer present which prevents water loss under heat stress, and may form micro-pores to aid water absorption. Bafoil et al. (2019) also worked with an *Arabidopsis* mutant *gtap5,* which is not able to make suberin or cutin, and saw that plasma was not able to improve the germination without these polymers in the seed coat, suggesting their importance. This waxy layer was even considered as a parameter in the experimental design of a study done by Park et al. (2018). They used two seed types, one with and the other without a waxy layer, and their results indirectly implied that this waxy layer plays a role in the effect that plasma treatment will have on the seeds. Considering that wax limits water loss and controls gas exchange, this suggests that plasma changes the seed coat permeability by altering this waxy layer.

Billah et al. (2020) pointed out that there may be exothermic reactions from the plasma that release heat and possibly melt the wax due to its low evaporation temperature of 37ºC, or the surface is eroded by reactive species. This was also pointed out by Holc et al. (2019). Wang et al. (2017) also suggested that through surface modification via etching, the seed is able to absorb water through increased hydrophilicity. This enhanced water absorption by modifying the seed structure is further supported by others who observed the degradation of cellulose on seed surfaces (Yamauchi et al., 2012). Currently, the analysis of components with FTIR-ATR is limited to cellulose since it is difficult to differentiate between different organic plant components like cellulose, hemicellulose, pectins, etc. Wang et al. (2017) used both FTIR-ATR to measure seed surface changes as well as FTIR to analyze the gas exhaust from the plasma treatment. They needed to omit wavenumbers i.e. peaks below 1500 cm^{-1} that were difficult to assign to specific functional groups during analysis but also suggested that the spectral bands are mainly attributed to cellulose rather than wax.

Junior et al. (2016) showed that water may be guided differently after plasma treatment due to surface modifications. They observed that the hilum increased the amount of water absorption, the micropyle had a more open configuration and in particular, the water absorption was improved mainly through the hilum rather than micropyle. In any case, by thinning the seed coat, it is logical to assume that water will be more readily absorbed.

Interestingly, the water absorption can be controlled by the plasma treatment using different gases and coating thickness, as mentioned by Volin et al. (2000). Depending on the working gas, 0.5 - 2 μm thick coatings were applied and their thickness modified imbibition. This suggests that the seed coat thickness can be mechanically modified by etching or by changing the chemical properties.

Despite adding to the thickness with an additional layer of coating, germination was improved in a few instances, which highlights the importance of chemistry, but it is difficult to separate the etching effect from the chemistry in this study. As it still remains, it is not yet known whether this increased permeability is principally due to mechanical mechanisms such as etching as Pawlat et al. (2018a) suggested, a combination of both mechanical and chemical as mentioned by Gómez-Ramírez et al. (2017), Tounekti et al. (2018), and Park et al. (2018), or solely due to chemistry.

SEM is an insightful tool, which can provide quick, qualitative results of the plasma-seed treatment. Nevertheless, caution should be exercised when interpreting SEM images because plant genetics influence the seed coat pattern. Otherwise, observed changes might already be pre-existing and should instead be attributed to biological variation rather than plasma treatment and therefore, using the same seed before and after treatment is recommended.

Pawlat et al. (2018a) noticed that seed shape influences the type of change affecting the seed and showed in their SEM images that the seed edge was torn off whereas the middle grew in sharpness. This brings attention to the fact that this process needs to be delicately handled. For example, Cui et al. (2019) used tape to prevent the movement of seeds during the plasma treatment but this may affect the seed coat, especially if it is done in the presence of moisture. As a result, it seems that there is a limit to how much information can be extracted from surface analysis using microscopy. Instead, it would be useful to further explore the chemical modifications since this is momentarily limited in the literature.

It may very well be that the chemistry is sufficient to affect downstream processes considering that an effect in growth parameters without visible seed coat modifications have been observed (Los et al., 2019). Mildažienė et al. (2017) also observed positive growth effects using vacuum and EMF without visibly changing the seed coat structure using SEM. It may also be the case that the results will depend partly on whether there is physical or physiological dormancy, meaning whether dormancy is due to the seed coat or embryo.

2.6 Chemical factors

On the one hand, etching may play more of a role in certain seed types with impermeable seed coats but, on the other hand, it may be that a specific concentration and/or mixture of reactive species from a higher power plasma is required to observe an effect. In the latter, mechanical damage might merely be a side effect, which too can contribute to enhanced water absorption, and might not be the primary factor. For this reason, chemical factors will now be discussed in the next section.

2.6.1 Nutrient absorption

In addition to analyzing mechanical damage, SEM can be coupled with energy dispersive X-rays (EDX) to look at the seed surface composition. Other methods such as X-ray Photoelectron Spectroscopy (XPS) and micro X-ray fluorescence spectroscopy (*μ*-XRF) can also be used to acquire information about the

elemental distribution. It has been observed by several authors like Pérez-Pizá et al. (2019) and da Silva et al. (2017) that lipid layers undergo chemical oxidation, which can therefore improve interaction with water.

Shapira et al. (2018) found that irreversible wettability is not due to electric charging, which could indirectly imply that it may be done chemically. Pérez-Pizá et al. (2019) also correlated the increased hydrophilicity with oxidized seed surfaces using nitrogen and oxygen plasmas. This perhaps suggests that hydrophilicity again is done through chemical rather than mechanical changes. This may not be entirely dependent on the gas type but rather on the production of an appropriate profile of reactive species at sufficient concentrations that will oxidize the outermost lipids. It has been shown by Gómez-Ramírez et al. (2017) using XPS and EDX that plasma treatment can oxygenate carbon and deposit nitrogen groups on the seed surface. They hypothesised that these elements, as well potassium, are later absorbed by the seed in the presence of water (Cakmak, 2005).

Ambrico et al. (2019) instead used *μ*-XRF and showed the concentration and redistribution of macro- and micronutrients such as potassium after plasma treatment. On the one hand, the diffusion of potassium into the seed interior may improve the germination through enzyme activation, help with water retention, or detoxify ROS (Wang et al., 2013). On the other hand, potassium at the surface can be interpreted as seed damage since it may be used as an indicator of cell membrane integrity (Miguel and Marcos Filho, 2002). Interestingly, Zhou et al. (2016) exposed seeds to plasma-treated water and they had the lowest leakage rate when measuring electrical conductivities, which is used to assess cell membrane integrity. This mobilization of nutrients perhaps is not lost to the environment but is loosened and absorbed immediately by the seed (Carvalho et al., 2009).

2.6.2 Gas exchange

When considering gases, it could be that oxygen on the surface is responsible for not only increased wettability but may also assist in seed respiration. Considering that oxygen is another requirement for germination, Sarinont et al. (2016) observed oxygen, NO, and nitrogen gas to be the most effective for plant growth but fumigation with oxygen gas already had an effect on growth parameters, albeit mild compared to plasma. This fumigation might be sufficient to improve plant growth, like in the case of nanobubbles (Ahmed et al., 2018). Although fumigation had a positive effect on plant growth, plasma application had a stronger effect, which may be due to the transport process being more efficient i.e. speed up the process by not relying on diffusion or provide more directionality. This highlights the presumably overlooked importance of oxygen, especially considering that Rahman et al. (2018) also saw more obvious changes in growth parameters when using an oxygen admixture instead of air.

This principle could likewise work for nitrogen gas but instead of modifying respiration, it may be directed towards other cellular processes, such as photosynthesis, since it is a major component of chlorophyll or protein synthesis (Leghari et al., 2016). As mentioned in the paper of Gao et al. (2019), the presence of CO and O_2^+ signals confirmed that chemical etching of the seed surface by plasma played an important role in stimulation of seed germination (Filatova et al., 2011). These chemical changes may then be responsible for changing the biochemistry and molecular events in the plant and therefore, these will be discussed in the next section.

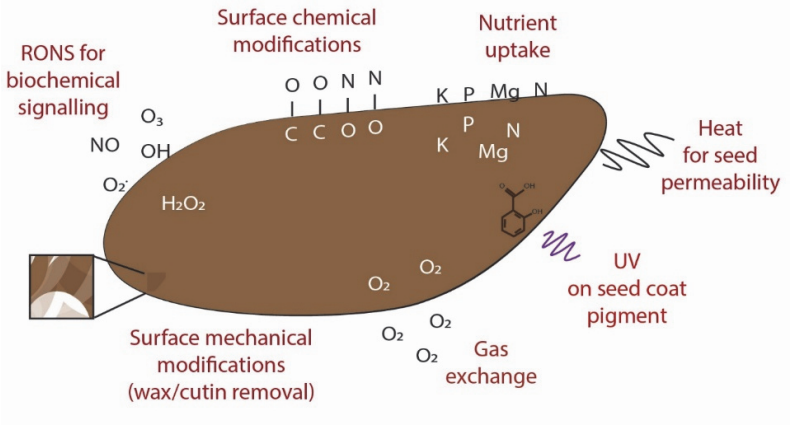

Figure 2.5. Summary of the possible mechanical, chemical, and biochemical interactions of plasma components with the seed surface.

2.7 Biochemical factors

2.7.1 RONS and seed coat interactions

Regardless of whether the changes to the seed coat surface might loosen an elicitor such as oligosaccharins, as mentioned by Iranbakhsh et al. (2018), many authors are in agreement and speculate that it is principally reactive oxygen and nitrogen species (RONS) that trigger biological processes.

Oxidation of the seed coat is very often observed, but this can be propagated internally since there can be an increase in maldonaldehyde (MDA), a product of lipid peroxidation, after plasma treatment, as seen by both Los et al. (2019) and Cui et al. (2019). This may be among the first steps in the signal transduction, considering that lipid peroxidation does not rely on enzymatic activity, which is very limited in dry seeds (El-Maarouf-Bouteau and Bailly, 2008).

Instead, Mujahid et al. (2020) mentioned that it may be the hydroxyl radicals which are responsible for the cell wall loosening. Additionally, Bafoil et al. (2019) calculated an increased expression of 23 genes coding for class III peroxidases after plasma treatment. These are proteins localized in the seed coat which regulate concentrations of hydrogen peroxide and precede the rupture for germination. Pauzaite et al. (2018) also suggested that ROS may alter the seed coat pigmentation, which is known to be linked to germination. The flavonoid biosynthetic pathways and abscisic acid, a hormone for dormancy, are regulated by the same gene locus. Although they had changes in the seed coat flavonoids, they could not find any clear connection in their study between flavonoids and seed germination parameters.

Others have seen that the seed coat pigmentation does in fact influence seed permeability and the rate of imbibition, where brown seeds had faster water uptake and reached a germination optimum sooner but were more susceptible to imbibition damage than black seeds (Siddiqui and Khan, 2010). Liu et al. (2019) also attempted to decipher between seed types using plasma-treated water and brought up in their discussion that seed storage proteins are typically oxidized during germination, which may facilitate the mobilization of the storage reserve. Therefore, it may be possible that plasma-derived ROS may be affecting the seed pigmentation first, and then downstream the germination rate. Additionally, considering that Mueller et al. (2009) suggested that ROS play a role in the cleavage of cell wall polymers, it may be that changes inside the

seed in turn modify the outer layers. The mechanical pressure on the endosperm needs to be relieved for the radical protrusion but this is based on cell wall loosening, which is linked to the action of ROS (El-Maarouf-Bouteau and Bailly, 2008). Once again, this may be another argument that external seed coat erosion might not be necessary for modifying germination.

Alternatively, it could also be that chemical modifications to the seed coat, for example through lipid oxidation, may lead to the carbonylation of proteins, rendering them more susceptible to cleavage and leading to the breakdown of aleurone layer (El-Maarouf-Bouteau and Bailly, 2008; Weber et al., 2015). Therefore, post-translational modifications, such as carbonylation and sulfhydryl group's oxidation, are also another mechanism by which ROS, specifically H_2O_2, can shift a seed from a dormant to non-dormant state, as shown by Oracz et al. (2007) and Valderrama et al. (2019).

2.7.2 ROS entry and involvement in seed development

Generally, it is not fully understood how external ROS are detected and how these signals are transduced in seed cells but this process may partly depend on the presence of water. Water is needed to trigger germination and increase respiration, a reaction which oxidizes sugars to release energy in the form of ATP. During respiration, ROS are produced as by-products and thus, ROS generation is the hallmark of dormant seeds transitioning into metabolically active seeds (El-Maarouf-Bouteau and Bailly, 2008). Where the moisture content is low, there is very little, if any, enzymatic activity but there may be hydrated pockets within the seed, permitting limited metabolic activity. It could be that this pocket of water may assist the diffusion and retention of ROS in the seed and therefore, it may be that plasma-derived ROS accumulate in these pockets and trigger signaling for intracellular programs. This process may be done more efficiently with hydrated instead of desiccated seeds due to the higher water content. Nevertheless, in dry conditions, ROS may have an effect that is simply paused until imbibition (El-Maarouf-Bouteau and Bailly, 2008) and this may be why there are long-term effects (Pauzaite et al., 2018) or why long-term storage of plasma-treated seeds can still have a positive effect on growth parameters relative to the untreated seeds (Sarinont et al., 2016). On the other hand, the effects of the plasma treatment may be continuously ongoing but are not morphologically obvious until imbibition and subsequent growth. Mildažienė et al. (2016) pointed out that "biochemical changes continue to occur in seeds at various levels, including hormonal balance, gene expression, oxidative processes, mRNA content, and protein translation during storage." These changes may occur across all of these levels after plasma treatment.

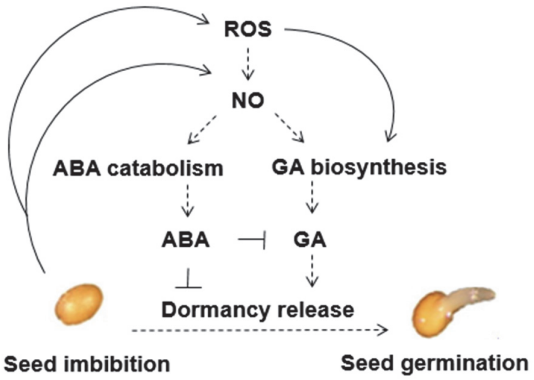

Figure 2.6. The role of ROS and NO in seed germination via hormone modulation (modified from Arc et al., 2013).

Although it is not known which ROS is/are responsible for the effect and how it/they enter, it is known that seeds have pores whose size is genetically regulated and this also may assist the diffusion of ROS into the seed (Souza and Marcos-Filho, 2001). H_2O_2 can interact with the surface and diffuse through the membrane but charged species, such as superoxide, are not able to bypass the membrane. They are dependent on voltage-dependent anion channels called porins, which are only found in the mitochondria (Parvaiz Ahmad, 2014). Despite its charge, superoxide can break down into hydroxyl and singlet oxygen and these may be able to bypass more easily (Grene, 2002). In some cases, RONS bypass the membrane using aquaporins, protein channels used for water transport (Yusupov et al., 2019). Transport also depends on the life stage of the plant since ozone can be taken up through the stomata in the leaves. Seol et al. (2017) observed an accumulation of ROS in chloroplasts, and suggested that through the micropores, ROS can travel down further into the plant tissue, from the epidermis into the mesophyll. Evidently, if it is too potent, chloroplast degradation occurs.

Considering the lifetime and complexity of the reactive species reactions, there is a bias for researchers to measure long-lived species, like H_2O_2, O_3, and NO, and therefore, most of the focus for this section will be dedicated to these species herein (Mhamdi and Van Breusegem, 2018).

2.7.3 Ozone

Surprisingly, little has been done to monitor and measure ozone during plasma treatments and it seems that many overlook the effect of ozone on seed germination, despite it being possible to enhance germination with optimized treatment parameters. On the one hand, ozone can impair plant growth by replacing CO_2 and reducing photosynthesis. This is done by inhibiting the opening of the stomata due to the reduced flow of potassium ions (Torsethaugen et al., 1999). In some cases, plants like mung beans are not able to overcome ozone stress with their antioxidant defenses (Chaudhary et al., 2015). On the other hand, ozone has been used to enhance seed germination (Avdeeva et al., 2018) or improve fruit quality (Rodoni et al., 2009). Ozone seemingly has an ambivalent role that is dependent on the concentration and length of treatment (Abeli et al., 2017) as it is the case for many other treatments i.e. hormone, nanoparticles, and heat. The authors mentioned that there is a variable response to the same ozone treatment based on the species and it is weak and transient. By using electron paramagnetic resonance (EPR), they showed that O_3 increased the concentration of radicals (carbon and oxygen species) in all tested species except one. Pawlat et al. (2018b) measured 0.01 ppm of ozone produced during plasma treatment and had an effect on the growth parameters but it is not clear whether this is due to ozone or other treatment parameters, such as the short heat treatment at 40ºC. Perhaps this ozone treatment might not have been sufficient, in terms of concentration and exposure time, but it is a good example to use to emphasize the importance of controls in plasma studies in order to clarify whether the effect is due to ozone, other reactive species, or other parameters.

It is especially interesting to differentiate this because ozone can trigger the production of ethylene, which then breaks down abscisic acid, the seed dormancy hormone (Emberson et al., 2018). Alternatively, it could be that ozone generates short-lived species inside the seed, which may be recognized as a signal for germination considering that the accumulation of ROS and peroxidation products is linked with seed dormancy alleviation (Oracz et al., 2007). As Sudhakar et al. (2011) pointed out, the production of hydrogen peroxide is observed in the early imbibition period of tomato seeds and nitric oxide, hydroxyl radicals, and superoxide radicals accumulate during seed germination in different species. For this reason, many scientists speculate

that H_2O_2 and NO are the reason for the observed effects using plasma treatments, whether or not it is the short-lived species such as superoxide, hydroxyl, or NO that are eventually converted into H_2O_2 (Cui et al., 2019).

Wang et al. (2017) used a nitrogen plasma in open air and suggested that nitrogen oxides, which are known to have a role in dormancy and germination signaling, are present after plasma treatment and may initiate these biological processes (Giba et al., 2003). Instead, Puac et al. (2014) looked at meristematic cells of carrots treated with a RF plasma for less than 2 minutes and suggested that at least H_2O_2 and superoxide can pass through the cell membrane. Reactive species may be one mechanism since they observed fluctuations in levels of redox quenching antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), which control and limit damage from excessive levels of ROS. This was also observed by Henselova et al. (2012) in maize using a DSCBD for less than 2 minutes. Simultaneously, these antioxidative enzymes may behave like sensors to detect ROS availability and redox perturbations so that an organism can respond appropriately (Noctor et al., 2017; Valderrama et al., 2019). There is also an overlapping relationship between NO and H_2O_2. NO positively regulates APX1 through a post-translation modification, *S*-nitrosylation. It enhances resistance to oxidative stress and improves immune responses (Yang et al., 2015) and furthermore, NO regulates itself and ROS (Romero-Puertas et al., 2016; Valderrama et al., 2019).

2.7.4 NO and H_2O_2

Rahman et al. (2018) suggested that plasma induces H_2O_2 formation, which is found in hydrated seeds and is involved in imbibition and early germination. They compared Ar/O_2 to Ar/air admixtures and observed higher concentrations of H_2O_2 with Ar/O_2 without inducing scavengers that counter H_2O_2 production. They concluded that plants treated with this admixture had better growth parameters due to the increased H_2O_2 concentration and did not see any changes in NO. Likewise, after plasma treatment, an increase in H_2O_2 concentration was correlated with positive effects on germination, whereas a decrease was correlated with negative effects on germination (Pauzaite et al., 2018). The same authors also showed for the first time the oscillatory dynamics of H_2O_2, which occurs in *Arabidopsis* and conifers but is not characteristic for other seed types like radishes and sunflowers. This already suggests that there can be differences between species, including on a molecular level.

Kang et al. (2020) interestingly did not see an effect and observed high variation in rice germination. Los et al. (2019) also saw no changes in H_2O_2 levels in wheat but still had growth enhancement. This suggests that there are either differences in the plasma that do not necessarily lead to changes in H_2O_2 and requires optimization, or that seeds respond differently, and it is not exclusively the action of H_2O_2 but instead nitrites and nitrates.

This latter point was heavily emphasized by Billah et al. (2020) who observed an increase in H_2O_2 but they think that nitrogen is the main contributor for enhanced growth in gram seeds. In contrast, Liu et al. (2019) compared different gases using direct and indirect plasma treatments to produce plasma-treated water and soaked a variety of seeds. They were tempted to believe that effects are more likely due to oxygen-derived species. Interestingly, NO can downregulate the signal for H_2O_2 and will activate genes for antioxidant enzymes, as pointed out by Iranbakhsh et al. (2017). Once again, this illustrates the difficulty in separating the effects of H_2O_2 and NO.

In the case of H_2O_2, it is considered a long-distance signaling molecule, which is highly interconnected with hormones, metabolism, and gene transcription. The signalling likely includes MAPK cascades, as pointed out by Babajani et al. (2019). ROS-mediated signalling includes calcium signalling, protein phosphorylation, and gene transcription, which are redox sensitive. The relationship between ROS and MAPK is not fully elucidated and both are able to regulate each other. These complexes phosphorylate transcription factors, kinases, phosphatases, or other proteins, which can then change enzyme activity or gene expression (Jalmi and Sinha, 2015).

Additionally, ROS may produce changes in calcium signaling and use different signatures, in terms of duration and amplitude, depending on the species and this will dictate what happens downstream i.e. root elongation (Wilkins et al., 2016). It was shown by Cui et al. (2019) that there was increased calcium in the roots of 4-day-old *Arabidopsis* seedlings grown from plasma-treated seeds and mentioned that this may eventually lead to plant growth if maintained at a low level. Since there is little evidence about signalling specific to plasma treatments, the focus now will shift from signal transduction to how hormones, metabolism, and gene expression are modified after plasma treatment, starting first with hormones.

2.8 Molecular factors

2.8.1 Hormones

It is difficult to differentiate between the action of ROS and hormones because ROS are highly interconnected with hormones like salicylic acid (SA) and jasmonic acid (JA), which are widely reported (Fig. 2.7). Information remains scarce with auxin and cytokinins, which are hormones that affect germination properties (Mhamdi and Van Breusegem, 2018). Nevertheless, “hormones modulate the effects of ABA/GA balance: auxin IAA (indole-3-acetic acid) is a negative regulator of germination; ethylene, cytokinins, brassinosteroids, and strigolactones can stimulate germination; SA and jasmonate (stress hormones) may affect germination positively or negatively depending on the situation” (Mildažienė et al., 2019).

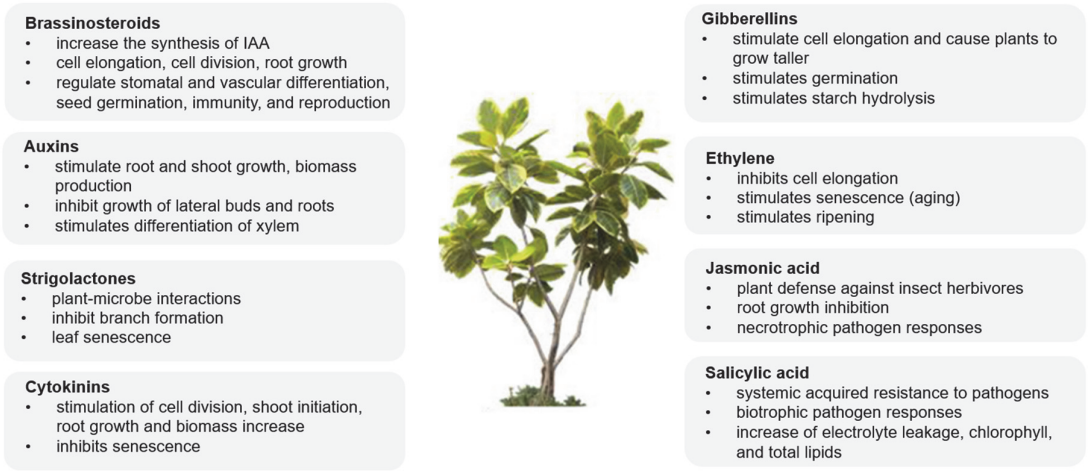

Figure 2.7. Selected hormones and their functions in plants (modified from Sytar et al., 2019).

There are authors like Kitazaki et al. (2014), who agree on the importance of reactive species. Specifically, they think it is the relationship between ROS and the hormones, which stimulates plant growth. It is true that the importance of hormones cannot be overlooked considering that the seed coat is a source of hormones for

the developing seed (Smýkal et al., 2014; Sabelli and Larkins, 2015). Mildažienė et al. (2017) also demonstrated how a dry seed undergoes subtle metabolic modulation by using proteomics. They showed that vacuum affected the auxin/cytokinin balance, cold plasma increased GA, and EMF decreased the amount of ABA and increased IAA and SA without changing GA. Despite this information, it was not possible to make a clear connection between phytohormones and germination kinetics. Ji et al. (2016) also observed an increase in GA in spinach seeds but did not measure any other hormones. The increase in GA may be the result of plasma-derived ozone (Sudhakar et al., 2011), although it is often speculated that specifically auxin and cytokinins are affected, which are hormones that increase and stimulate cell division, proliferation, and elongation. Pérez-Pizá et al. (2018) showed that with plasma treatment, there was a decrease in ABA and increase in IAA, a hormone which increases growth by regulating enzyme activity. They also detected H_2O_2, which coincided with an increase in ethylene after 24 hours of imbibition.

It may be that plasma-derived ROS regulate hormone production so, for example, increased auxin levels could be the reason for increased lateral root growth, as pointed out by Wang et al. (2018). The authors correlated increased auxin levels in tomato with lateral root growth under drought stress. Stolárik et al. (2015) found changes in auxin and cytokinins with a 2-minute plasma treatment in peas. They specifically found an increase in IAA, oxIAA, and zeatin, the most common cytokinin, and correlated this with increased growth parameters. This is logical since these two hormones work together; auxin is responsible for DNA replication and cell cycle initiation, whereas cytokinins trigger cell division and mitosis (Heldt and Piechulla, 2010).

Furthermore, it seems that plasmas may somehow modulate concentrations of hormones like auxin since this hormone also affects xylem differentiation (Fabregas et al. 2015). It has been observed that plasma treatment modified the root and stem diameters and altered tissue differentiation of xylem and phloem (Safari et al., 2017; Moghanloo et al., 2019; Gao et al., 2019; Seddighinia et al., 2019). These changes in root morphology may then enhance nutrient exchange (Jiang et al., 2018), which was also proposed by Zahoranová et al. (2016). Moreover, Babajani et al. (2019) pointed out that auxin transport may also be affected by NO, which is often produced in plasmas. Additionally, it could very well be the case that auxin biosynthesis is upregulated because it is known that abiotic or biotic stresses increase the shikimate pathway, which produces the precursor for IAA.

2.8.2 Metabolism

As stated previously, there is a link between hormones and metabolism and both primary and secondary metabolism, which are involved in growth and defense respectively, are altered after plasma treatment (Ljung, 2013) (see Figures 2.8 and 2.9).

Regarding primary metabolism, increased ATP and ethanol levels were observed, which suggests increased anaerobic respiration (Zhang et al., 2017). Moreover, higher concentrations of protein and sugar were observed by Islam et al. (2019) and Billah et al. (2020) due to an increase in reserve utilization enzymes. Billah et al. (2020) explained that this could be because H_2O_2 transduces the signal for soluble sugar synthesis and therefore, more soluble sugar and protein are seen after plasma treatment (Singh et al., 2019). Moreover, exogenous treatment with H_2O_2 can stimulate germination by breaking dormancy through the oxidative pentose phosphate pathway, which provides reducing power and carbon for the new growth (Tian et al., 1998).

This pathway also links primary and secondary metabolism because it provides the precursor for the shikimate pathway, which is important for plant defense.

Regarding secondary metabolism, an increase in enzymatic antioxidants, like catalase (Moghanloo et al., 2019), superoxide dismutase (Iranbakhsh et al., 2018), phenylalanine ammonia lyase (PAL), and peroxidases (Babajani et al., 2019), as well as non-enzymatic antioxidants, like total soluble sugar and proline, to better tolerate stress have been observed, likely due to the increased concentration of ROS. Furthermore, Ghasempour et al. (2020) showed an increase in phenols, chlorophyll, flavonoids, and alkaloids with plasma treatment. Pauzaite et al. (2018) saw changes in flavonoids after plasma treatment, while others have observed increased phenolic compounds (Scholtz et al., 2015; Ji et al., 2016; Filatova et al., 2020).

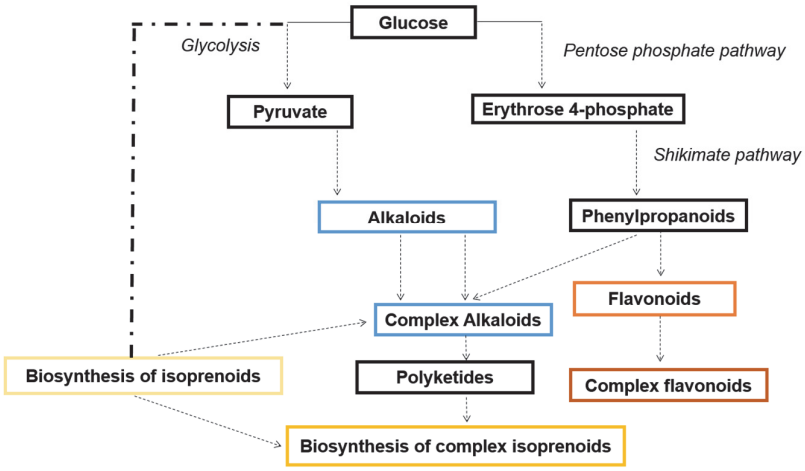

Figure 2.8. Pathways involved in the production of plant defense compounds (modified from Isah et al., 2018).

2.8.3 Defense

ROS may also trigger defense compounds which can originate from the shikimate and phenylpropanoid pathways to induce the production of precursors. For example, H_2O_2 can activate the shikimate pathway (Noctor et al., 2015; Moon and Mitra, 2016). ROS can also make the plant readily available in a redox state, as stated by Filatova et al. (2020). Iranbakhsh et al. (2017) suggests that the defense response, triggered by ROS and/or UV, modulates the hormone balance since they saw an increase in PAL, a key enzyme in the phenylpropanoid pathway. This pathway also produces protective proteins called pathogenesis-related proteins and depending on whether they are acidic or basic, they can be upregulated by salicylic acid and ROS or methyl jasmonate and ethylene, respectively (Jain and Khurana, 2016).

Although Perez et al. (2019) used plasma-treated water, they looked at several genes involved in plant defense in an infected tomato plant and saw an increase in the gene expression of *pal* (phenylalanine ammonia lyase) but not *pr1a, pr4, pr5* (pathogenesis related proteins) or *erf1* (ethylene response factor). The gene *pal* is involved in the phenylpropanoid pathway and makes defense compounds, like phytoalexins and phenolic compounds, and therefore, this information complements the increase in phenolic compounds seen experimentally by others who were previously mentioned in the metabolism section.

2.8.4 Gene expression

ROS can also affect gene transcription either directly or indirectly through hormone conjugates, as mentioned by Stolárik et al. (2015). Redox sensitive transcription factors are widespread among animal, bacteria, and plants (Grene, 2002), and these factors can be modified through the formation of disulfides upon sensing ROS (El-Maarouf-Bouteau and Bailly, 2008).

Unsurprisingly, there are others who think plasma also modifies gene expression, although molecular information concerning plasma-seed treatments is very limited. Hayashi et al. (2011) suggested that ROS generated in plasma with water vapor may be a method to control the redox state of the plant by changing the thiol quantity (oxidizing cysteine to cystine with OH radicals). This has an important role in gene transcription and therefore, can change the plant response.

Iranbakhsh et al. (2020) observed an increase in the transcription factor WRKY1 and other enzymes involved in secondary metabolism in plasma-treated hemp seeds. WRKY is of particular interest since it is a family of transcription factors involved in many biotic and abiotic stress responses, such as fungus, cold stress, salt stress, and drought tolerance. Furthermore, they are known for regulating phenolic compounds and there are specific factors, such as AtWRKY23, that regulate auxin. Moreover, there are also interactions between MAPK cascades and this WRKY family and can activate PR proteins (Phukan et al., 2016).

The other genes that have been studied were by Guo et al. (2017) who observed an increase in late embryogenesis abundant (LEA) chaperone during stress. Ji et al. (2016) saw increased expression of a hydrolytic enzyme called pullulanase in spinach seeds. Ghasempour et al. (2020) saw an increase in gene expression for DAT, an enzyme in the biosynthesis of vinblastine and vincristine, which are alkaloids with anti-cancerous properties. Islam et al. (2019) showed changes in gene expression of ascorbate peroxidase and catalase, but not superoxide dismutase with an air plasma in rapeseed.

These few preliminary studies demonstrate that plasma treatment can change gene expression for both primary and secondary metabolisms for growth and defense, respectively.

2.8.5 Epigenetics and genetics

In the short-term, these changes as a result of plasma treatment may be due to epigenetics by regulating DNA cytosine methylation, which inactivates gene expression (Hayashi et al., 2016; Mira et al., 2020).

Zhang et al. (2017) checked the methylation of genes involved in ATP synthase, an enzyme needed to produce energy for the cell, and TOR kinase, an enzyme which may increase metabolism and biosynthesis for energy and biomass production. They saw decreased methylation, meaning that the expression of these genes increased. Nevertheless, it cannot be ruled out yet that there may be changes to the DNA, considering that genotoxic effects from plasma treatment have been observed (Kyzek et al., 2017).

Although it was not verified, Mildažienė et al. (2017) found many similarities between cold plasma and electromagnetic treatment in protein expression but this may also be done epigenetically or through post-translational modifications. Despite the authors attributing the changes to radiation, it may be the action of hydroxyl radicals or other ROS since they too are produced from high energy radiation (Tuteja et al., 2001).

Additionally, it remains possible that the DNA repair process is triggered, for example by ozone, and this may be responsible for the improved germination kinetics (Kurek et al., 2019; Pandiselvam et al., 2019).

Lastly, the plant genome itself may play a role in sensitivity to plasma treatment. Kobayashi et al. (2020) treated *Arabidopsis* seedlings and saw that the ecotypes Col and L*er* responded differently to the same plasma treatment, although the results were not statistically significant. Lo Porto et al. (2019) also pointed out that asparagus germination is strongly influenced by ecotype, although they did not include this in their study. Therefore, the genetic component should not be overlooked when considering plasma-seed treatments. Studies should be done on multiple generations since plasma treatment can have long-lasting effects in the same generation, as observed by Sarinont et al. (2016), where the growth enhancing effects remained even after 17 months of storage of plasma-treated radish seeds.

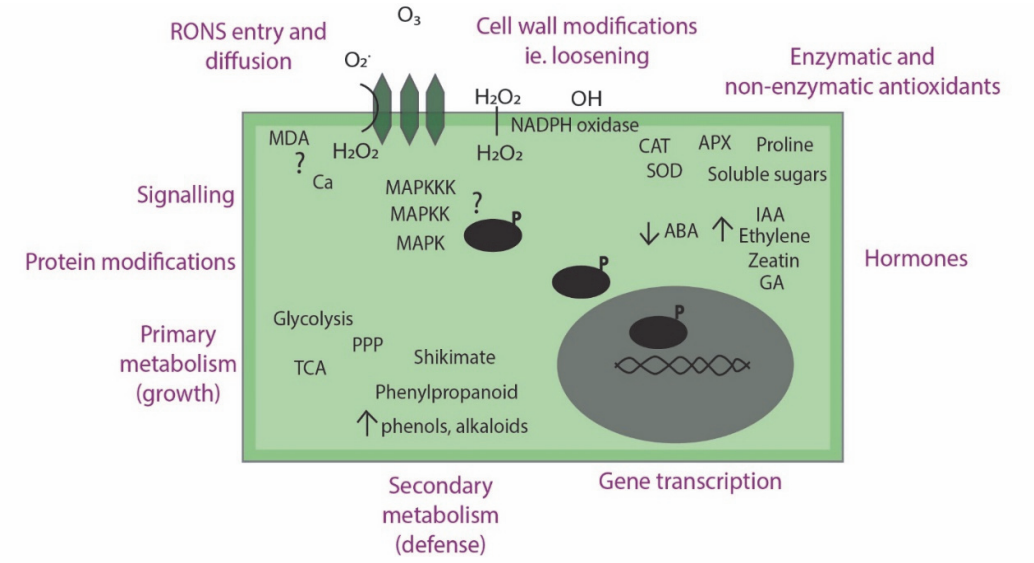

Figure 2.9. A summary of hypotheses and current evidence of plasma-seed treatments on a molecular scale.

In summary, it remains difficult to infer whether these effects are primarily attributed to the action of a single mechanism like mechanical scarification, chemical modifications or changes in biochemistry through ROS; even within ROS, each species behaves and functions differently. Additionally, variables such as the state of dehydration or hydration of the seed may influence the interaction and retention of ROS.

Ozone specifically in the presence of UV radiation may undergo reactions to eventually generate superoxide and hydroxyl radicals in the seed coat or these short-lived species may directly interact with the seed coat. This could be the critical point that determines how the external physical and chemical stimuli are transformed into internal biological stimuli.

The seed coat pigments can be altered and seed coat embedded enzymes, like superoxide dismutase, NADPH oxidases, or peroxidases, can transform these species into signalling molecules, like H_2O_2, which can more easily diffuse through the membrane or the apoplastic space, and then transduce this signal either through secondary messengers, like calcium and MAPK cascade, or be transmitted without the assistance of enzymes. Once the signal is sent, the hormonal balance may be modulated between GA and ABA to break dormancy, to increase auxin and cytokinin to accelerate the germination process, and increase ethylene, SA and JA for stress or disease resistance.

In parallel, metabolism may be modulated through the addition of water and enhanced gas exchange to modify enzyme activity in order to break down food reserves but at the same time, increase enzymatic and non-enzymatic antioxidants to shield against this sudden burst of ROS either externally derived or internally from metabolism. As a result of this stressful stimulus, metabolism in other defense pathways, like shikimate and phenylpropanoids, may be then activated to produce precursors in anticipation of future stressful events, such as hormones or protective proteins.

Depending on how the plant interprets the plasma and its unique composition, a spectrum of different responses can be elicited by the plant. For example, ozone from the plasma might trigger an ethylene response, ion bombardment may cause wounding and trigger ROS production, or the presence of H_2O_2 may trigger an apoptotic response. Figure 2.10 summarizes how different plasma components can trigger different plant responses.

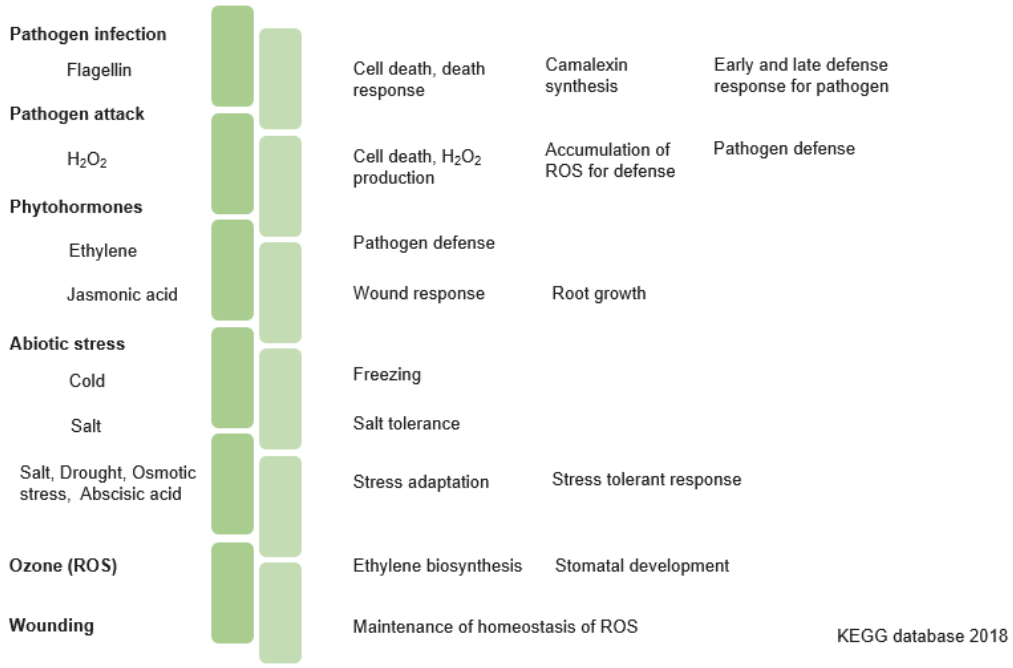

Figure 2.10. A collection of plant responses to abiotic and biotic stresses (modified from KEGG, 2018).

Although information is known about how each plasma component could possibly affect the growing seed, it is unclear whether this is done through one or multiple mechanisms and whether it is done synergistically. Furthermore, as evidenced throughout this chapter, many of the plasma components can have similar effects on the plant that can enhance germination properties, disease and stress resistance, and yield.

Chapter 3

Dielectric barrier discharges and plasma-seed treatment design

Abstract

In this chapter, plasma ignition mechanisms, such as Townsend breakdown and streamer formation, power supplies, and methodology for calculating power are briefly summarized. The design and development of several plasma sources are described and explained, with a focus on surface dielectric barrier discharges. The final plasma-seed treatment is described and characterized with power, ozone, humidity, and temperature measurements at the end of this chapter.

This chapter is partly based on the article cited below. I contributed towards the plasma source design, design of the plasma-seed treatment, and performed a majority of the characterization experiments, except for the power measurements.

SDBD operation in a humid environment: reversible and permanent degradation

Manuscript in preparation

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Keywords: SDBD, humidity, oxidation, dielectric, extinction, reproducibility

3.1 Introduction to plasma sources

There are many different configurations and geometries for plasma devices. Han et al. (2019) and Laroussi et al. (2020) describe the types of plasma treatments in detail, whereas Šimončicová et al. (2019) describe the types of plasma treatments for biological applications. Here, the most common plasma sources are shown in Figure 3.1 and are briefly described. From left to right, the planar dielectric barrier discharges (DBD) in various forms, DBD plasma jets, low pressure plasma in a vacuum chamber, and less common devices, such as gliding arcs and corona arrays, are shown. A DBD generates plasma by time-varying high voltage (kV) between two electrodes; the dielectric barrier prevents arcing between electrodes which could otherwise occur following electrical breakdown of the gas. The planar DBD may generate the plasma in the volume of gas between opposing electrodes (VDBD), or on the surface of a dielectric adjacent to the electrode edges (SDBD), or with embedded electrodes (diffuse coplanar surface barrier discharge (DCSBD). In a plasma jet, there is usually a gas flowing in a thin tube with a DBD excitation which can be pulsed DC, continuous AC, or RF. A corona discharge plasma jet (CDPJ) uses a parallel array of plasma jets with gas shielding. Low pressure plasmas include microwave plasma generated by a magnetron in the GHz range, and RF plasma (capacitively-coupled or inductively-coupled) which is usually sustained in the MHz range. A gliding arc device generates a controlled discharge propagating along two diverging electrodes in a gas flow at atmospheric pressure. In a corona, plasma is formed at the high voltage tip of a sharp edge and forms diffuse plasma toward the ground electrode; corona arrays have also been combined in a VDBD with a needle-to-plane matrix electrode.

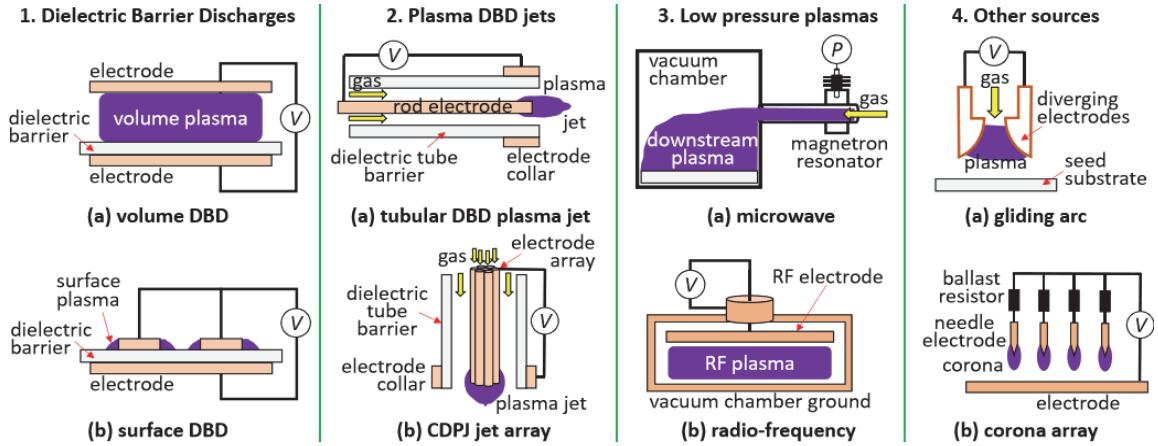

Figure 3.1. Sources of non-thermal plasma. V represents a pulsed or AC voltage source; P represents microwave power (courtesy of Dr. Alan Howling).

These devices can be used to treat seeds or seedlings directly with the plasma or indirectly at a distance away from the plasma. They can also be soaked or watered using liquids exposed to plasma. These liquids are considered as plasma-activated media (PAM) and if water is used, it is called plasma-activated water (PAW) or plasma-treated water (PTW). Comparing the gaseous and aqueous treatments, similar effects on macroscopic plant properties have been reported (Sivachandiran et al., 2017).

3.2 Dielectric Barrier Discharge

The focus of this thesis work is on DBDs, particularly SDBDs for the treatment of seeds. As briefly described in Chapter 2, non-thermal plasma is used in biological applications because of its unique property of attaining interesting, high-temperature chemistry at an overall low gas temperature. This phenomenon is dependent on the collision frequency, which is low in non-thermal plasmas. The efficient, inelastic collisions result in ionization yet inefficient, elastic collisions result in poor transfer of kinetic energy and an overall low temperature to generate a weakly ionized plasma (i.e. less than 1%). The electrical gas breakdown follows two mechanisms: Townsend breakdown and streamer formation.

3.2.1 Townsend breakdown

The Townsend breakdown operates on the principle that there are omnipresent free electrons near the cathode, generated by UV or cosmic irradiation. Once they interact with an electric field, the electrons accelerate and accumulate energy as they move towards the anode. The energy would need to be high enough for an elastic collision to become an inelastic collision, which is partly dependent on the electron mean free path. The longer the distance an electron can travel, the more energy it can accumulate and use to ionize a gas atom or molecule. Through the ionization from electron-molecule collisions, more electrons are freed so that an electron avalanche is gradually formed exponentially. From the cathode to anode, the freed electrons can be quantified as the number of newly formed electrons per electron per unit length and is known as the Townsend ionization coefficient alpha. As electrons are freed, positive cations are formed, which instead travel towards the cathode. It is possible to release new electrons from the ion-surface collision in a process called secondary electron emission or this can be done through photoionization from the UV photons. This is referred to as the Townsend ionization coefficient gamma (Kogelschatz, 2003; Kuechler, 2009).

This ionization efficiency and Townsend coefficients are taken into account in Paschen's Law where the efficiency is dependent on the mean free path. The path is dictated by the pressure and number of molecules in a given volume, the product of the pressure and gas gap distance, as well as the gas type and electrode material (Paschen, 1889). Overall, a sufficient density of molecules is required to have enough collisions to result in ionization, however, a high density of molecules can shorten the mean free path and make it difficult to ensure enough ionization energy. Within this range, as shown in Figure 3.2, a voltage which makes the gas conductive needs to be applied, which is higher than the voltage breakdown of the gas. Monoatomic gases, like argon, are more efficiently ionized compared to diatomic gases, like oxygen and nitrogen, because they lack ro-vibrational states, which would otherwise dissipate energy in low-energy, inelastic collisions. However, air as a working gas is less expensive for upscaling.

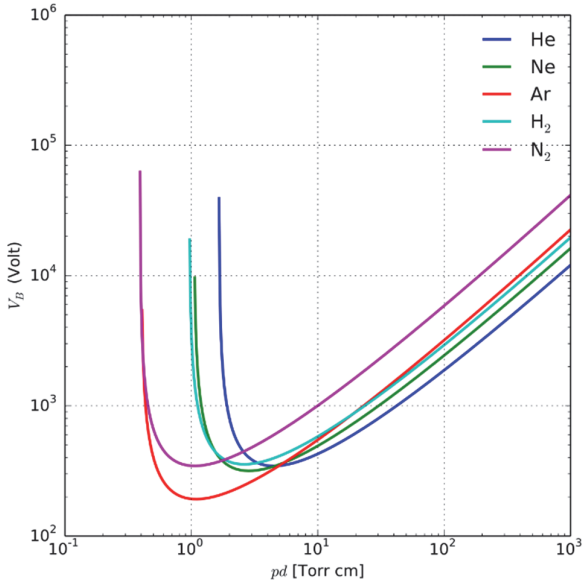

Figure 3.2. The electrical gas breakdown according to Paschen's Law. Voltage is a function of the product of the pressure and gap distance (Lieberman and Lichtenberg, 2005).

3.2.2 Streamer formation

In the event that there are sufficient number of electrons (~10^8), the avalanches transition into a streamer where the electrons collect at the head and the ions remain in the tail, as shown in Figure 3.3. This creates a difference in charge and therefore an electric field. This field further enhances the ionization where avalanches are multiplied, and eventually can connect the electrodes. This appears as lightning strikes or what is known as filamentary mode in plasmas. This process is faster than an avalanche and care must be taken that these streamers do not develop into sparks and eventually arcs, which can catastrophically melt the apparatus (Fridman, 2008).

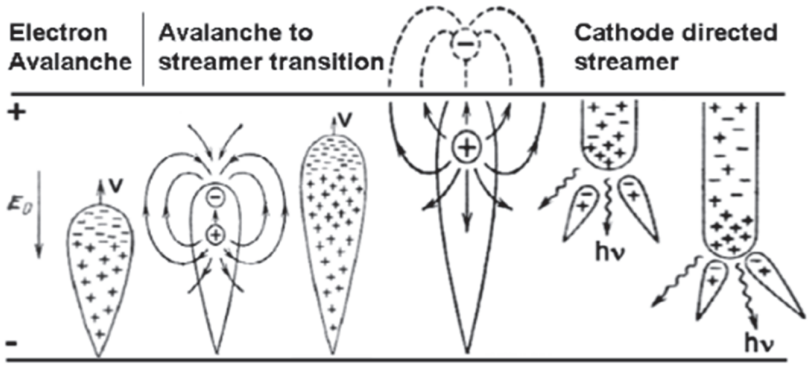

Figure 3.3. Townsend breakdown mechanism with avalanches transforming into a streamer (Fridman, 2005).

Therefore, the function of the dielectric barrier is to accumulate the charges, cancel out the local electric field, and extinguish the streamers. When the avalanche is being extinguished as a streamer, it generates so-called microdischarges. By distributing the charges evenly and minimizing the memory effects from the charge accumulation, a homogenous plasma can be attained, which is generally desired in biological applications as opposed to a filamentary plasma.

To ignite a plasma discharge, a sinusoidal time-varying voltage, like an alternating current (AC) power supply, or a pulsed power supply can be used. With an AC power supply, the polarity is inversed and therefore, charge accumulation is very short-lived (lifetime of DBD microfilaments: 10 – 20 ns). With a pulsed power supply, it can be arguably more energy efficient because there are two reactions for one action (Laroussi et al., 2004). First, there is a short voltage rise time, which is in the orders of nanoseconds or microseconds, and it is extinguished eventually by the charge accumulation and therefore, the local electric field. As the voltage drops, the electrons are dispelled spontaneously into the gas without any additional power input, causing a second discharge. This effect can be mimicked by using a duty cycle, such as an AC power supply with a modulation frequency to control the on/off time. Arguably, the advantages of using these methods are on one hand, avoiding excess heat during the plasma-seed treatment and on the other hand, influencing the chemistry where RONS have the opportunity to distribute themselves homogeneously (Kogelschatz et al., 1997; Meiners et al., 1998; Mildren et al., 2001).

3.2.3 Plasma characterization: power calculations with Lissajous figures

The plasma is defined by the microdischarges and this is influenced by the power density, which is the amount of energy applied over a given area. Microdischarges are very short-lived and operate within a nanosecond timescale. These rapid current pulses are very difficult to measure accurately with current probes and therefore, a solution was developed by Manley (1943). A capacitor probe is used in series between the DBD and the ground to measure the charge, which is the time integral of the current, in a given period in order to measure the energy-per-cycle. In so-called Lissajous figures, a parallelogram is produced where charge is plotted against the voltage and the energy-per-cycle can be calculated from the area within this parallelogram. Instantaneous power, written as P(t), is the product of the instantaneous voltage and current, V(t) x I(t), whereas energy is the sum or integral of power with respect to time. The energy dissipated in one cycle is the integral of P(t)dt over one cycle, which is measured in a Lissajous figure. The time-averaged power is the energy-per-cycle multiplied by the number of cycles per second i.e. the energy in the Lissajous figure multiplied by the frequency (Figure 3.4).

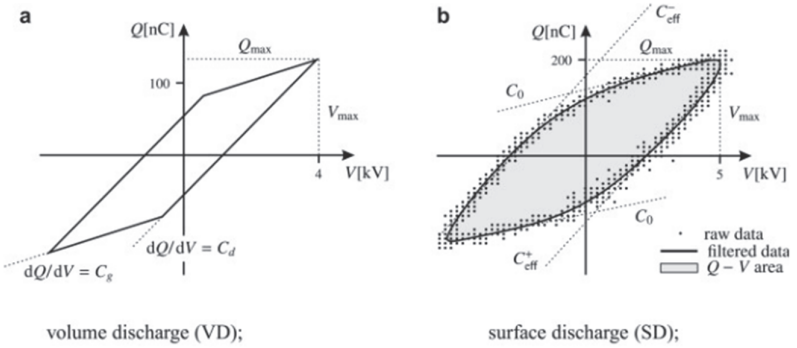

Figure 3.4. Lissajous figures used to calculate the plasma power for (left) a volume DBD and (right) a surface DBD (Kriegseis et al., 2011).

This parallelogram is formed because there are two phases in the plasma. The DBD acts as a capacitor by storing charge, and is then followed by the gas breakdown and formation of microdischarges. These two events occur sequentially for both increasing and decreasing voltages. The capacitor in series can capture the charge of the accumulated microdischarges. Afterwards, the power can be calculated using the energy consumed in one period, by multiplying the average energy-per-cycle over a defined number of cycles by the frequency, where the number of cycles is determined by the number of sweeps performed.

3.3 Design and characterization of the first DBD and plasma-seed treatment prototypes

Due to the plethora of plasma sources, a literature search was conducted and combined with previous, personal experience with commercial and custom-built devices to determine the design criteria for the plasma-seed treatment. These qualities included: operation at atmospheric pressure, air as an input gas with possibly a gas flow, type of electrode material, shape of electrode geometry, adjustable height, possibility to change seed movement and location, option to use plasma diagnostics, and option to use various waveforms and operating parameters. Therefore, a SDBD was preferred over a VDBD due to the flexibility in treating the substrate, regardless of its geometry and size. Moreover, the possibility to modify the distance between the plasma and seed substrate would allow for the comparison of direct, indirect, and remote treatments. With a VDBD, the treatment would be largely restricted to direct treatments in most instances because the plasma typically encompasses the substrate.

The first SDBD prototype was built using copper striped electrodes on a copper plane, separated by Kapton as a dielectric (Figure 3.5). It was embedded in a 3D printed casing with dimensions that fit a square Petri dish to be able to treat *Arabidopsis* seedlings planted in agar or to treat seeds directly or indirectly.

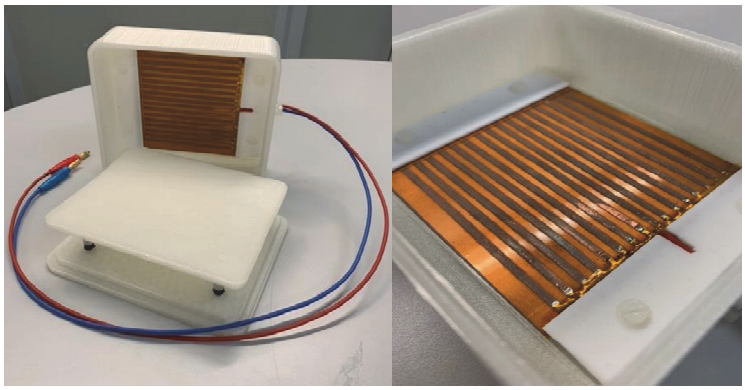

Figure 3.5. The first SDBD used in plasma-seed experiments with copper electrodes and Kapton dielectric.

The dimensions of the plasma device were 10 x 10 cm copper ground electrode, layered with 0.6 mm thick Kapton tape, and then covered by 16 high voltage copper electrodes (3 mm thick and 9 cm long with a 3 mm gap between each electrode). It was ignited using either an AC or nanopulse power supply at 1 kHz and 3 kV breakdown voltage. The temperature of the ground electrode was measured using thermocouple and was 28°C after a 10-minute treatment. The power was calculated using the Lissajous method and was approximately 5 W when powered by AC. However, it was not possible to calculate it when powered by nanopulse because of the following reasons: irregularity in the system's capacitance, the imperfect pulse and noise from the first nanopulse power supply used, insufficient frequency response of the voltage probes for the fast ramp speed, and possible plasma-ignition-time changes between pulses. All plasma-seed treatments with this prototype were performed in open air under ambient conditions (40 - 60% RH, 23ºC).

In the event of an arc or other damage to this prototype, it would need to be completely replaced by hand which would then introduce variation and possible errors between experiments. Therefore, a more sophisticated approach needed to be taken to ensure reproducibility so the design of this first prototype was the basis for the next generation of SDBDs which were outsourced for manufacturing. Different combinations of electrode widths, dielectrics (F4R or Kapton), and electrode coatings (gold coating on copper electrodes or

solderresist on copper electrodes) were designed and manufactured (Table 3.1). The parameters used to achieve breakdown voltage were 1 kHz using a 200 nanosecond pulsed power supply and measured from peak-to-zero (V_{p-0}). The temperatures of the SDBDs without and with the copper heat sink were measured after 2 and 10 minutes, respectively, using a thermal imaging camera (FLIR).

Table 3.1. Voltage breakdown and temperature characterization of the next generation SDBDs.

SDBD type	Breakdown voltage (V_{p-0})	Full plasma voltage (V_{p-0})	Temperature	Temperature with copper
Coplanar, rigid F4R	2.8 kV	3.8 kV	32.5°C	26°C
Interdigitating, rigid F4R	2 kV	5.7 kV	75°C	not selected
Interdigitating, flexible kapton	1.5 kV	5.5 kV	110°C	not selected
Coplanar, flexible kapton	2.4 kV	2.8 kV	34°C	30°C
Interdigitating, flexible, solderresist	2.3 kV	6.2 kV	60°C	not selected
Coplanar, flexible, solderresist	n/a	n/a	not operable	not selected
Coplanar, rigid, solderresist	3.4 kV	4.3 kV	32°C	28°C
Interdigitating, rigid, solderresist	2.5 kV	6.5 kV	75°C	not selected

As an example, the coplanar, rigid printed circuit board was made of a 0.3 mm thick FR4 plate with gold-coated copper electrodes on each side. The copper electrodes were coated with electroless nickel immersion gold (ENIG), a nickel plating which was covered by a layer of gold. The gold layer protects the copper electrodes against corrosion, while the nickel underneath enables easier soldering. FR4 is a dielectric material made of glass fibres in an epoxy resin. It was designed to have the high voltage electrodes cut in the shape of 2 mm spaced and 2 mm wide fingers and a 0.1 mm thick copper plane as the ground electrode. The diameter of the circular SDBD was 7.4 cm to mimic the shape of standard Petri dishes with a 9 cm diameter.

From these 8 different combinations of SDBDs in Table 3.1, the SDBDs with the lowest temperatures and breakdown voltages using a nanopulse power supply were identified and selected for the plasma-seed treatments. It was evident that the coplanar SDBDs remained at a lower temperature and therefore, interdigitating SDBDs were not included in any of the experiments within this thesis. Furthermore, the ignition functioned better without solderresist and thus, coplanar rigid F4R and coplanar flexible Kapton SDBDs were chosen, as shown in Figure 3.6. The first two SDBDs from the left are referred to as SDBD1 and SDBD4 respectively, for the rest of this chapter.

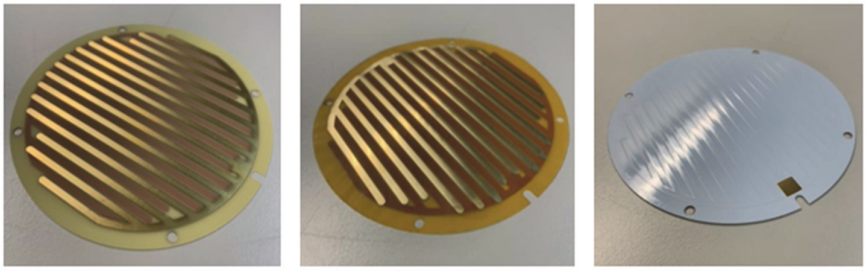

Figure 3.6. SDBD prototypes for plasma-seed treatments. From left to right, a SDBD with FR4 (SDBD1), kapton (SDBD4), or solderresist. The first two from the left were used for subsequent plasma-seed experiments.

SDBD1 and SDBD4 were powered either with an AC power supply (Trek Model 615-10) which generates a sinusoidal voltage at 1 kHz, with variable amplitude, and variables voltage between V_{pp} or V_{p-0} = 1−6 kV, or with a custom built nanopulse power supply, which generates a voltage at 1 kHz with a 200 ns pulse. At the time, the electrical limitations of the power supply were a maximum of 7 kV for long pulses or 10 kV for short pulses.

The parameters and experimental conditions using these two SDBDs to treat *Arabidopsis* seeds or seedlings are described in Table 3.2. The goal was, first and foremost, to find an effect which alters the root length, shoot length, or germination rate of *Arabidopsis*. Afterwards, this would be followed by studying the plasma components separately to identify the mechanism behind the plasma-induced plant effect i.e. similar but separate temperature, ozone, or electric field treatment.

Table 3.2. Parameters for SDBD1 and SDBD4 treatments on *Arabidopsis thaliana* seeds.

DBD	Power supply	Treatment type (V_{p-0})	Time points
Coplanar, rigid F4R or coplanar, flexible kapton	Nanopulse	direct at 3.4 kV	0.5, 1, 2, 5, 10 min
Coplanar, rigid F4R or coplanar, flexible kapton	Nanopulse	indirect at 3.4 kV and 1-3 mm gap distance	1, 2, 5, 10, 15 min
Coplanar, rigid F4R or coplanar, flexible kapton	Nanopulse	direct at 1.9 kV (only electric field and no plasma)	0.5, 1, 2, 5, 10 min

Seeds treated with SDBD4 were considered as a higher priority than those treated with SDBD1 because of the lower breakdown voltage. Therefore, subsequent germination and seed surface analysis experiments were solely performed with SDBD4 (see supplementary section, Chapter 4). The voltage waveform of SDBD4, the actual voltage arriving to the SDBD, and the corresponding temperature are given in Figure 3.7 and Table 3.3.

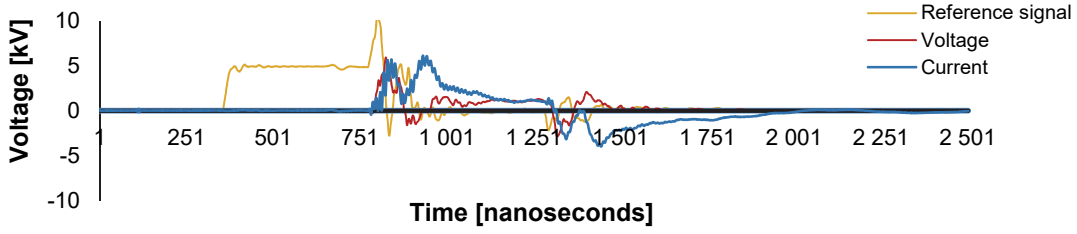

Figure 3.7. Voltage and current waveform of SDBD4 using nanopulse power supply (courtesy of Dr. Gennady Plyushchev).

Table 3.3. Voltage and temperature measurements of SDBD4 with Kapton dielectric powered by the nanopulse.

Voltage (V_{p-0})	3.5 kV	4 kV	4.5 kV	5 kV	5.5 kV	6 kV	6.5 kV
Actual voltage (V_{p-0})	2.3 kV	2.7 kV	3 kV	3.3 kV	3.5 kV	3.8 kV	4 kV
Temperature at 5 min	24ºC	--	28 ºC	--	30 ºC	--	32 ºC

In the meantime, the electric field was measured in order to simulate a similar, but separate treatment without the plasma for subsequent experiments with seeds. Using COMSOL, the simulations revealed that the electric field is very close to the electrodes and therefore, only the seeds resting on the electrode edges would experience the effect of a strong E-field of 1 MV/m for 1 kV.

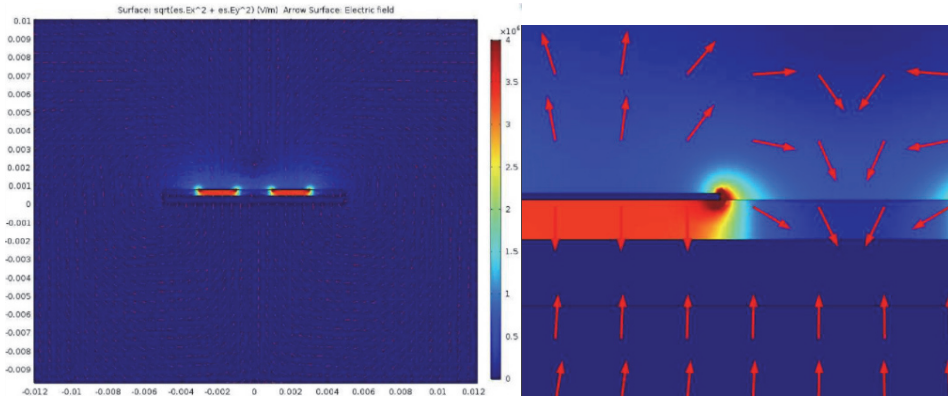

Figure 3.8. Electric field simulations of SDBD4. The small electric field around the electrode edges demonstrates the importance of seed location on the SDBD for direct and indirect treatments (courtesy of Dr. Riccardo Agnello).

However, there was again the question of reproducibility because of the contradicting germination and bioassay results (see Chapter 7). This was eventually linked to an empirical observation of the plasma brightness dimming over time, and the build-up of a white oxidation layer around the metallic electrode edges. These effects were further investigated and in conclusion, the SDBD was aging over time with prolonged use. It seemed to be mainly attributed to humidity, where the damage was either reversible or irreversible depending on the experimental conditions. The effects of increasing humidity are shown in Figure 3.9 where exposure to 50% or more relative humidity dims the plasma, giving a patchy and non-uniform appearance.

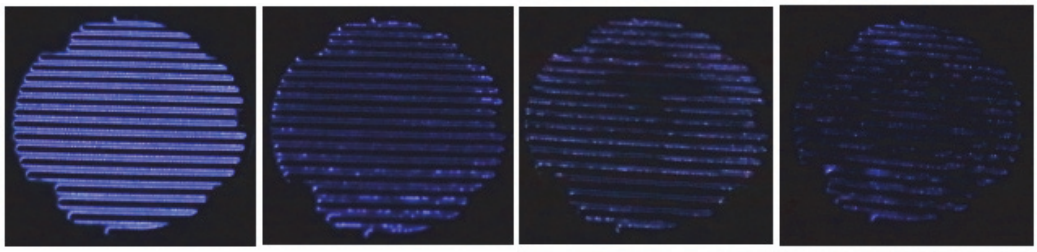

Figure 3.9. SDBD plasma appearance in ambient and humid conditions. From left to right, increasing humidity from 36% to 86% RH results in extinguished plasma. Photographs of the plasma visible emission after 30 minutes continuous operation at relative humidities of 36%; 50%; 71%; and 86%. The humidifier was off for the ambient air relative humidity of 36% (courtesy of Marion von Allmen).

After observing the SDBD deterioration and nanoparticle deposition from the electrodes (see Chapter 4), experiments were instead done with the Sihon Electronics mesh, stripes, and honeycomb printed SDBDs in an attempt to minimize or avoid altogether the nanoparticle deposition. Moreover, in hopes of improving the reproducibility of plasma-seed treatments, the hypothesis was that they would more robust and similar between replicas since they are commercially manufactured.

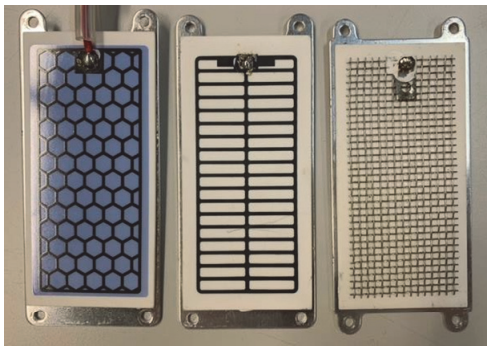

Figure 3.10. Commercially available Sihon Electronics SDBDs used for the current plasma-seed treatments. From left to right, honeycomb, stripes, and mesh patterned SDBDs are shown.

The Sihon Electronics SDBDs were used initially for direct plasma-seed treatments, without enclosure and under ambient air and humid conditions of 40 - 60% RH, to ensure sufficient interaction between the seed and the plasma. However, the operating parameters of the plasma-seed treatment were soon after optimized and changed from a direct to an indirect treatment for the following germination, FTIR, and RNA sequencing experiments (see Chapters 4, 5, and 6).

3.4 Development and characterization of the current plasma-seed treatment

At the end of 2019 and onwards, the plasma-seed treatment was modified and optimized into a completely different SDBD plasma-seed treatment. This was done by conducting the experiment within an enclosed, in-house built reactor, adding a controlled gas flow, monitoring and controlling the humidity, and changing the waveform by replacing the nanopulse with a modulated AC power supply. Each of these features will be elaborated in the next section. The investigation included both mesh and stripes SDBD but was later narrowed down to the stripes SDBD (see Chapter 5).

Since humidity was an issue with SDBD4, the behaviour of the Sihon Electronics SDBDs under relative humidity levels was first investigated. As shown in Figures 3.11 and 3.12, it too revealed that low humidity needs to be used for optimal plasma brightness, assuming the treatment is done without additional gas flow.

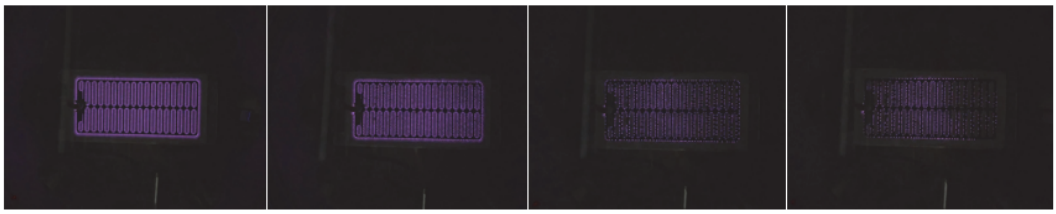

Figure 3.11. Sihon Electronics stripes SDBD powered with AC. Plasma is increasingly extinguished with increasing humidity. From left to right, SDBD operation is in 20%; 30%; 40%; and 50% relative humidity.

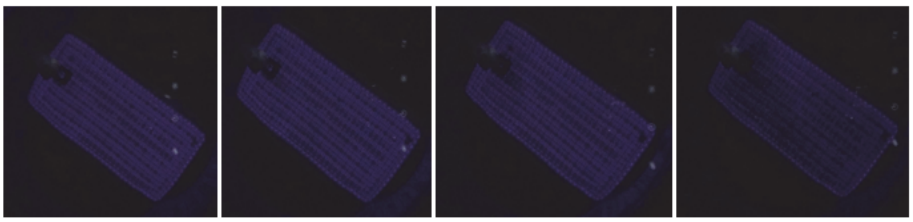

Figure 3.12. Sihon Electronics mesh SDBD powered with AC. Plasma is partially extinguished with increasing humidity but maintains plasma brightness better than the stripes SDBD. From left to right, the SDBD operation is in 20%; 30%; 40%; and 50% relative humidity.

Preliminary experiments rarely controlled the humidity until the humidity issue was investigated with an ultrasonic humidifier connected to an automatic regulator (HumidiKit, Incubator Warehouse). The humidity controller measures the relative humidity inside the reactor and is used to set the desired humidity value. Two parameters can be changed: the intensity of the humidifier, which was kept at minimum, and the relative humidity threshold of the regulator. Interestingly, in open air, there were no issues provided that the SDBD was not previously saturated with high RH. Through these types of experiments, it was determined that introducing a gas flow would be beneficial for monitoring and maintaining uniform plasma ignition. Since there were fluctuations in the humidity levels using the HumidiKit, a mass flow controller was installed and dry, synthetic air was used with 2 L/min as a standard operating parameter and 1.5 - 3% RH for the plasma-seed treatments.

The temperature of the SDBDs was first measured using a thermocouple attached to the ground electrode for safety reasons and the electrode was initially in contact with a copper disc heat sink (Figure 3.13).

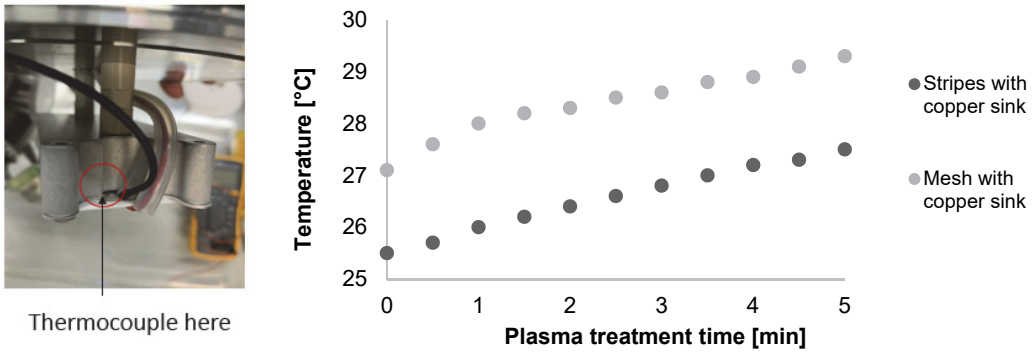

Figure 3.13. Temperature measurements of Sihon Electronics stripes and mesh SDBD using a thermocouple attached to the ground electrode. Minor temperature increases observed with a copper heat sink.

As mentioned previously, a thermocouple was used, however, the copper heat sink was later removed to maintain a consistent setup to that of our collaborator, Dr. Paolo Ambrico, where our goal was to ensure treatment reproducibility independently. For the sake of simplicity, a thermal camera (FLIR E85) was later used instead to take infrared images of the SDBD front electrode and dielectric during plasma treatment. Without the copper heat sink, the temperatures were higher but still acceptable for a plasma-seed treatment with a short treatment time and 10% duty cycle (Figure 3.14). The continuous wave operation reached temperatures as high as 58.3°C using an 80 s treatment time, whereas a maximum of 31.8°C, with only a temperature change of approximately 4°C, was reached with a 10% duty cycle.

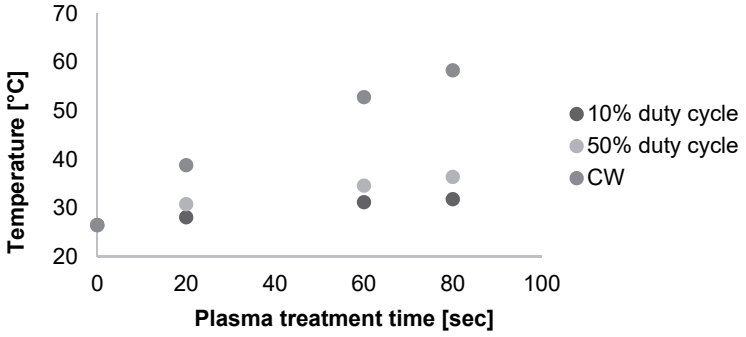

Figure 3.14. Infrared temperature measurements of Sihon Electronics stripes SDBD front electrodes. SDBDs were powered by a 10% duty cycle, 50% duty cycle, or continuous wave, without a copper heat sink.

3.4.1 The final plasma-seed treatment description

In conclusion, the SDBD electrode assembly comprised of a high-voltage printed electrode in a stripe pattern on an alumina dielectric plate, placed on an aluminium ground electrode. The airtight stainless steel reactor chamber, 18 cm diameter and 11 cm high, was used to confine the SDBD air plasma and its gaseous products. The *Arabidopsis* seeds were placed on Teflon cylinders, several mm below the SDBD plasma, or on ceramic plates to reduce the seed-plasma gap as shown schematically in Fig. 3.15b. The feed gas system of a Bronkhorst mass flow controller provided the flow from a bottle of dry synthetic air (80:20 N_2:O_2) into the sidewall of the reactor as shown by the transparent tube in Fig. 3.15a. The exhaust gas flowed to the intake of a ventilation extraction system.

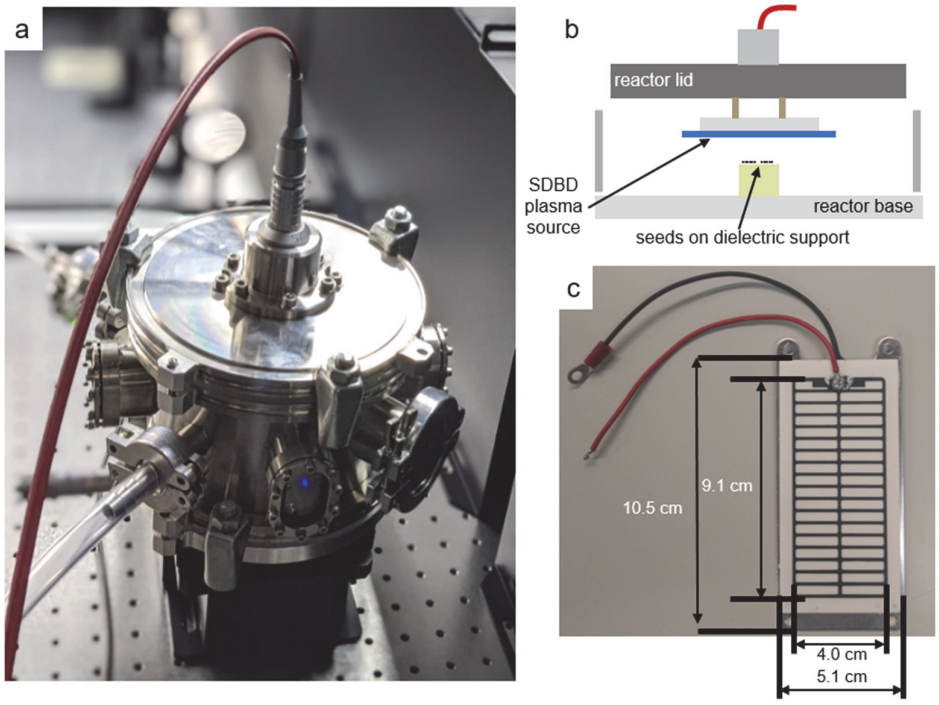

Figure 3.15. The latest plasma-seed treatment setup (a) Stainless steel reactor with high voltage coaxial cable connection (b) the interior of the plasma-seed treatment with the inverted SDBD positioned above the seed substrate (c) the high voltage stripe SDBD electrode printed on an alumina dielectric plate. The ground electrode is an aluminium plate behind the dielectric (schematic and reactor photo courtesy of Max Leftley and Lorenzo Ibba, respectively).

3.4.2 The final plasma-seed treatment operating parameters and characterization

The operating parameters are listed in Table 3.4. The default parameters were 1 min delay with flow flushing before treatment, 10 kHz, 8 kVpp, 60 s plasma treatment time, 3.7 mm distance between seeds and plasma, and 2 L/min of dry synthetic air (80:20 N_2:O_2). The reactor total volume was 2.8 L with an internal gas volume of about 1.0 L. For the flow rates of 2, 4, and 5 L/min, the gas residence times were therefore 30, 15, and 12 s respectively, which are of the same order as the treatment time of 20, 60, and 80 s. Therefore, the time dependence of each species is a complex convolution of its production rate by the plasma, any secondary reactions in the gas or on surfaces, and its loss rate by convection in the air flow (Sakiyama et al., 2012). This depends also on how long the seeds are left in the reactor after the plasma treatment.

Table 3.4: Operating parameters for plasma-seed treatments with default, standard values.

Parameter	Parameter values	Standard or Default value
Voltage	6.5, 7.5, 8.5, 9.5 kV	8 kV
Frequency	5, 7, 10 kHz	10 kHz
Distance	3, 3.7, 4.3 mm	3.7 mm
Flow rate	2, 4, 5 L/min	2 L/min
Time	20, 60, 80 s	60 s

The source voltage waveform for all excitation frequencies was a burst of 2 sinewave cycles with a 500 Hz on/off power modulation, provided by a Rigol DG4102 signal generator amplified by a Matsusada AMPS-20B20-LC(5m) power supply. At 10 kHz sinewave frequency and 8 kV peak-to-peak, 2 cycles modulated at 500 Hz corresponded to a 10% duty cycle, similar to the technique used by Ambrico et al. (2020). The waveform is consistent and reproducible over time, although it is distorted by the high voltage amplifier (Figure 3.16).

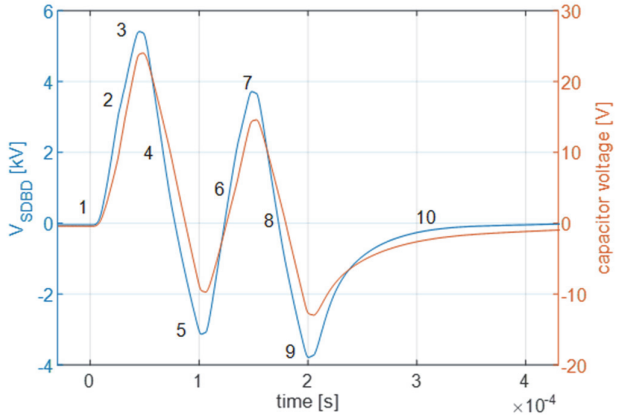

Figure 3.16. The voltage waveform of the SDBD plasma-seed treatment for nominal 8 kV peak-to-peak. The blue curve shows the voltage measured across the SDBD; the orange curve shows the voltage across the 68 nF series capacitor used to measure the charge on the electrode. The waveform distortion is caused by the transient response of the high voltage amplifier when using high frequency bursts. The numbers are used as references for Fig. 3.17 to highlight specific key points (courtesy of Lorenzo Ibba).

The SDBD voltage and the capacitor charge deduced from Fig. 3.16 were used to measure the power dissipated in the plasma using the Lissajous figure method (Manley, 1943). Since the 2 cycles were not identical due to the transient distortion by the high voltage amplifier, the resulting Lissajous figure shown in Fig. 3.17a is not conventional. The locus is not a parallelogram because the waveform is not a continuous sinusoidal wave, but a burst whose voltage converges to 0 every 2 cycles. This produces the discontinuity at the origin visible in the figure. Nonetheless, the area within the locus of the voltage vs charge contour for each cycle represents the energy-per-cycle dissipated in the plasma and was reproducible. Accounting for the 500 Hz modulation frequency of the burst, the time-averaged dissipated power for different voltages is shown in Fig. 3.17b. The power was calculated to be 1.2 - 3 W for nominal voltages, ranging from 4 to 9 kVpp. If the entire striped area of the SDBD is taken into consideration, the corresponding power density is 0.03 - 0.08 W/cm^2 and therefore, was operating in ozone mode (see Chapter 5).

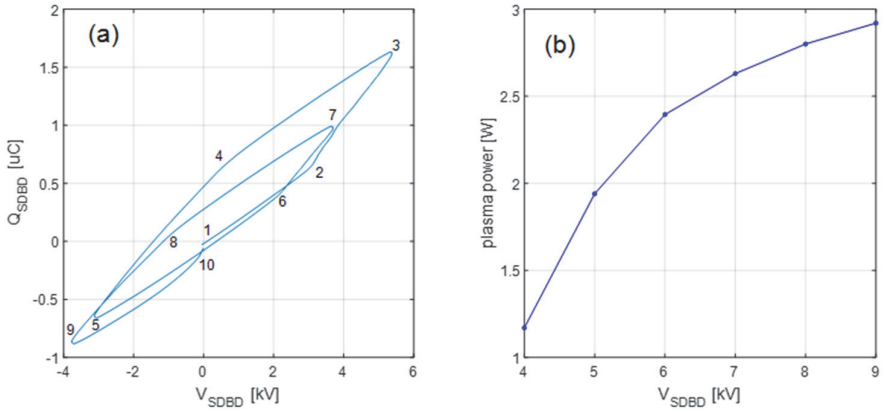

Figure 3.17. (a) Lissajous figure of the SDBD plasma for the two-cycle waveform at 8 kVpp nominal voltage (b) power calculation for nominal voltages 4 - 9 kVpp. The numbers are used as references for Fig. 3.16 to highlight specific key points linked to the waveform (courtesy of Lorenzo Ibba).

Humidity was measured using a Vaisala model HM42 probe and ranged between 1.5 - 3% RH. This low humidity is consistent with the use of dry synthetic air and only small outgassing of humidity from the reactor walls. Ozone measurements were taken independently and at a later time with Eco Sensors model UV-100 ozone analyzer (see Chapter 7). The highest ozone absorbance at 80 s corresponded to 160 ppm at the time of measurement, however, there may be fluctuations in the values due to the aging of the DBD electrode.

The temperature was measured at the centre of the SDBD with a FLIR E85 infrared camera and the measurements for 20 s, 60 s, and 80 s were 28.1°C, 31.2°C, and 31.8°C respectively using 10% duty cycle, whereas using the original AC continuous power source provided with the SDBD was 38.8°C, 52.8°C, and 58.3°C respectively. The 10% duty cycle was selected for lower temperature plasma treatment to avoid overheating the seeds. The advantage of using pulsed power duty cycle control is that high voltages can be used to ensure plasma ignition, whilst nevertheless maintaining a low time-averaged power for ambient temperature experiments. High flow rates may also be useful to cool the seeds by convection, but this dilutes the gas species, which are the active components of the plasma treatment.

Chapter 4

Surface analysis of plasma-treated seeds: which component of the plasma affects the seed surface and how?

Abstract

Surface characterization of plasma-treated seeds has made significant progress over the last decade. Most papers in the literature use scanning electron microscopy (SEM) and contact angle goniometry to investigate surface modifications. However, very few papers address the chemical modifications to the seed coat after plasma treatment. Here, a summary of the methods used to analyze plasma-treated seeds is presented, such as SEM, contact angle goniometry, energy-dispersive X-ray spectroscopy (EDX), X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). Using SEM, EDX, ATR-FTIR, and XPS, untreated *Arabidopsis thaliana* Col-0 seeds were analyzed and compared to seeds treated with heat, ozone, or plasma. SEM revealed erosion and XPS revealed surface oxidation exclusively in plasma-treated seeds. This suggests that the synergy of different plasma components or another component of plasma is responsible for change instead of the individual heat or ozone treatments, and this may be attributed to the etching ability of plasma. Overall, in these set of experiments, it was concluded that XPS was the most useful surface analysis technique on *A.thaliana*.

This chapter is based on the perspective paper cited below. I conceptualized the study, designed the experiments, worked alongside facility members for each diagnostic, analysed the data, wrote the manuscript, and handled the submission process.

Frontiers in Materials

Advantages and limitations of surface analysis techniques on plasma-treated *Arabidopsis thaliana* seeds

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4.1 Introduction to surface analysis methods for seeds

Biological applications of low temperature, non-thermal plasmas have recently become widespread from medicine, environmental remediation, and sterilization, to agriculture. The interest in plasma agriculture continuously increases as scientists demonstrate the potential of improving plant growth parameters, such as germination, crop yield, and disease resistance when seeds are treated with plasmas (Attri et al., 2020). However, it remains unclear which plasma properties are responsible for these plant growth effects. To gain a better understanding, surface characterization of plasma-treated seeds has the potential to provide both structural and chemical information.

Scanning electron microscopy (SEM) targets a beam of primary electrons which are aimed at the sample under vacuum. Electrons are collected to produce a high-resolution image so that surface morphology information can be obtained; these electrons are either backscattered primary electrons reflected from the sample, or secondary electrons displaced from the sample by the primary electrons.

Seed coat erosion in plasma-treated seeds is the most common effect observed by SEM (Stolárik et al., 2015; Junior et al., 2016; Ambrico et al., 2017; da Silva et al., 2017; Li et al., 2017; Wang et al., 2017; Gao et al., 2019) but this does not necessarily occur in every instance. For example, Mildažienė et al. (2016) noticed etching only on the seed surface facing the plasma, while other authors did not remark any (Sera et al., 2010; Bormashenko et al., 2012; Kitazaki et al., 2014). Cui et al. (2019) showed SEM images of *Arabidopsis* seeds after various plasma treatment times and observed detached epidermis with increasing exposure. However, adhesive tape was used to hold the seeds during the plasma treatment which may or may not have affected the results. This inconsistency in seed coat erosion may be partly due to the seed type, as described by Molina et al. (2020), who noticed that nasturtium seeds were less prone to etching than wheat seeds under the same experimental conditions (Molina et al., 2018). SEM can also be used to observe surface roughness where Pawlat et al. (2018a) interpreted the more creased appearance as increased microroughness. Alternatively, atomic force microscopy (AFM) can be used to observe and measure poration or surface roughness. Volkov et al. (2019) used AFM to show an increase in poration on pumpkin seeds after plasma treatment and Holc et al. (2019) found that plasma removed the wax layer from the garlic tissue via etching.

Another method coupled to SEM is energy-dispersive X-ray spectroscopy (EDX). EDX uses high energy electrons to collect information about the chemical composition of a sample by capturing X-rays. The electron beam can displace an electron from a low energy level and when this vacancy is replaced by an electron from a higher energy level, the excess energy is released as an X-ray, which characteristic for each element. Therefore, EDX can be used to identify the elements and provide both the relative concentration and spatial distribution in a cartography map.

EDX has been used on plasma-treated seeds, for example, by Cui et al. (2019) on *Arabidopsis* seeds. They observed an increase in O concentration from approximately 38% to 45% and a decrease in C concentration from 62% to 55% in the untreated and plasma-treated seeds, respectively, indicating carbon oxidation. Using cartography images, Gómez-Ramírez et al. (2017) and Molina et al. (2018, 2020) observed a migration of potassium from the interior to the exterior in cross-sections of quinoa, nasturtium, and wheat seeds after dry air plasma, and helium plasma treatment, respectively. Molina et al. (2020) attributed the initial movement of potassium to the polar-containing groups on the seed coat. Gómez-Ramírez et al. (2017) pointed

out that, with the addition of water vapor, the potassium and nitrogen-containing groups decreased, presumably because they were absorbed by the seed and therefore could improve seed germination.

In contrast, X-ray photoelectron spectroscopy (XPS) is the inverse of EDX where X-rays are used, instead of an electron beam, and the results are obtained from the kinetic energy of the electrons ejected from the first few nanometers of the sample, instead of characteristic X-rays. Furthermore, XPS provides information about chemical composition, empirical formula, chemical state, and electronic state of the elements. Higher penetration depth can be achieved with other methods, like hard XPS.

Similar trends observed with EDX have been confirmed by XPS, generally revealing an altered C/O ratio, increased potassium and sometimes, increased nitrogen concentration after plasma treatment. Depending on the seed type and plasma setup, the changes can range from a 5% to 30% increase in oxygen. Specifically, there is an increase in C-O, O-C=C bonds at the cost of C-C or C-H bonds. Gomez-Ramirez et al. (2017), Molina et al. (2018, 2020), and Holc et al. (2019) all demonstrated this with quinoa, nasturtium, wheat grains and garlic, respectively. Molina participated in two separate studies where nitrogen increased in quinoa, but not in wheat grains. However, different plasmas were used, where quinoa was subjected to an air plasma and wheat grains to helium plasma with impurities. In both circumstances, the seed surfaces were oxidized. Similarly, the oxidation of hydrocarbons was also detected on plasma-treated thermally modified wood by Talviste et al. (2019) using a diffuse coplanar surface barrier discharge (DCSBD) air plasma. Therefore, it seems that oxidative etching plays a role in plasma-seed interactions, whether the plasma is removing the top layer to expose layers that are more oxygen-rich, or by oxidizing the surface directly.

XPS can investigate the depth of the sample within the first few nanometers, whereas attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) has a higher penetration depth and therefore, can analyze several layers.

ATR-FTIR provides information about the inorganic and organic compounds at the surface using infrared spectroscopy and has a penetration depth in the μm range. An infrared beam directed at the sample is partly absorbed at the seed surface during total internal reflection on a transparent substrate. Depending on how much is absorbed, there is a characteristic spectral fingerprint for each compound, where specific chemical groups can be recognized according to their wavenumber.

Kitazaki et al. (2012) did not observe any differences on radish seeds after plasma treatment, however, other authors have similar findings to the XPS results. Zahoranova et al. (2018) found an increase in C=O and decrease in hydrocarbons after treating maize seeds and suggested that lipids were removed from the seed surface. Svubova et al. (2020) did not see such a pronounced effect but saw a slight decrease in the C-H bond stretching of lipids, suggesting lipid oxidation. Despite omitting wavenumbers below 1500 cm^{-1} which were difficult to assign to specific functional groups, Wang et al. (2017) suggested that the seed is able to absorb water due to improved hydrophilicity via oxidative etching and the partial cellulose degradation. This enhanced water absorption is supported by Yamauchi et al. (2012) who witnessed the degradation of cellulose on seed surfaces. Holc et al. (2019) and Molina et al. (2020) suggested two scenarios: plasma may be removing the hydrophobic layers and exposing the cellulose and other hydrocarbons, or remodelling the hydrophobic wax through heat and/or chemical reactions.

Lastly, contact angle measurements are a straightforward method to determine if the seed surface is more hydrophilic because it is used to quantify the wetting of a solid by a liquid. The sessile drop method is the simplest approach where a water droplet is dispensed on the surface and the contact angle, in other words the tangent line at the solid and liquid interface, is measured. If the angle is small and water droplet is flat, then the surface is hydrophilic. If the angle is large and the water droplet is round, then the surface is hydrophobic. Quantitative results often are done with a goniometer or tensiometer where the Young-Laplace equation is selected with the tangent line drawn from the baseline of the drop to the edge and are averages of several replicates. Most authors include this measurement in their studies to determine if the seed surface is more hydrophilic and simultaneously use this information to see if the water uptake rate has changed.

Many authors over the last decade have observed that plasma treatment increases seed surface hydrophilicity. Bormashenko et al. (2012) were among the first to check this and showed improved wettability of lentil, beans, and wheat seeds after an air plasma treatment. Velichko et al. (2019) compared an argon plasma jet in open air with an atmospheric air dielectric barrier discharge (DBD) in terms of germination and contact angle of plasma-treated wheat grains. Both methods decreased the contact angle, although it was more strongly reduced by the air DBD. Likewise, Alves Junior et al. (2016) observed increased hydrophilicity in *Mimosa* seeds with a helium plasma jet in air and Hoppanová et al. (2020) observed increased hydrophilicity in cereal seeds with a DBD in open air. Da Silva et al. (2017) demonstrated that air plasmas increase the hydrophilicity due to the absorption of radicals, such as OH and N_2^+. Conversely, it is possible to increase the hydrophobic properties by changing the gas source to carbon tetrafluoride (CF_4) or octadecafluorodecalin (ODFD) (Volin et al., 2000), although this is achieved by deposition rather than by modifying intrinsic properties.

In the context of plasma agriculture, contact angle measurements improve our understanding of water interaction and uptake. Arguably, an XPS analysis provides the chemical composition and surface modifications such as oxidation, which would increase wettability, so it may not be necessary to do contact angle measurements. However, XPS may not be readily available to many scientists so it is understandable if many use the sessile drop method for straightforward qualitative and sometimes, quantitative results. Clearly, this is best done on a flat surface and may not be possible with every seed type due to the range of shapes and sizes as in our case, i.e. if 1 µl of water is the minimum volume, a seed like *Arabidopsis* is too small, or if the seed surface is very rough, it is not possible to obtain quantitative measurements.

The relative merits of these various surface analysis techniques listed above were investigated by using them as diagnostics in a model experiment. The aim in the following experimental investigation was to gain insight into which physical phenomena are relevant in the plasma treatment and that would have an effect on the seed coat surface using a model plant organism, *Arabidopsis thaliana* Col-0. Changes to the seed coat and other downstream processes may be due to ozone (chemical modifications to the seed surface), heat (mechanical modifications of wax), or plasma (mechanical and chemical modifications to the seed surface). Therefore, ozone and heat were applied as separate treatments and compared to the plasma treatment in an effort to identify the individual effect of each component on the seed. Based on these results, it seems to be a synergy of the plasma effects, and not its individual components, which is responsible for any modifications to the seeds. Finally, through this experience, certain surface analysis techniques can be preferentially recommended as they provide more fruitful results than other techniques.

4.2 Experiment description

Commercially available surface dielectric barrier discharges (SDBD) by VOSOCO, Sihon Electronics with alumina dielectric and printed electrodes, in a honeycomb or striped geometry, were used for the plasma treatment (Figure 4.1A and B, respectively).

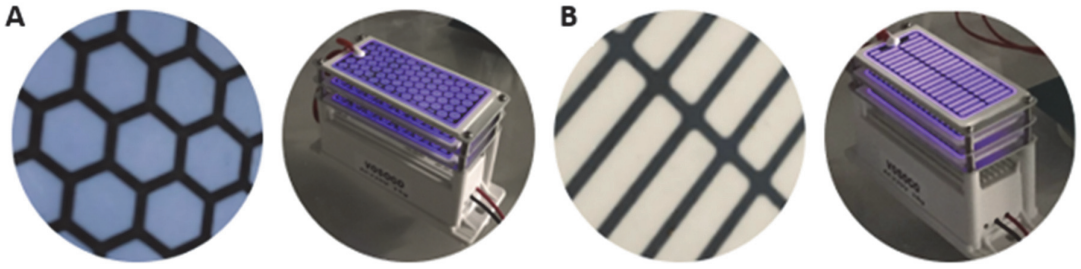


Figure 4.1. A detailed and an overall view of a SDBD plasma source with A) honeycomb or B) stripe electrodes.

The SDBD was powered by a 12 kHz sine wave with 8.8 kV peak-to-peak voltage, for a power of ~75W over a stack of three alumina plates, each of 105 mm x 50 mm area. Treatments were performed in an open air environment with no gas flow (ambient temperature, pressure, humidity ~40%). A plasma treatment time of three minutes was chosen because the first visible plasma effects on the seed surface, seed coat erosion, were observed (Figures 4.4 and 4.5). To identify the effect of the individual components, heat and ozone treatments were carried out separately in the absence of a plasma. The heat treatment parameters were selected in such a way to mimic a similar temperature rise of the dielectric/electrodes with plasma on. The temperature reached 80ºC after 3 minutes, but to ensure sufficient heat transfer from the hot plate to the seed, the treatment time was extended to 10 minutes. The ozone treatment parameters were based on measurements with plasma on using an ozone analyzer UV-100 (Ecosensors). The ozone generator, developed by Sterilux, was an enclosed compartment with three, 172 nm V-UV lamps (Thill and Spaltenstein, 2020). Since it could only reach 200 ppm of ozone, the treatment time was also extended to 10 minutes. Arguably, because the plasma treatment was carried out in open air, the overall ozone concentration was not so important provided that a similar ozone concentration was maintained at the seed surface. All treatments were performed directly on the seeds initially spread uniformly over the SDBD.

4.3 Results and comparison of surface analysis techniques

4.3.1 Qualitative assessment of germination in plasma-treated *Arabidopsis* seeds

First, seed germination mainly after plasma treatment was analysed qualitatively using a light microscope on the same batch of seeds harvested in May 2019. The germination was not negatively affected by the plasma treatment (~100% germination probability). Likewise, the germination after the ozone or heat treatments was not affected but the images are not shown here. Finally, there were no obvious indications of abnormalities in the early stages of germination and growth (Fig. 4.2).

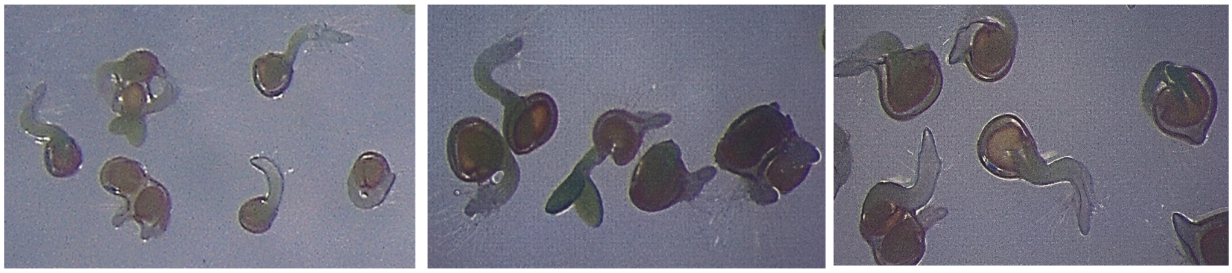


Figure 4.2. Light microscopy images show that seed germination was not negatively affected after plasma treatment. (Left) control, untreated seeds; (Center) 3 min plasma-treated seeds using honeycomb electrodes; (Right) 3 min plasma-treated seeds using stripes electrodes.

4.3.2 Scanning electron microscopy and atomic force microscopy

The SEM imaging was performed on dry seeds since soaked *Arabidopsis* seeds release mucilage; this made it difficult to obtain a high quality image, as shown in Figure 4.3. It is clearly evident that the cells are protruding forward and are inflated relative to dry *Arabidopsis* seeds, as shown in Figure 4.5.

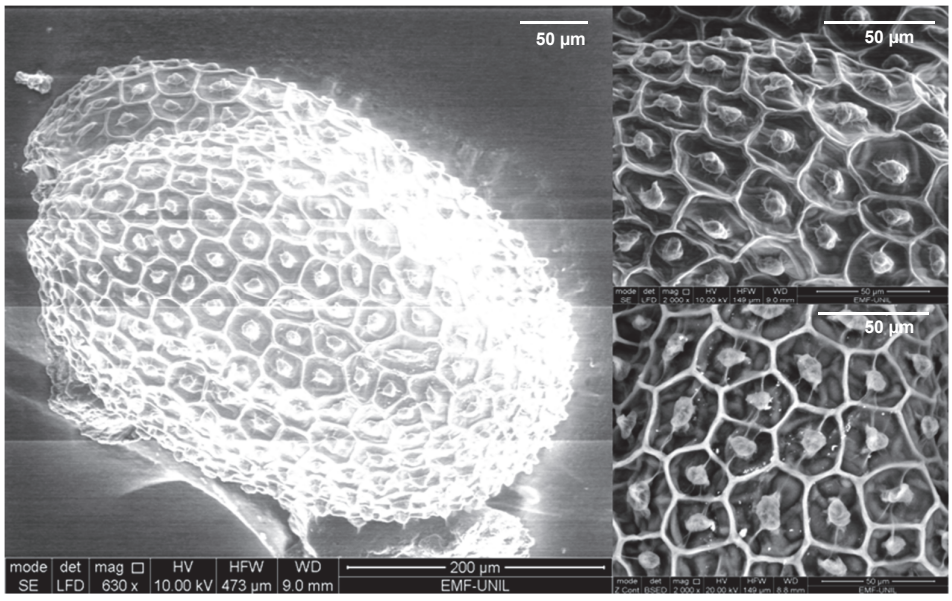


Figure 4.3. SEM images of *Arabidopsis thaliana* seeds kept on water agar for 2 days. Seed coat structure changes with cell expansion. Released mucilage makes it difficult to obtain a high quality image and therefore, for ease of handling, dry seeds were preferentially used.

Seed erosion was observed after plasma treatment, but not with heat nor ozone alone (Figures 4.4 and 4.5). Erosion was particularly observed with the stripes electrodes and to a smaller degree with the honeycomb electrodes.

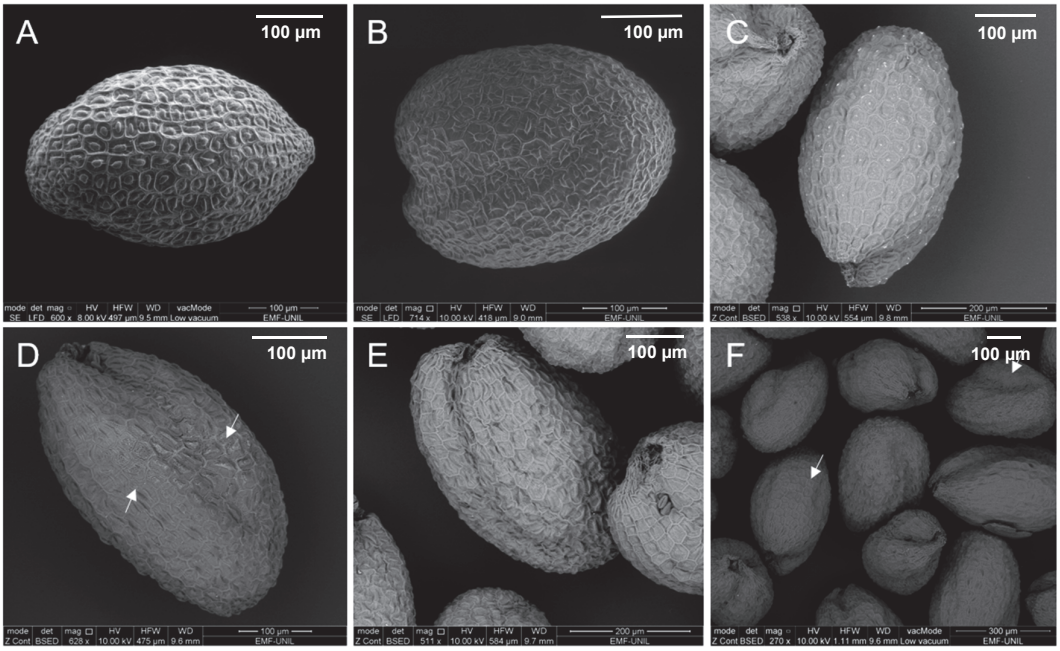


Figure 4.4. SEM images of *Arabidopsis* seeds which are A) untreated, B) heat-treated for 10 min at 80˚C, C) plasma-treated for 3 minutes using honeycomb electrode DBD configuration, D) plasma-treated for 3 minutes with stripe electrode DBD configuration; the white arrows point to eroded areas, E) ozone-treated for 10 min at 200 ppm, showing the biological diversity in seed coat patterns F) ozone-treated for 10 min at 200 ppm of multiple seeds at lower magnification. Higher magnification is shown in Fig. 4.5.

These results reinforced the previously reported observations of seed coat erosion in some, but not all, cases. Molina et al. (2018, 2020) showed differences in seed coat erosion based on different seed types, but in our study, the same seed type was used with different plasma geometries. This highlights that seed type is not the only parameter to consider whether there will be erosion and the type of plasma treatment needs to be considered as another parameter. As pointed out by Mildažienė et al. (2016), this may depend on the seed position and whether it is in direct contact with the surface or close to the plasma. It was observed that the plasma ignited in this hexagonal geometry of the honeycomb electrode (see Fig. 4.1A) positioned the seeds in the centre of the hexagon, perhaps due to electrostatic forces or air flow, during the plasma treatment. This distancing from the plasma can possibly explain the lack of erosion whereas the stripe electrodes, the seeds were more evenly distributed, interacting with the plasma and therefore resulted in erosion. It is worthy to note that it is important to monitor the seed position before, during, and after plasma treatment.

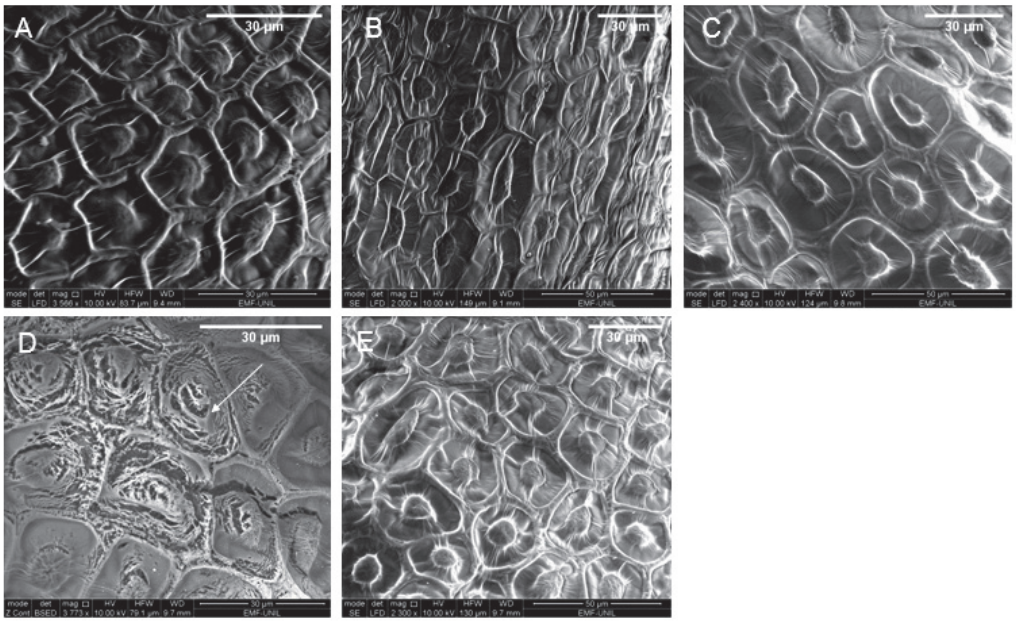


Figure 4.5. SEM images of *Arabidopsis* seeds which are A) untreated, B) heat-treated for 10 min at 80˚C, C) plasma-treated for 3 minutes using honeycomb electrode DBD configuration, D) plasma-treated for 3 minutes with stripe electrode DBD configuration; the white arrow points to the eroded area, and E) ozone-treated for 10 min at 200 ppm. Images at a lower magnification are shown in Fig. 4.4.

Furthermore, caution should be taken when interpreting information from SEM images because plant genetics (natural variation) and dehydration of the seeds can influence the seed coat pattern. As shown in Figs. 4.4F, the white arrows point out variations in the seed coat in the same seed sample despite undergoing the same ozone treatment simultaneously. Therefore, observed changes on different seeds could be attributed to biological variation rather than the applied treatment. This emphasizes the necessity of checking the seed sample prior to use and if possible, observing the same single seed before and after treatment.

The samples also need to be handled carefully to avoid overexposure to the SEM electron beam because the beam can cause damage to the sample and may be confused as an effect of plasma treatment. Furthermore, since SEM analysis is performed in vacuum, moisture is removed (unless operating under low vacuum conditions where not all moisture is removed). This then may not exactly reproduce the same experimental set up and, as a result, there may be cracking in the seed coat. However, this can be verified to some extent by comparison with the control seeds assuming that all seeds have similar moisture content. Alternatively, AFM can produce topography images without surface damage and the morphology can be measured in *x*, *y,* and *z*, but it was not technically feasible to image *A.thaliana* seeds due to the deep contours and seed roundness. AFM remains as an option for flat seed types such as pumpkin seeds, whereas SEM can be used on all seed types in a qualitative manner (Volkov et al., 2019).

Overall, SEM can provide detailed surface topology information within seconds or minutes but is otherwise limited in explaining the effect of plasma on seeds. Authors such as Mildažienė et al. (2017) observed positive growth effects using vacuum and electromagnetic fields without visibly changing the seed coat structure using SEM. This suggests that SEM is not sufficiently specific since it remains unclear whether erosion is necessary to alter plant growth. Thus, it is arguably more interesting to focus on the seed coat chemistry, which may be more explicit concerning downstream processes that change the growth parameters

(Los et al., 2019). Analyzing the chemical changes on the seed coat after plasma treatment can be done with ATR-FTIR, EDX, or XPS.

4.3.3 Attenuated total reflection Fourier transform infrared spectroscopy

ATR-FTIR analysis was performed but the data did not reveal any relevant information between the untreated and treated seeds. With our seed type, it was difficult to ensure that sufficient pressure was applied to guarantee proper contact and a strong signal without crushing the seed. When the appropriate conditions were identified, there were no detectable differences between the untreated seeds, and the heat-, ozone- or plasma-treated seeds in the ratio between the C-H stretch around 3100 – 2850 cm^{-1} and C=O stretch around 1800 – 1650 cm^{-1} (see Figure 4.6).

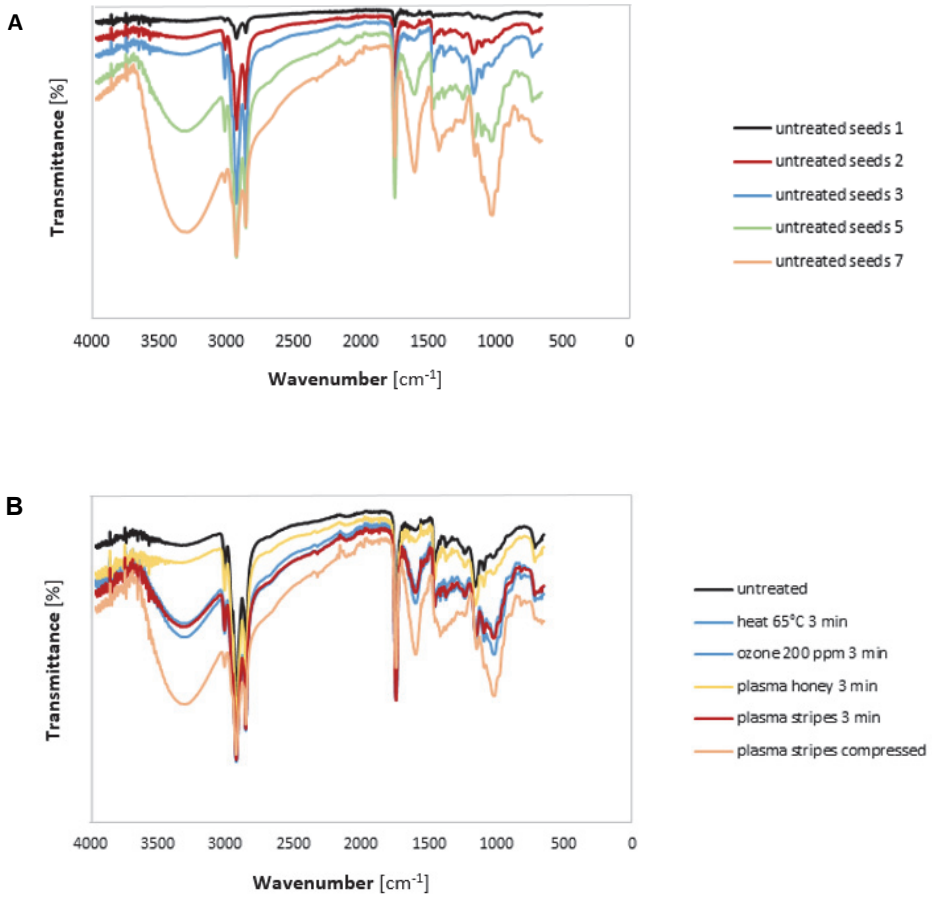


Figure 4.6. ATR-FTIR spectra of *Arabidopsis* seeds A) spectra of untreated seeds under increasing pressure show that changes are only due to increase in pressure B) spectra of untreated, heat-treated, ozone-treated, plasma-treated seeds demonstrate no relevant changes.

Cui et al. (2019) showed an increase in the O-H stretch with increasing plasma treatment time. However, in our case, it was clear that incrementally increasing the pressure was responsible for the O-H stretch around 3400 – 3200 cm^{-1}, suggesting that compressing the seed may release its water content. Furthermore, Sera et al. (2021) also had a reference spectra with more water content and in one among three species of pine seeds, there was no obvious difference. Therefore, it may be important before the analysis to dehydrate the seeds to have consistent results. In our case, the expected oxidation of the hydrocarbons was perhaps not observed because ATR-FTIR penetrates into the bulk material, whereas XPS measures only the

first nanometers of the sample, where plasma is expected to make surface changes. Therefore, for newcomers to the plasma agriculture field, it is recommended to work with specialists to interpret the overlapping bands. When done properly, it is interesting to see how the plasma etches through the tissue layers (Molina et al., 2020). Although ATR-FTIR does not damage the surface, finding the correct amount of pressure may be simpler for hard seed types, like maize and pine, but more difficult for delicate, small seeds like *Arabidopsis*. Although Cui et al. (2019) performed ATR-FTIR on *Arabidopsis*, the authors selectively showed only the O-H stretch change due to plasma treatment, but in our instance, the change was due to increased pressure. Under our conditions, similar information by the ATR-FTIR can be obtained with XPS on delicate seeds if simply checking for oxidation or nitration.

4.3.4 Energy-dispersive X-ray spectroscopy

With EDX, it is possible to retrieve the distribution of elements. However, considering that plasma treatment affects the surface and not the bulk material, this technique may not necessarily provide any information about the seed interior unless a cross-section is taken, as done by Gómez-Ramírez et al. (2017) and Molina et al. (2018, 2020). Furthermore, difficulties may be encountered in performing absolute measurements, partly due to the curved surface of the seed, and in identifying the source of any changes in element concentration i.e. carbon may be taken up from the adhesive paper on the sample holder.

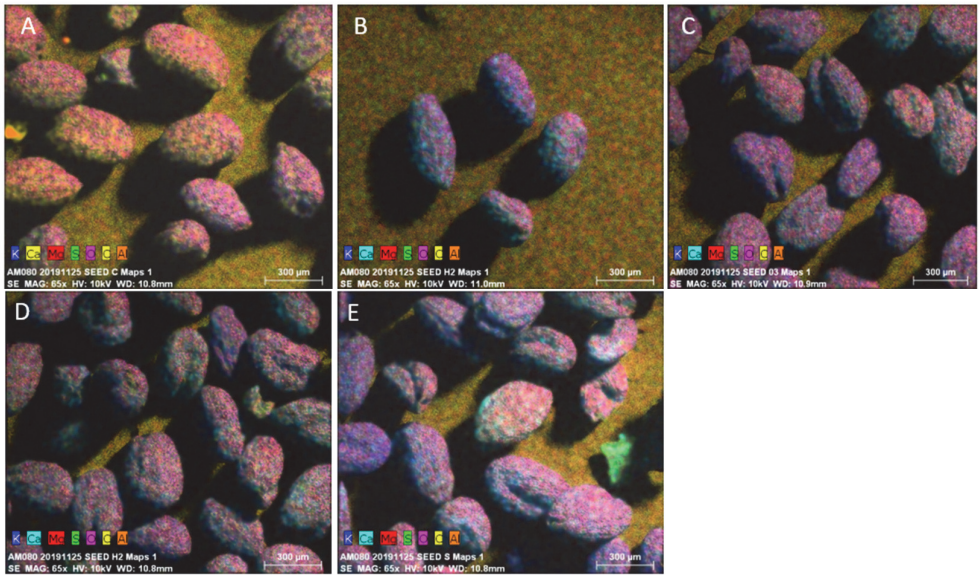


Figure 4.7. EDX maps of *Arabidopsis* seeds which are A) untreated, B) heat-treated for 10 min at 80˚C, C) ozone-treated for 10 min at 200 ppm, D) plasma-treated for 3 minutes using honeycomb electrodes, E) plasma-treated for 3 minutes using stripe electrodes.

The EDX maps did not reveal any obvious changes other than perhaps an increase in potassium in heat-, ozone- and plasma-treated seeds compared to the untreated seeds, but this information was not enough to confidently draw any conclusions. Nevertheless, an important aspect of this analysis revealed that nickel was unexpectedly detected on the seeds after plasma treatments when initially using a different SDBD, SDBD4 with gold-plated copper electrodes on Kapton or epoxy dielectric (Figure 4.8, see Chapter 3). This indicates that one should be mindful of nanoparticle contamination of the biological sample from the electrodes (Kasote et al., 2019; An et al., 2020).

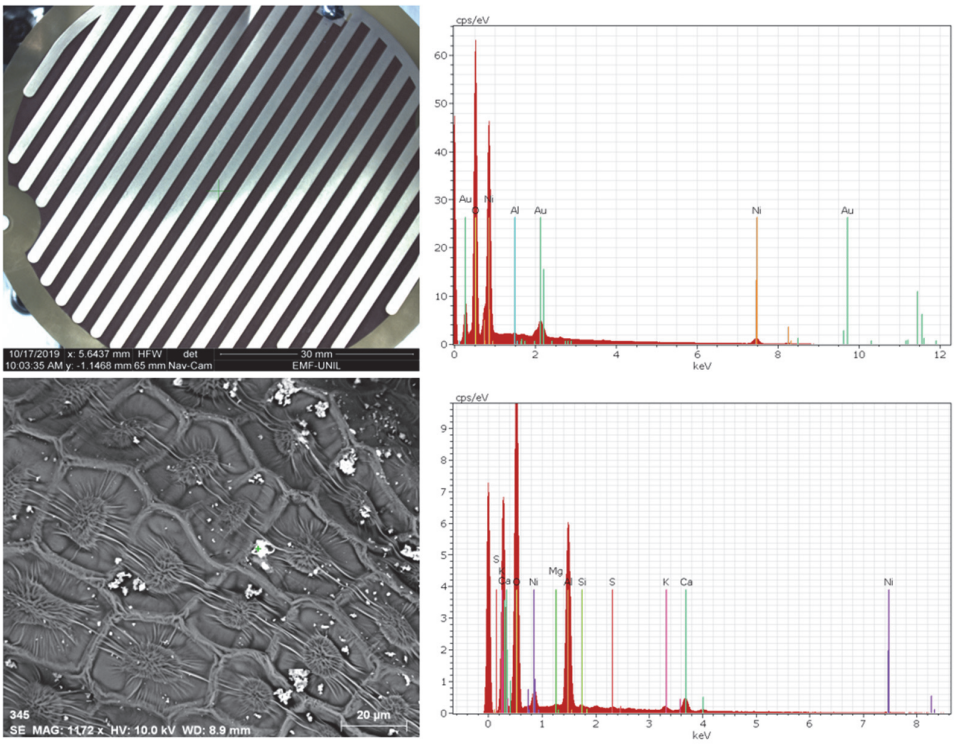
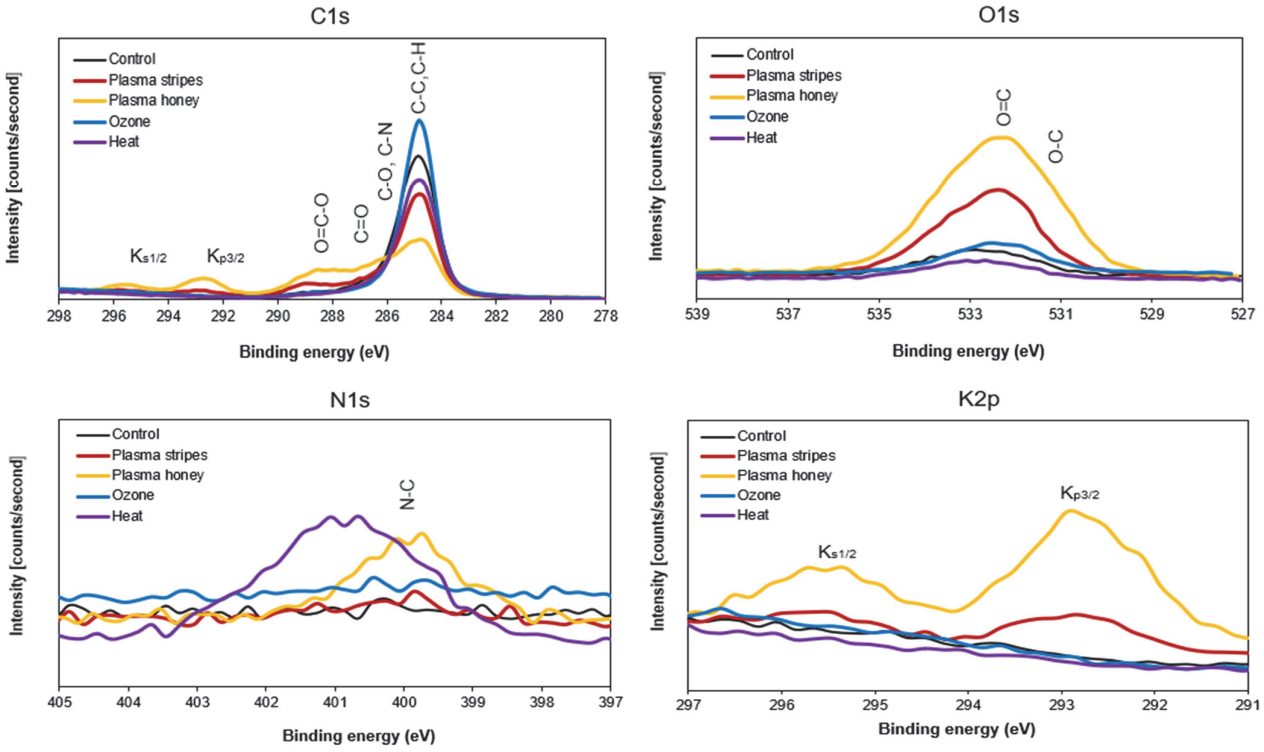


Figure 4.8. SEM-EDX images of (top row) SDBD4 with Kapton dielectric and (bottom row) plasma-treated *Arabidopsis thaliana* seed surface. Nickel deposition found on the seeds originates from the gold-plated copper electrodes of SDBD4.

4.3.5 X-ray photoelectron spectroscopy

To avoid this contamination, SDBD4 was changed to the stripes and honeycomb electrodes described earlier in this paper, and XPS was used as an alternative method to obtain absolute values of the elements, which could later be compared to the EDX measurements (Figure 4.9). The elements analyzed were carbon, nitrogen, oxygen, potassium, magnesium, calcium, sulfur, and silicon. However, only the first four are shown since the others remained mostly unchanged.

In line with Gomez Ramirez et al. (2017), Molina et al. (2018, 2020) and Holc et al. (2019), the oxidation of carbon, shown by the decrease in C-H bonds and increase in O=C-O, C=O, C-O, and increased potassium peaks were the obvious changes in seeds after plasma treatment. Here, the oxygen content increased between 15% or 30% depending on the electrodes used, which was in agreement with the 5% to 30% increase in oxygen observed by other authors. Additionally, there was a qualitative increase in C-O and O-C=C bonds at the expense of C-C or C-H bonds in the C1s spectra (Figure 4.9). These changes, however, were not observed with the ozone or heat treatments.



Treatment	% C1s	% N1s	% O1s	% K2p
Control	91.34	1.31	6.40	0.07
Heat 65˚C 3 min	93.24	0.00	6.25	0.10
Ozone 200 ppm 3 min	90.44	0.31	7.12	0.26
Plasma stripes 3 min	75.20	0.09	21.37	0.43
Plasma honeycomb 3 min	59.04	3.14	34.76	1.86

Figure 4.9. XPS spectra with atomic concentration table of carbon, nitrogen, oxygen, and potassium in untreated, heat-treated, ozone-treated, and stripes or honeycomb electrode plasma-treated *Arabidopsis* seeds.

4.4 Conclusions

There are therefore two clear conclusions: first, that the XPS surface analysis technique gives the most significant and unambiguous results; and second, that plasma with either stripes and honeycomb electrodes is responsible for oxidizing the seed surface. Concerning heat or ozone, these individual treatments may not be sufficiently reactive during the 3-minute treatment time to cause changes to the seed surface, however, they have the potential to do so with longer treatment time. Likewise, higher potassium concentrations were mainly in plasma-treated seeds; possibly indicating that an intense plasma treatment leads to damage since potassium should be found intracellularly, not extracellularly. Interestingly, there was no erosion found with the honeycomb electrodes, suggesting that either correlation is not causation or that there is a time window to capture and measure increasing levels of potassium which was missed for the stripes electrodes. For example, Svubova et al. (2020) did not observe erosion despite detecting chemical changes.

Considering that all treatments were done in open air, the concentration of ozone was difficult to determine and therefore may not be precise. However, it is plausible that the surface would become more reactive due to plasma's well-known ability for etching and sputtering, explaining the lack of obvious changes with ozone treatment alone. The etching mechanism may indeed, as others have postulated, remove lipid layers to facilitate oxidation (Zahoranova et al., 2018; Svubova et al., 2020). Although ozone has a role in seed treatments and the potential to oxidize surfaces, it may not be sufficiently long enough to induce changes.

This suggests that the combination of heat and ozone within the plasma or, indeed, other plasma properties altogether, such as electric fields or ions, are responsible for yielding these surface changes on the seed coat, rather than the individual components, such as heat or ozone, acting alone.

Chapter 5

In situ FTIR characterization of plasma gas chemistry: what is responsible for the accelerated germination?

Abstract

Despite the numerous successful results of plasma-seed treatments reported in the literature, it remains elusive which plasma treatment parameters are required to improve plant growth because of the plethora of physical, chemical, and biological variables. Here, we searched for the optimal conditions in our surface dielectric barrier discharge (SDBD) setup using a parametric study and attempted to understand the relevant species in the plasma treatment using *in situ* Fourier transform infrared (FTIR) absorption spectroscopy. Our results suggest that treatment time and voltage are key parameters in our study for accelerated germination, however, there is still no clear conclusion about the causative agent(s). Speculations point towards nitric oxide (NO) as a contributor.

This chapter is based on the article cited below. I contributed to the design of the parametric study, performed all of the germination experiments, temperature, humidity, and ozone measurements, supervised the FTIR data collection by a Master thesis student, performed the data analysis, wrote the manuscript, selected the journal, and handled the submission process.

International Journal of Molecular Sciences

An *in situ* FTIR study of DBD plasma parameters for accelerated germination of *Arabidopsis thaliana* seeds

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Keywords: plasma; *Arabidopsis thaliana*, FTIR, germination, reactive oxygen and nitrogen species, surface DBD

5.1 Introduction to the germination parametric scan

After the analysis of seed surfaces, the SDBDs shown in Chapters 3 and 4 were enclosed in a reactor to search for a germination phenotype. Simultaneously, a parametric study was being co-designed and synchronized with a collaborator, Dr. Paolo Ambrico in Bari, since previous experiments did not reveal any reproducible changes in plant growth. Initially, both the stripes and mesh SDBDs were included in the study with varying parameters, but were later narrowed down to the stripes SDBD.

The design of the parametric study contained the plasma source characterization, the seed preparation protocol, and the plasma-seed treatment operating parameters (i.e. time, frequency). The values of the operating parameters were varied within a defined range in order to determine which combinations would result in plant growth changes. This was done systematically, as summarized in Figure 5.1.

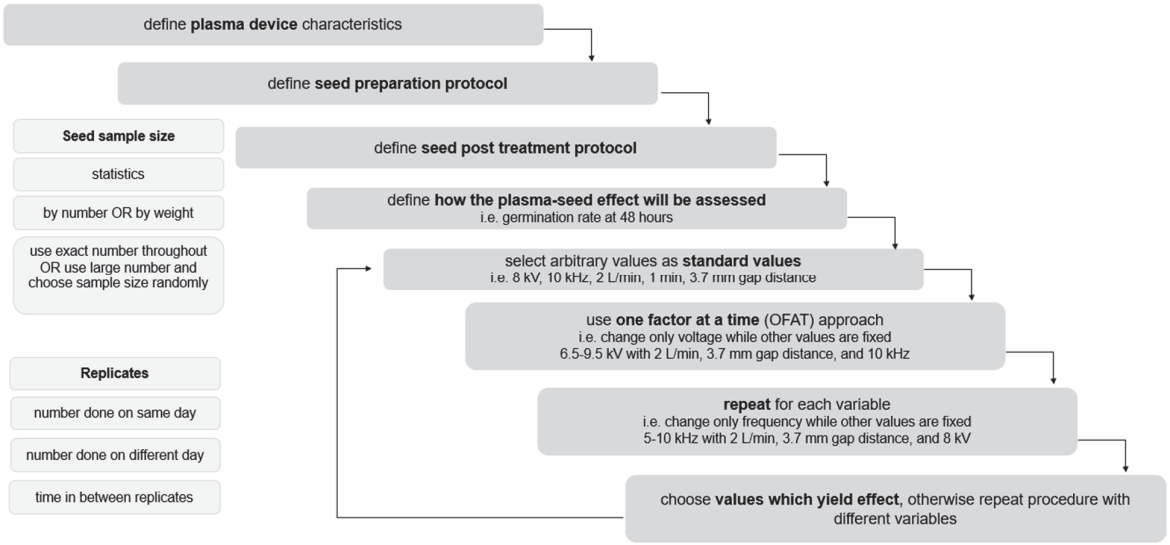

Figure 5.1. The workflow for plasma-seed treatment optimization.

For the germination experiments, *Arabidopsis thaliana* Col-0 seeds were cultivated in a plant chamber room and harvested in May 2019 using seeds from the Department of Plant Molecular Biology at the University of Lausanne. Therefore, the seeds at the time of the germination experiments were 18 - 20 months old. Seeds were stored in the dark in Eppendorf or Falcon tubes at 4ºC for the experiments performed in Bari, or were stored at room temperature for the experiments performed in Lausanne.

In Bari, the following conditions were used: a 1 min wait time before treatment, 500 Hz, 500 ns pulse, 8 kV, 1 min plasma-seed treatment, 2 L/min gas flow, 50% relative humidity, and 4.33 mm plasma-gap distance. The sample size of each treatment was 30 seeds and was performed in triplicate. The plasma-treated *Arabidopsis* seeds were sown on water agar and grown in the dark. All measurements were performed at 48 hours after sowing. As shown in Figure 5.2, there were no statistically significant results obtained in Bari, other than with the mesh SDBD powered by nanopulse power supply at 500 Hz.

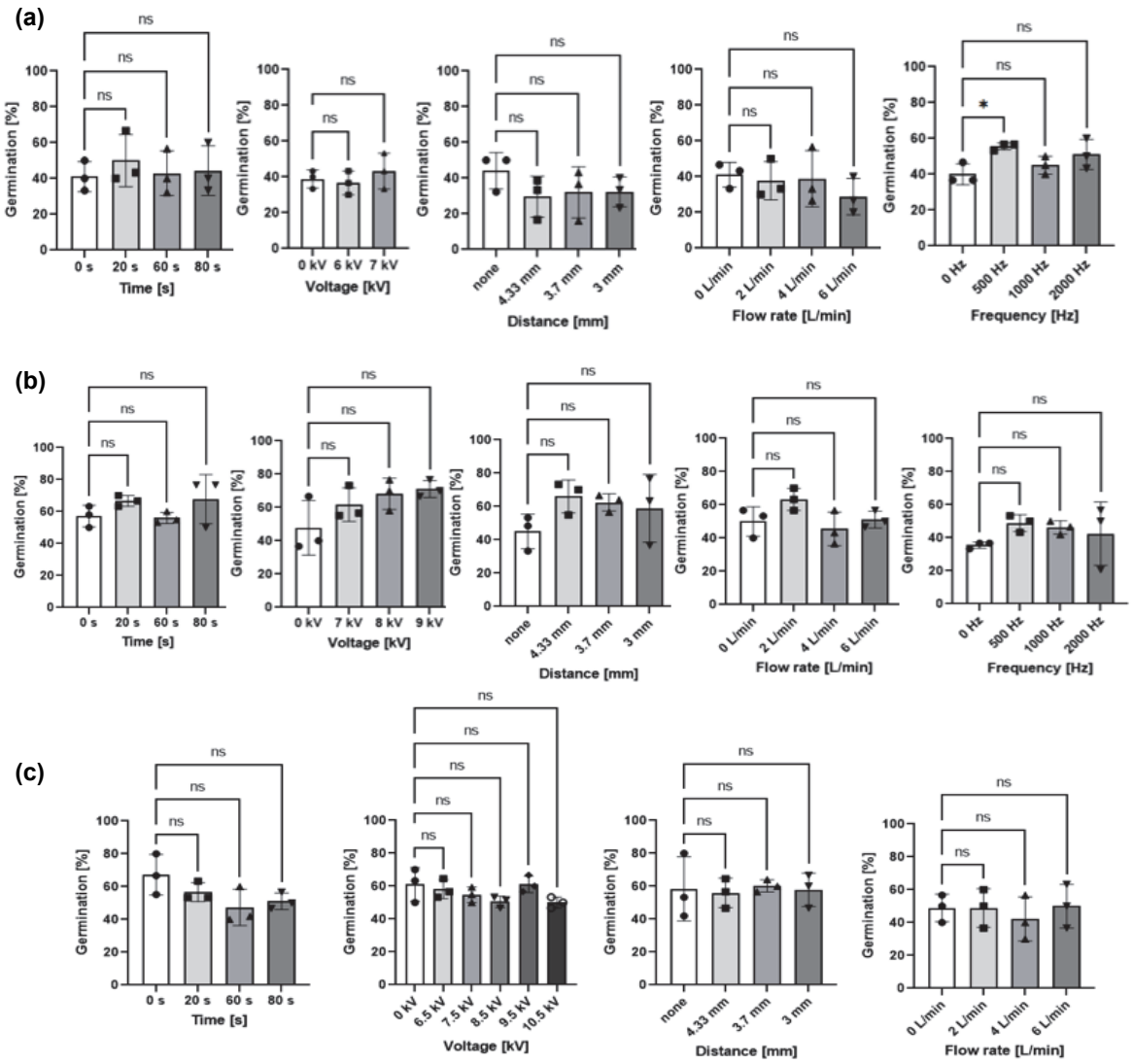

Figure 5.2. Germination rate results of plasma-treated *Arabidopsis* seeds at 50% RH from the parametric study for (a) nanopulse with mesh SDBD (b) nanopulse with stipes SDBD (c) AC with mesh SDBD. Germination was measured at 48 hours with seeds grown on water agar in dark conditions at 23°C with 65% RH in the incubator. Experiments are an average of triplicates.

Due to the initial lack of results in Bari, the plasma-seed treatment was further modified in Lausanne and simplified by minimizing the relative humidity levels. This was done because empirical observations seemed to suggest that plasma treatment of seeds previously exposed to high humidity were negatively affected. Furthermore, to ensure significant results were not due to biological variation, experiments were reproduced twice on different days. Otherwise, the protocol established in Bari was followed as closely as possible for reproducibility reasons. In Lausanne, the default operating parameters were as before, except with the following changes: 3.7 mm plasma-seed gap distance, 3 kV for the nanopulse power supply since it delivered almost double the value than what was displayed, 8 kV for the AC power supply, less than 3% RH, a total of 6 replicates, and the seeds were grown using a 24-hour light cycle. The results of the parametric study done in Lausanne are shown in Figure 5.3.

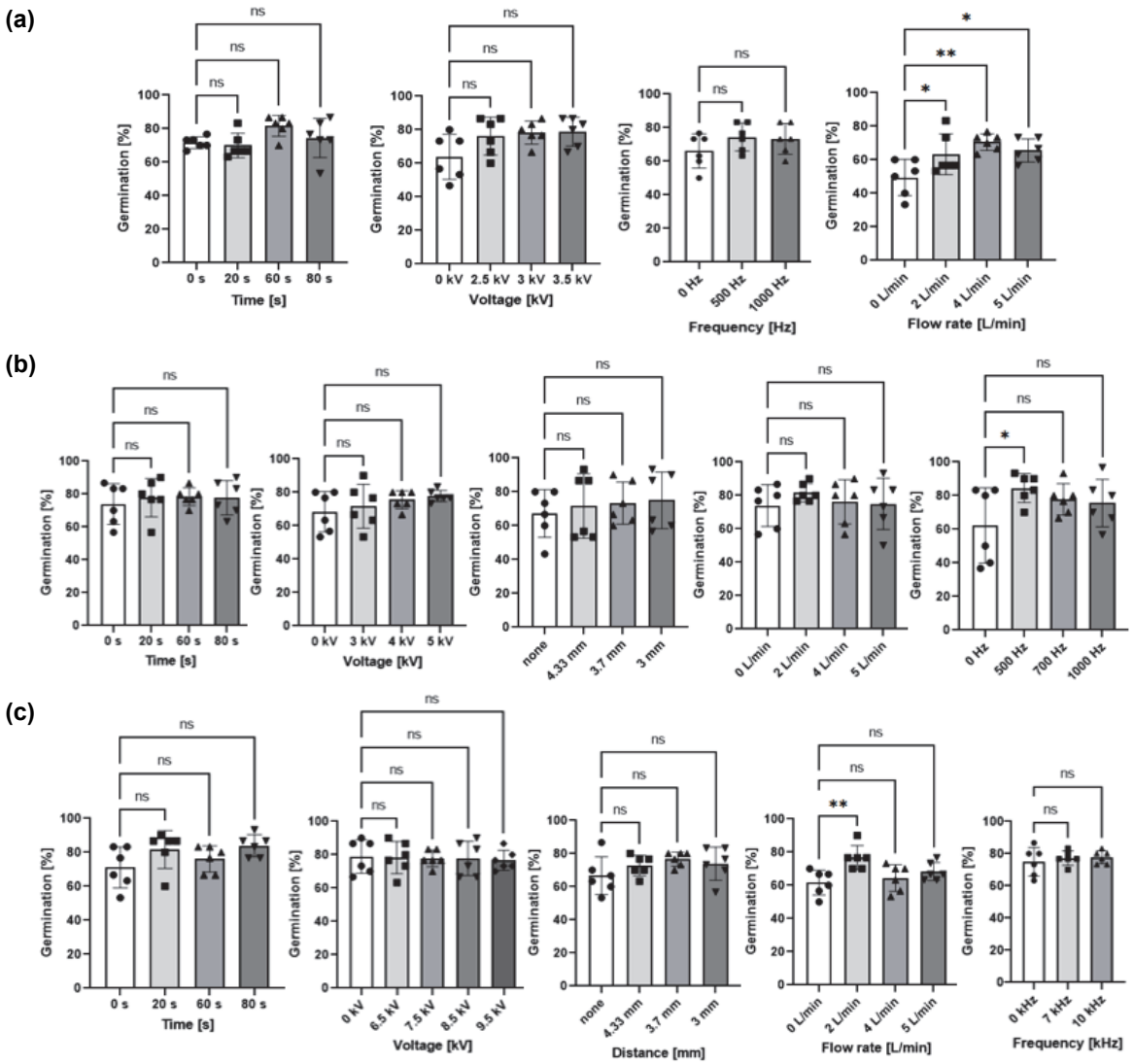

Figure 5.3. Germination rate results of plasma-treated *Arabidopsis* seeds at 3% RH from the parametric study for (a) nanopulse with mesh SDBD (b) nanopulse with stripes SDBD (c) AC power supply with mesh SDBD. Germination was measured at 48 hours with seeds grown on water agar in continuous light conditions at 23°C with 65% RH. Experiments are an average of two triplicates performed independently for a total of 6 replicates. Asterisks denote statistical significance where * signifies p <0.05; ** is p <0.01; and * is p <0.001. "ns" indicates "not significant".**

Statistically significant results were observed in multiple combinations; nanopulse with mesh SDBD, AC with mesh SDBD, and nanopulse with stripes SDBD for gas flow rate and frequency. However, there were differences in the initial germination rate of both untreated, control seeds, and plasma-treated seeds, which was later attributed to seed storage, seed age, or SDBD aging. It became evident that the germination rate was decreasing over time. It then became clear that the decrease in the first Eppendorf tube was likely due to the repetitive humidity exposure when a second, unopened Eppendorf tube of the same seed batch had a high initial germination rate. Since there were appreciable differences between experiments using the seeds in the first tube and second tube, it was difficult to make a conclusion with confidence in some cases. Nevertheless, this finding highlighted the importance of seed storage and handling for plasma-seed treatments.

Exceptionally, there was one set of experiments using the stripes SDBD powered by an AC power supply that was the promising. All of the experiments used seeds only from the first tube and there was the least amount of variation between the replicates and experiments in the untreated and plasma-treated seeds.

Therefore, this data set became the basis for further investigations using FTIR presented below, and RNA sequencing (Chapter 6). Here, we found the optimal plasma conditions for germination in our setup using a parametric study, and attempted to understand which were the relevant species in the plasma treatment by using in situ Fourier transform infrared (FTIR) absorption spectroscopy.

5.2 Results

5.2.1 Germination rate of plasma-treated *Arabidopsis thaliana* Col-0 seeds

Germination rates were measured for parametric scans of plasma treatment time, SDBD voltage, air flow rate, seed-to-SDBD distance, and AC excitation frequency. The default operating parameters were a plasma treatment time of 60 s (6 s plasma ON time), peak-to-peak voltage 8 kV, 2 L/min flow rate of dry synthetic air, seed substrate distance 3.7 mm, and 10 kHz frequency with a power on/off modulation at 500 Hz and 10% duty cycle, corresponding to a burst of 2 cycles per modulation period. The 10% duty cycle was used to avoid heat shock of the seeds (Ambrico et al., 2020). Figure 5.4 shows the germination rate of seeds measured after 48 hours, compared to the control, untreated seeds with no plasma exposure (labelled as 0 values in the bar graphs).

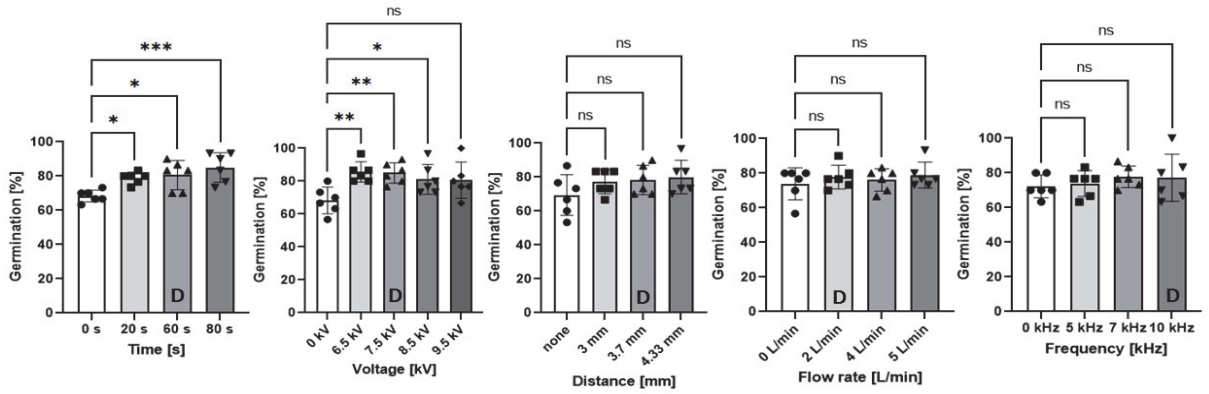

Figure 5.4. Germination rate results of plasma-treated *Arabidopsis* seeds from the parametric study. Germination was measured at 48 hours with seeds grown on water agar in continuous light conditions at 23°C with 65% RH. Experiments are an average of triplicates performed twice independently for a total of 6 replicates. Asterisks denote statistical significance where * signifies p <0.05; ** is p <0.01; and * is p <0.001. "ns" indicates "not significant". The label "D" in each graph denotes the default parameter value.**

Seeds were not sterilized nor subjected to seed preselection before plasma treatment. After plasma treatment, 30 seeds were sown immediately, or a few hours after (within the same day at the latest), on water agar plates (20 g/L, using distilled water, pH of approximately 6.7) and kept in a phytotron (AR-36L2 PlantClimatics GmbH) under continuous light using Osram L 18W 77 G13 Fluora with a 24 h light cycle at 23˚C and 65% humidity. Germination was recorded at 48 hours and seeds with roots were counted by eye. Germination rate was calculated as the number of seeds with roots divided by the total number of seeds and was converted into a percentage.

Differences between the two groups were assessed using ordinary one-way ANOVA. Each treatment group was compared to their respective control, and the bar graphs represent two independent experiments with 3 replicates each, for a total of 6 replicates. GraphPad Prism 9 (GraphPad Software, Inc.) was used for statistical analyses. All p-values < 0.05 were considered to be significant. Only significant p-values are shown in the graphs. A Welch two-sample t-test set to 95% confidence interval was used to compare untreated seeds to the default parameters of plasma treatment.

As marked by the asterisks in Fig. 5.4, scans of treatment time or voltage yielded statistically significant increases in the germination rate for 20, 60, 80 s treatment times (2, 6, or 8 s plasma ON), and for 6.5, 7.5, 8.5 kVpp voltages. In contrast, modifying the flow rate, gap distance, or frequency gave no significant changes, although none of the plasma treatments caused the germination rate to fall. The longest plasma treatment time of 80 s had the highest statistical significance for the germination acceleration among all the combinations tested. This suggested that the plasma duration was too short in retrospect, perhaps because of the low 10% duty cycle, and further improvement might have been achieved with even longer plasma treatment time.

Compiling all of the data across the five experimental scans in Fig. 5.4, the mean germination rate of the five triplicate control groups (no plasma treatment) was 70.4%±8.1%, and the mean germination rate for the five triplicate default parameter sets was 79.8%±9.0%. The standard deviation error bars are indicative of the natural variance of the seed germination results, and the statistical significance for the effect of the default plasma on the germination rate is represented by p= 0.0001. The significance is high due to the averaging across a large number of samples.

5.2.2 In situ FTIR analysis of plasmas using parametric scans

Fourier Transform Infrared spectroscopy (FTIR) is a technique used to analyse the chemical composition of a sample based on the characteristic vibrational states of the atoms and molecules. These vibrational states correspond to the stretching and relaxing of bonds, which absorb energy from a photon at a specific wavenumber. This wavenumber is specifically in the infrared region of the electromagnetic spectrum and typically ranges from 500 to 2500 cm^{-1} in experiments; the intensity can be recorded as an absorbance or transmittance value. An IR beam passes through a beam splitter mirror which travels towards one fixed and one movable mirror and the beams are reflected back to the beam splitter mirror. Because the path difference of the two optical pathways can be changed with the movable mirror, it is possible to generate constructive or destructive interference, where amplitudes of the waves can be additive or subtractive. This beam passes through the sample and the photon absorption or transmittance is captured by the detector.

Since the spectra is performed in the time domain, Fourier transform is used as a mathematical conversion to transform the spectra from the time domain into the frequency domain; the wavenumber is plotted on the x-axis since frequency and wavenumber are directly proportional. Depending on the wavenumber and the absorption or transmittance, the identity of the compound can be resolved.

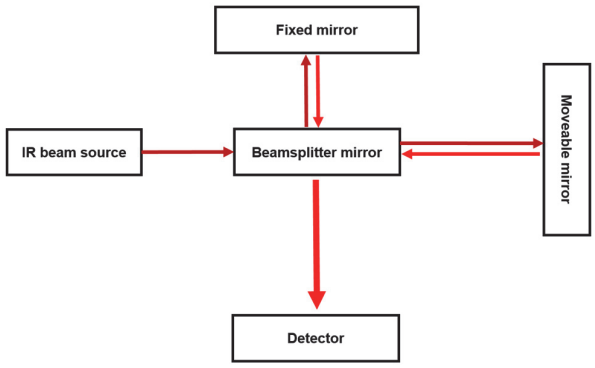

Figure 5.5. A simplified FTIR experimental setup.

Since it was observed that particular plasma conditions yielded a statistically significant effect on germination, the plasma chemistry was compared between these conditions to identify what could be responsible for this effect. Typical absorption spectra in the mid-infrared range 2500 – 500 cm^{-1}, with 4 cm^{-1} resolutions, and 20 s acquisition periods, are shown in Fig. 5.6. These measurements were taken independently of the germination experiments (without seeds) but under the same conditions using exclusively dry synthetic air.

The entire reactor, including gas flow control and electrical diagnostics, was mounted within the sample compartment of a Bruker Vertex 80V vacuum FTIR absorption spectrometer. The importance of in situ FTIR measurement is to avoid any possible alteration of the gas during recirculation of the exhaust to a downstream FTIR spectrometer. In situ measurement means that molecules are measured at their source, before any contact with surfaces, and instantly after their production without any transit delay when reactive species could be altered by secondary gas reactions. The FTIR spectra, therefore, represent the plasma species which would be directly in contact with the seeds. However, these preliminary in situ FTIR spectra do not appear to be any different from the conventional downstream FTIR measurements, showing that there are apparently no different species to be considered in the plasma treatment of the seeds with sufficiently high density to be detectable by this single-pass FTIR method.

The optics bench of the vacuum FTIR spectrometer was sealed by a 49.5 mm-diameter KBr window on each side of the sample compartment. To protect these windows, and to confine the gaseous plasma products within the reactor, supplementary KBr windows of 22.5 mm diameter were installed on opposing reactor ports. The infrared beam, of waist diameter approximately 1.5 cm, passed through these windows, and between the SDBD and seed substrate which are only a few mm apart. The penalty for in situ measurements, in this case, was the small solid angle which reduced the transmitted infrared intensity.

During the plasma, a strong absorption peak continuously grew at approximately 1352 cm^{-1}, with a proportionately much smaller peak at 833 cm^{-1}. These peaks remained permanently after the plasma was extinguished, and they were due to an irreversible surface transformation of the KBr reactor windows. We tentatively attribute this to a form of KNO_3 (Brooker et al., 1968; NIST), probably due to an attack from NO_x species produced in the plasma.

Additionally, because of the electrical connections and gas tubing to the reactor, the sample compartment remained open, hence the IR beam also traversed the ambient air outside of the reactor over a short distance. Variations in the laboratory atmosphere were responsible for the spurious CO_2 signal in the IR spectra of Fig. 5.6.

Otherwise, molecules, such as ozone (O_3), nitrous oxide (N_2O), and nitrogen dioxide (NO_2), were monitored in situ and the absorbance spectra for different treatment times, voltages, and air flow rates are shown here as they had the clearest trends.

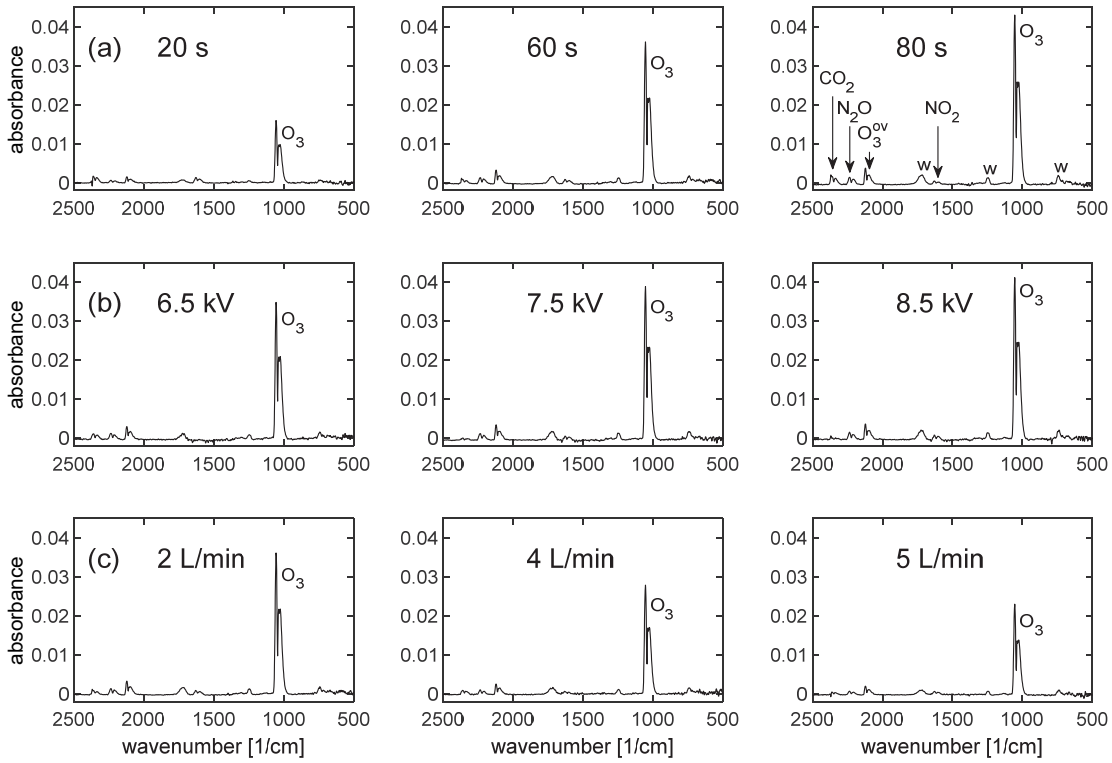

Figure 5.6. FTIR spectra of the plasma for different values of (a) plasma treatment time (b) SDBD peak-to-peak voltage (c) air flow rate, plotted at the moment of maximum absorbance. Longer plasma treatment time and higher voltage both increase the ozone concentration, whereas a higher flow rate decreases the ozone concentration, as expected. The species identified are labelled in the top right-hand graph; O_3ov indicates overtones of ozone, and "w" indicates non-gaseous compounds which were tentatively attributed to surface reactions on the KBr windows.

In Figure 5.6, the ozone increased strongly with time, less strongly with voltage, and decreased with air flow rate, as could be expected intuitively. The germination rate in Fig. 5.4 increased correspondingly with the time series for ozone, but did not follow the ozone trends for the voltage nor the flow rate series. Furthermore, the germination rate was the highest at 80 s treatment time (8 s plasma ON), corresponding to the highest concentration of ozone, although similar ozone levels were produced in other parameter combinations, like Figure 5.6(c), without having the same effect on germination.

Figure 5.7 shows that the time evolutions of N_2O during the series of treatment time and flow rate are qualitatively similar to ozone. Therefore, neither ozone nor N_2O were correlated with the germination rate. In summary, from the FTIR observation of these gas species, the apparent lack of correlation means that it remains unclear which agent influenced the germination rate.

It is instructive to note that, if the experiment had been designed to measure only the time series, then it would have been trivial - but incorrect - to infer that the increase in gas species in Fig. 5.6(a) was responsible for the increased germination rate in the time series of Fig. 5.4. This demonstrates the importance of experiments designed for scans over several independent parameters, and not just one single variable.

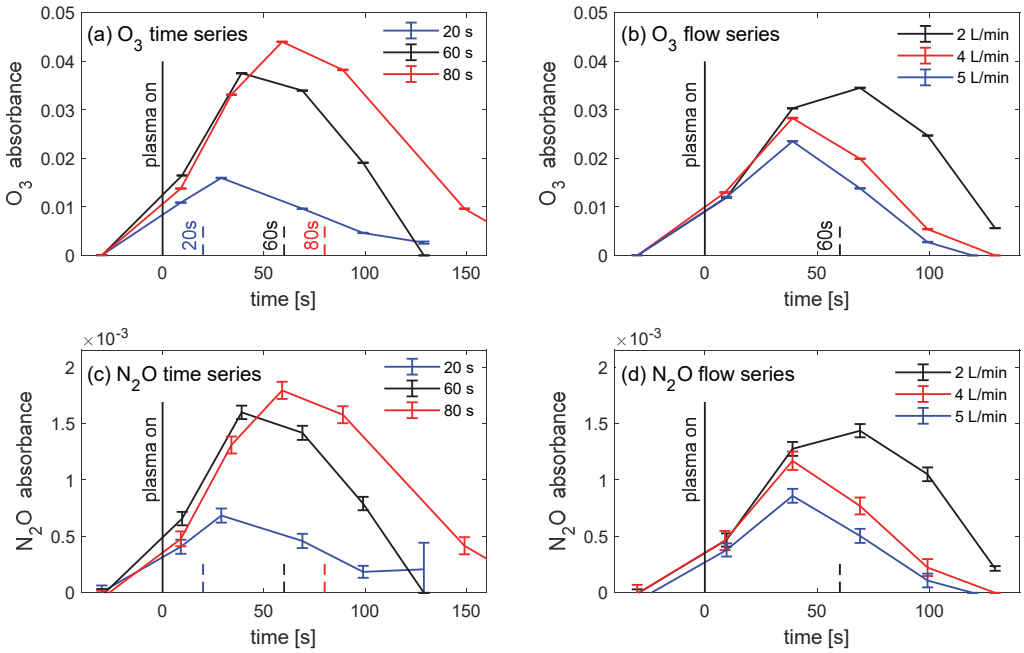

Figure 5.7. Time-dependent absorbance measurements of O_3 and N_2O from the series of treatment times (20, 60, and 80 s plasma duration) and flow rate (2, 4, and 5 L/min). The densities of both molecules increase with the plasma treatment time, and decrease with the air flow rate, as expected. The O_3 and N_2O molecules follow similar trends for each series. The measurements for the default parameters are shown in black.

Moreover, if only the time series results had been selectively reported in this work, then this biased selection would deceptively imply that the increase in gas species was responsible for the increase in the germination rate. This showed the importance of objective reporting of all correlations, whether positive, null, or negative.

5.3 Discussion and Conclusions

Few studies have combined FTIR and plasma-seed treatments although, to our knowledge, this has not been done using in situ FTIR. Plasma diagnostics using in situ FTIR have been performed in dry and humid air (Sakiyama et al., 2012; Yoon et al., 2017; Dascalu et al., 2021), and in specialized gas chemistries (Pan et al., 1998; Nair et al., 2007; Jia et al., 2016; Jia et al., 2017), although independently of seeds and germination rate studies.

Germination rate is a relatively quick and convenient biological measurement, but it may not always be the appropriate indicator; field data are perhaps more relevant. For example, Koga et al. (2015) showed that the harvest mass of *Arabidopsis* strongly increased after DBD plasma treatment. Indeed, short-duration plasma had a long term beneficial effect on harvest mass (Koga et al., 2015; Sarinont et al., 2016), without showing a strong change in germination rate. Even if no improvement in germination was observed, it could still be possible that other changes in the seed development may occur after plasma treatment. Therefore, studies should be careful before making conclusions and to remedy this, additional measurements, like harvest mass or RNA sequencing, can be used to confirm results.

Using FTIR, it is possible to measure species such as NO_2, N_2O, NO, CO_2, HNO_3, HNO_2, water, CO, N_2O_5, and O_3 in an air plasma. Kyzek et al. (2019) and Tomekova et al. (2020) measured the plasma chemistry

of a DCSBD using either ambient or synthetic air. Interestingly, ambient air conditions resulted in predominance of NO_x species but found mostly ozone followed by N_2O_5, N_2O, and HNO_3 when using synthetic air. Our results are comparable in terms of the presence of similar species except that in our case, ozone was the dominant molecule, likely because synthetic air was used but it could also be possibly due to the power. The steady increase of ozone with time in the measurements of Fig. 5.7(a) is consistent with the observation of Shimizu et al. (2012) that ozone increases monotonically with time for SDBD power densities below 0.1 Wcm^{-2}. The SDBD power was always below 0.08 Wcm^{-2}, therefore the experiments were clearly in ozone mode, before secondary reactions lead to significant concentrations of NO_x species. N_2O_5 is reported for longer discharges (Sakiyama et al., 2012; Yoon et al., 2017), depending on conditions, and so was not detected here. Furthermore, ambient air is often in the range of 40 - 50% RH and it has been shown by authors such as Koga et al. (2015) and Sarinont et al. (2016) that humidity is an important factor in improving harvest. However, our results suggest that it is possible to have some effect in dry conditions, although the FTIR spectra have fewer radical types (none with H) because of this. Future work will explore the differences in FTIR spectra upon the addition of seeds and varying levels of humidity.

Based on Wang et al. (2017), Kyzek et al. (2019), and Tomekova et al. (2020), the presence of nitrogen oxides seems to be sufficient to trigger changes in germination. However, Tomekova (2020) found the most DNA damage using pure nitrogen. Our results indicated clear changes in ozone, N_2O, and NO_2 concentrations, but other nitrogen species were not detectable.

Ozone and NO_x species are known to affect seed dormancy and germination (Cohn et al., 1984; Sudhakar et al., 2011; Avdeeva et al., 2018; Rather et al., 2020). However, in this work, there was no clear conclusion as to whether any particular molecule is responsible for the accelerated germination. Therefore, perhaps the effect is not due to gas chemistry but rather to ions or electrons, or other short-lived species, such as NO, which possibly play a role if they reach the seed surface. Kyzek et al. (2019) detected NO using FTIR absorbance spectroscopy in higher power plasma where NO_x species were dominant; the NO absorbance was far smaller than for NO_2 and N_2O, which would be consistent with the absence of NO in the spectra of Fig. 5.6. Laser-induced fluorescence (LIF) is another diagnostic that can detect short lifetime species, such as O, NO, OH, N, and HO_2, but these are so short lived and very reactive that they disappear in only a short distance from the plasma (Sakiyama et al., 2012). There are multiple candidates which are either long or short lifetime species which may have an effect on germination and it is not yet clear which are responsible for the plasma effect. For short lifetime species, it is best to use LIF because single-pass FTIR is at the detection limit and cannot measure primary reactive species (radicals, ions, or metastables). Preliminary LIF studies in the present setup confirmed the presence of NO within the first few mm from the SDBD. This suggests that reactive, short-lifetime species are responsible, and not ions, because an indirect plasma treatment was used. Two key points should be emphasized; multiple diagnostics should be used to cross check results within the same study and that our FTIR methodology needs to be adjusted so that NO is no longer under the detection limit. It may be possible that NO is partly responsible for the effect on germination and therefore, future studies will need to explore this further.

Nonetheless, the FTIR parametric study improved our understanding of how each variable influences plasma chemistry. Overall, similar species were detected across all spectra but it was useful to confirm trends such as an increase in reactive species with time and a decrease in concentration with increased flow rate.

Cimerman et al. (2021) showed that there is an increase in ozone with an increase in voltage, which is in agreement with our results and Yuan et al. (2018). However, Liu et al. (2017) increased voltage and decreased their air flow rate to shift from ozone to NO_x mode. With a higher air flow rate, in both instances, ozone decreased but N_2O increased in their setup and not in ours. Perhaps this did not occur in our study because the operating ranges are small (6.5 - 8.5 kV in comparison to 16 - 26 kV), but this remark is important to note for those who would like to work in a specific regime. It may be possible that operating with such high voltages resulted in an elevated temperature and this contributed to higher concentration of N_2O. Chen et al. (2019) pointed out that higher electrode temperatures suppress the generation of NO_2 and promote N_2O generation. Likewise, it is known that increasing temperatures can decrease ozone concentration (Al-Abduly and Christensen, 2015). It is noteworthy that most energy is dissipated as heat in the system and only a fraction of energy is used to generate reactive species, as pointed out by Abdelaziz et al. (2016), and therefore, power will partly determine the efficiency of reactive species generation. Our DBD operates in very low time-averaged power ranges of approximately 3 - 3.5 W, principally because of the 10% duty cycle, as shown by the Lissajous figure (see Chapter 3), which could explain the dominance of ozone and the low concentration of N_2O.

In conclusion, single-pass in situ FTIR measurement provides simultaneous monitoring of many biologically significant radicals during plasma-seed treatment, and is potentially more relevant than monitoring downstream exhaust gas. However, it is too early to say if there are any different radicals, or new behaviour, observed using in situ FTIR. The experiments demonstrated the importance of performing scans over multiple independent variables to avoid erroneous interpretations. Currently, it remains unclear which agents influence germination and therefore, further monitoring of reactive species should improve our understanding of how to tailor plasma-seed treatments.

Chapter 6

Molecular analysis of plasma-treated seeds: how do plants respond to plasma?

Abstract

In this chapter, RNA sequencing is used to characterize the changes in gene transcription in *Arabidopsis thaliana* (L.) Heynh. seeds 6 days after exposure to surface dielectric barrier discharge plasma treatment. Here, we provide an overview of all pathways which are differentially expressed where few genes are upregulated and many genes are downregulated. Our results reveal that plasma treatment time is a parameter which can activate different pathways in plant defense. An 80 s treatment upregulates the glucosinolate pathway, a defense response to insects and herbivores to deter feeding. In contrast, a shorter treatment of 60 s upregulates the phenylpropanoid pathway which reinforces the cell wall with lignin and produces antimicrobial compounds, a defense response to bacterial or fungal plant pathogens. It seems that plasma elicits a wounding response from the seed in addition to the redox changes. This suggests that plasma treatment can be potentially applied in agriculture to protect plants against abiotic and biotic stresses. With a 24-hour delayed extraction in seedlings grown from plasma-treated seeds, increased nitrile synthesis was observed. A glucosinolate response was confirmed again, yet the results revealed the breakdown of these glucosinolates into a specific catabolic product. Lastly, it remains unclear which genes could be used as molecular markers specific to plasma treatment, but a few are proposed that could respond to oxidative stress.

This chapter is largely derived from the two articles cited below. I performed the analysis for the potential oxidative stress gene candidates, carried out the experiments for the validation of the selected candidates, performed the nanoluciferase experiment, conceptualized the experimental design for the RNA sequencing study, carried out the germination experiments and RNA extractions, analyzed the data, contributed towards data visualization, developed a tentative hypothesis of how plants interpret plasma treatment and designed it schematically (Fig. 6.11), wrote the manuscripts, selected the journal, and handled the submission process.

International Journal of Molecular Sciences

RNA sequencing of *Arabidopsis thaliana* seedlings after non-thermal plasma seed treatment reveals upregulation in plant stress and defense pathways

and

Catabolism of glucosinolates into nitriles revealed by RNA sequencing of *Arabidopsis thaliana* seedlings after non-thermal plasma-seed treatment

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Keywords: non-thermal plasma, RNA sequencing, *Arabidopsis thaliana*, oxidation-reduction, plant defense, secondary metabolism, glucosinolates, phenylpropanoids, wounding, nitriles

6.1 Introduction to reactive oxygen and nitrogen species and potential marker genes

Reactive oxygen species, in other words, oxygen-derived free radicals, are an area of interest that is on one hand, challenging to understand due to their short (less than a second) lifetime but absolutely important as they play an instrumental role in biological systems i.e. signaling (Sies and Jones, 2020). They are short-lived but they trigger long-lived physiological or pathological reactions, for example through lipid peroxidation (Ayala et al., 2014). The immune response is largely due to ROS signalers such as superoxide. It is now known that ROS can be either beneficial or harmful but when harmful, it results in oxidative stress. Oxidative stress occurs when the concentration of the reactive species becomes too high, or (non)-enzymatic scavengers, called antioxidants, are too low. This leads to the radicals reacting and damaging cellular macromolecules, like DNA, RNA, proteins and lipids, resulting in oxidation, enzyme inactivation, or DNA mutations (Bhattacharya and Saha, 2015). There are several tracking tools that exist which range from qualitative methods using fluorescence to quantitative methods, such as genetically engineered probes (Mhamdi and Van Breusegem, 2018) or marker genes specifically responding to ROS. Considering plasma produces many of these reactive oxygen species, as well as reactive nitrogen species, it is important to better understand these components. Here, the focus will mainly be on reactive oxygen species, largely due to the amount of data available.

When considering which genes could be responsive to RONS, whether plasma-induced or not, it is helpful to first consider what is known before adding the complexity that is plasma. There are studies such as Petrov et al. (2012) that identified cis-elements, DNA sequences which regulate the transcription of neighbouring genes, for several hundred abiotic stress conditions. This was done by using genome-wide microarrays to identify gene groups induced by singlet oxygen, superoxide radicals, and H_2O_2. Abiotic stress is a well-known inducer of oxidative stress, however, one challenge is that there are general oxidative stress markers and specific markers to each ROS (Gadjev et al., 2006). This response will also partly depend on the subcellular synthesis site, as well as, the length and intensity of the stress signal. Not all reactive species are the same in that motifs can be found for singlet oxygen and superoxide, but not hydrogen peroxide because it is involved in many clusters. Therefore, finding a promoter region or genes specific to just H_2O_2 is more difficult and thus, this also makes it generally challenging to identify a gene expression profile specific to plasma treatment. Furthermore, the relationships between the reactive species amplifies the difficulty in identifying marker genes i.e. superoxide dismutase (SOD) converts superoxide into H_2O_2.

Despite the complexity, the concept of identifying a gene signature after plasma treatment was explored here. The idea was to first verify as a proof-of-concept that there is little to no heat shock during a plasma treatment using a transgenic *Arabidopsis thaliana* promoter line developed by Baptiste et al. (2021). Afterwards, the idea was to investigate whether it would be possible to identify a specific promoter for plasma-generated ROS, and then develop a reporter line as a tool for quantifying the oxidative stress response in the plant after plasma treatment.

To check the heat-shock response, a transgenic reporter line called HIBAT was used. It has a heat shock protein, hsp17.3B, cloned next to nanoluciferase which is 500 bp and requires no ATP, has more stability than the firefly version, is smaller, and very specific. The experiments with the first SDBD prototype and an indirect treatment style showed no increase in heat shock proteins. The results in Fig. 6.1 show the expression of nanoluciferase in untreated, plasma-, and ozone-treated seedlings. The positive control, heat treatment at

37°C, indicated that the plasma treatment did not trigger the expression of heat shock proteins. This is important because heat and oxidative stress can overlap (see Appendix for methods).

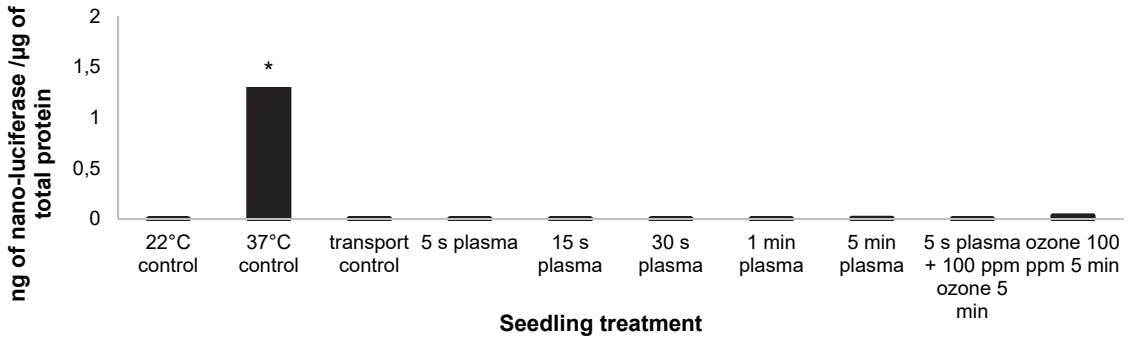


Figure 6.1. Quantification of nanoluciferase with heat, plasma, and ozone treatment. Nanoluciferase expression only increases with heat treatment, and not with plasma or ozone treatment.

The lack of nanoluciferase expression after plasma treatment was likely due to the short treatment times and indirect treatment style since the plasma was a few millimeters away from the seedlings. Therefore, if the temperature of the device was around room temperature and the substrate was away from the plasma, this suggests that the transcription and translation of heat shock proteins is not a concern under these experimental conditions. However, as mentioned before, heat and oxidative stress share common targets so it does not exclude the possibility of crosstalk.

After observing that heat is not a concern in specific plasma conditions, individual genes were screened in an attempt to identify the ones which respond the strongest to reactive species. An unbiased method was used to screen all 33,000 genes in *Arabidopsis* and was then followed by a brief analysis of the cis-elements. Genevestigator, a collection of microarray data specifically from Affymetrix ATH1 Genome Array, was used for the screen and only included studies with Col-0 seedlings exposed to heat, individual ROS such as hydrogen peroxide and ozone, or conditions that would generate ROS, such as high light and methyl viologen. The goal was to select genes which did not respond to heat (less than 0.1-fold change), and only responded to oxidative stress (greater than 3-fold change). The names of the reference studies used in Genevestigator were AT00025, AT00120, AT00185, AT00413, AT00495, AT00500, AT00645, and AT00654.

To narrow down the number of candidates, genes were selected further if oxidative stress was in the top 10 perturbation list and if there was no increase in gene expression under heat stress. The list was reduced to 80 candidate genes and was again, narrowed down based on the corresponding proteins location, preferably the cytoplasm, using the *Arabidopsis* subcellular database (SUBA) and were then validated experimentally using quantitative polymerase chain reaction (qPCR) (see Table 6.1).

Table 6.1. Information for each gene candidate and its corresponding perturbation and location.

Selected Gene Candidate	Name	Perturbation	Location
AT1G55920	Serine acetyltransferase 1, chloroplastic	H_2O_2	chloroplast, cytosol, plastid
AT1G74360	Probable LRR receptor-like serine/threonine protein kinase	Ozone	mitochondrion, endoplasmic reticulum
AT1G76680, AT1G76690	12-oxophytodienoate reductase 1	H_2O_2, ozone	cytoplasm
AT2G29420	Glutathione S-transferase U7	H_2O_2, ozone	cytosol
AT2G43820	UDP-glycosyltransferase 74F2	Ozone	plasma membrane, extracellular
AT3G25010	Receptor-like protein 41	ozone, UV-B	extracellular
AT4G04610	5'-adenylylsulfate reductase 1, chloroplastic	H_2O_2	chloroplast
AT4G22530	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	Ozone	nucleus, cytoplasm
AT5G38710	Proline dehydrogenase 2, mitochondrial	Ozone	mitochondrion
AT5G51830	Probable fructokinase-7	ozone, H_2O_2	cytosol
AT3G49620	Probable 2-oxoglutarate-dependent dioxygenase DIN11	ozone, H_2O_2	mitochondrion, cytosol

qPCR is a quantitative method for measuring gene expression by using polymerase chain reaction (PCR). Briefly, PCR is a method of DNA amplification which begins with a high temperature of around 95ºC to break apart the double-stranded DNA into single strands. This then allows the primers to anneal at a lower temperature, such as at 60ºC, and elongate the fragment at around 72ºC. This process is repeated for a fixed numbers of cycles, hence the amplification of DNA.

qPCR is useful because it compares the relative increase or decrease of gene expression across different samples and treatments. The workflow for qPCR entails extracting RNA from the cells and converting the transcripts into complementary cDNA strands since RNA is very sensitive to degradation. This cDNA strand is then amplified using PCR and the relative count is captured by the amount of fluorescence emitted by the probe. Many genes can be targeted by designing gene specific primers and the cDNA amplification can be detected using either a general DNA binding dye, called SYBR green, or specific probe, called Taqman. The advantage of using SYBR green is its versatility since it can be used on many transcripts, but its non-specificity does not guarantee accurate quantification. In contrast, Taqman probes are intentionally designed to bind and amplify specific transcripts. A quencher blocks the fluorescent tag of the probe but after binding the appropriate transcript, it is cleaved which then allows the fluorescent signal to be emitted and quantified. Regardless of the probe type, if there is increased gene expression, then this higher copy number of transcripts emit more fluorescence which can then overcome the set fluorescence threshold at lower cycle numbers. Using this information, quantification can be done, often with the double cycle threshold (CT) method.

As shown in Fig. 6.2, there were 4 genes selected out of 33,000 (AT2G29420, AT3G49620, AT5G51830, and AT4G22530) largely based on the fold change from qPCR experiments, tissue area, age, and location of expression. These genes are predominantly linked to the cysteine pathway where AT2G29420

is a glutathione S-transferase U-7 involved in glutathione mediated detoxification, AT3G49620 is a probable 2-oxoglutarate-dependent dioxygenase involved in ethylene biosynthesis, and AT4G22530 is a S-adenosyl-L-methionine-dependent methyltransferases superfamily protein involved in homocysteine production. The exception, AT5G51830, is a probable fructokinase-7 involved in sucrose and D-sorbitol degradation.

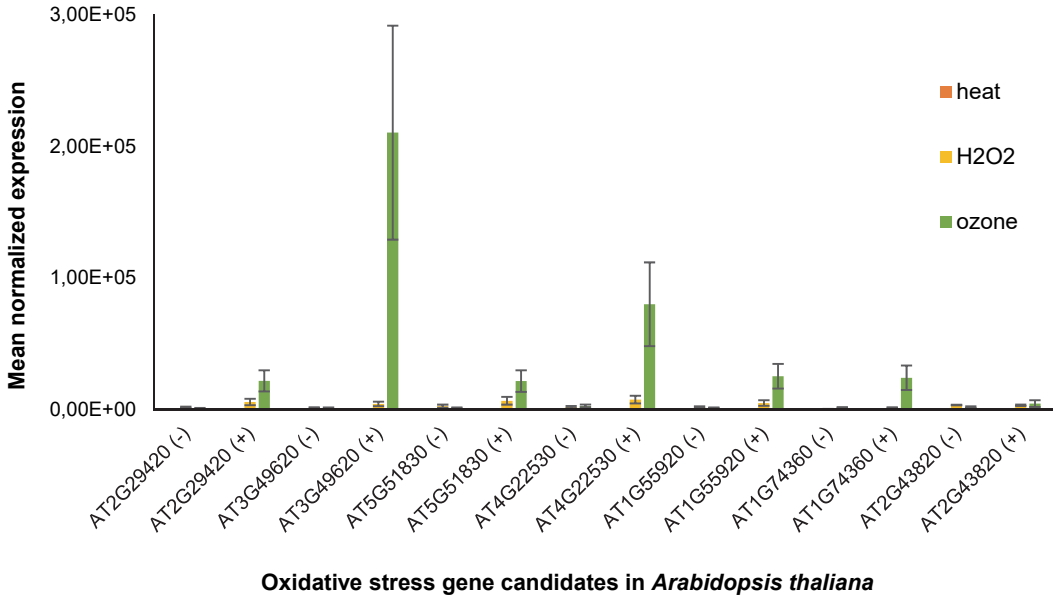


Figure 6.2. Potential oxidative stress gene markers. Responsiveness to heat, ozone or H_2O_2 treatment are shown for each gene.

However, qPCR is very targeted approach, meaning that the genes in question need to already be known in order to study them, whereas RNA sequencing enables an unbiased, exploratory approach. This is especially useful if the response of an organism to a treatment is unknown and unpredictable. There were a few qPCR experiments attempted on seedlings grown from plasma-treated seeds using the candidate genes mentioned above, but there were no changes in their expression. It could be that the expression indeed did not change since there were no changes in the plant macroscopic properties after plasma treatment. Nevertheless, qPCR proved to be an inefficient method because it remained unclear whether it was due to an insufficient plasma treatment, or simply the selection of unresponsive genes.

For this reason, RNA sequencing was performed immediately on seedlings, grown from plasma-treated seeds, with accelerated germination. These set of experiments were performed using the most recent, optimized plasma-seed treatment (see Chapter 5). Out of curiousity, the presence of the oxidative stress gene candidates was checked in the RNA-seq data, but they were largely absent with the exception of AT1G76680. It could be that these candidate genes are bound to a specific plant age and are thus only valid in 2-week-old seedlings. Since the RNA-seq data was gathered from 1-week-old seedlings, it might not be possible to directly translate this information to different plant ages. Nonetheless, this highlighted the difficulty in predicting which genes would be relevant after plasma treatment, especially across different experimental setups. Therefore, effort was redirected to identifying differentially expressed genes after plasma treatment.

6.2 Introduction to gene expression studies on plasma-treated seeds

After plasma treatment, it has been shown that seed germination can be accelerated or plant properties, such as root length, shoot length, harvest mass, or stress and disease resistance, can be increased (Guragain et al., 2021; Li et al., 2021). Despite the numerous successful results obtained in other studies, it remains elusive which plasma treatment parameters are required to affect plant growth parameters, mainly because of the plethora of physical, chemical, and biological variables (see Chapter 7). Therefore, the focus of the plasma agriculture community has recently shifted to the analysis of gene expression, gene methylation, and protein expression in plasma-treated seeds or plants using high throughput methods (Mildažienė et al., 2019; Tamošiūnė et al., 2020ab; Suriyasak et al., 2021).

Currently, the most common method for analyzing gene transcription is qPCR where specific genes of interest are targeted, but very few studies analyze genes in an unbiased manner using microarrays or RNA sequencing (RNA-seq) (Ji et al., 2018; Guo et al., 2017; Rahman et al., 2018; Adhikari et al., 2019; Islam et al., 2019; Perez et al., 2019; Adhikari et al., 2020; Ghasempour et al., 2020; Iranbakhsh et al., 2020; Ghaemi et al., 2020; Ebrahimibasabi et al., 2020; Sajib et al., 2020; Li et al., 2021; Ka et al., 2021).

With the current plasma-seed studies, these gene expression findings are often only relevant to a specific plant and may not be applicable elsewhere. This, therefore, limits our ability to understand the molecular mechanisms of plasma-seed treatments. Although the plant choice is well justified based on local or global economic importance, there is often only one or a few studies of each plant type, which makes it difficult to compare the results and find commonalties. However, there are a few recently published studies using transcriptomics which provide a global overview of gene expression changes after plasma treatment (Tong et al., 2020; Cui et al., 2021; Han et al., 2021; Wang et al., 2021).

A surface DBD (SDBD) with two electrodes separated by the dielectric was used where the plasma is formed in the gas at the edges of high voltage patterned electrode to treat *Arabidopsis thaliana* (L.) Heynh. as a seed substrate (see Chapters 3 and 5). *A. thaliana* has its entire genome sequenced and thus unraveling the molecular mechanisms of the interactions with plasmas is more feasible. Since it is a plant model organism from the *Brassica* family, these findings could be potentially applied to agriculturally relevant crop plants. This study is among the first ones using RNA-seq in *A. thaliana* to characterize the gene transcription changes in these seeds 6 days after SDBD plasma treatment using dry synthetic air. Here, an overview is given of all pathways with differentially expressed genes, where a few genes were upregulated and many genes were downregulated. A hypothesis is proposed in the Discussion and Conclusions as to how plants could react to non-thermal plasma.

6.3 Germination rate of plasma-treated *A. thaliana* seeds

As shown in Chapter 5, germination rates were measured for parametric scans of treatment time, voltage, flow rate, gap distance between the plasma and seed, and frequency. Here, the operating parameters were either 60 or 80 s plasma treatment time, 8 kV peak-to-peak, 2 L/min flow rate of dry synthetic air, seed substrate distance 3.7 mm, and 10 kHz frequency with a power on/off modulation at 500 Hz and 10% duty cycle, corresponding to a burst of 2 cycles per modulation period. The 10% duty cycle was used to avoid heat shock of the seeds. Figure 6.3 shows the germination rate of seeds measured after 48 hours, compared to the control, untreated seeds with no plasma exposure (indicated as 0 s).

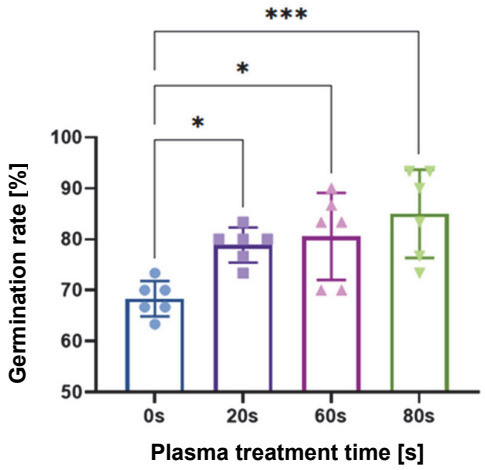


Figure 6.3. Germination rate results of plasma-treated *Arabidopsis thaliana* (L.) Heynh. seeds, demonstrate that 80 s has the strongest effect followed by 60 s treatment. Control experiments, i.e. no plasma treatment, are indicated with 0 s. Experiments are an average of triplicates performed twice independently for a total of 6 replicates. Asterisks denote statistical significance where * signifies p <0.05; ** is p <0.01; and * is p <0.001.**

As marked by the asterisks in Fig. 6.3, scans of treatment time yielded statistically significant increases in the germination rate for 20, 60, and 80 s times (total plasma treatment time), which corresponds to 2, 6 and 8 s plasma ON time. The longest plasma treatment time of 80 s had the highest statistical significance for the germination rate; therefore, this parameter set became the focus of the following section. However, data from 60 s is added and mentioned where appropriate. For detailed information about RNA sequencing methodology, please refer to the Appendix.

6.4 Global RNA-seq analysis of young seedlings after 60 s or 80 s non-thermal plasma-seed treatment

To obtain the RNA sequencing data, RNA is extracted and converted to cDNA if it is of sufficient quality which is determined by the 18S and 28S rRNA quality. Since this includes all forms of RNA, the mRNA needs to be exclusively separated and used to analyze transcriptional changes, meaning that the rRNA must be removed. Afterwards, libraries are prepared where the mRNA is fragmented, converted into cDNA, and then amplified. The transcripts have an adapter, or in other words a bar code, attached to the ends. Since RNA-seq is a multiplex method, this is useful for tracking the transcripts and knowing which samples they originate from. The adapters are then attached to a flow cell and each nucleotide complementary to the transcript is added sequentially. To know which base is added, each nucleotide has a uniquely assigned fluorescent tag to be able to discriminate between different bases. This process is done for all transcripts simultaneously and the transcripts are amplified.

Once the raw data is obtained as read counts, the location of the reads is mapped using the reference genome or transcriptome of the organism. These reads are filtered since mRNA can be degraded or contaminated, which is indicated by the GC content, and therefore, this step serves as a quality control check. The quality is checked further using bioinformatics to calculate an artificial average gene expression or compare sample clustering using Pearson coefficient or Euclidean distance. If there is sufficient separation between different conditions and tight clustering under the same conditions, the relative increase or decrease in gene expression in the treated sample relative to the control can be calculated; either on a gene or pathway level using fold change (log2) or the percentage of differentially expressed genes belonging to a pathway. To understand how proteins and metabolites change, proteomics and metabolomics should be used since trends are not linear.

To obtain gene expression profiles, RNA-seq data were generated from 6-day-old seedlings grown in continuous light. Each biological replicate was a pool of 30 seedlings grown in the same agar plate to reduce biological variability. An average of 33 million raw reads of 150 base pairs (bp) were produced for 80 s, while, after filtering steps, ~31 million of clean reads (~94 %) per library were retained (Table S1) and ~95.4% were mapped to the *A. thaliana* reference genome (Table S2). Similar values were obtained for 60 s.

To check if the RNA-seq data from different conditions were similar, a principal component analysis (PCA) was performed on the normalized gene expression values (Fig. 6.4). The PCA analysis showed similar clustering among the replicates of each condition. One replicate of 80 s treatment slightly grouped with one control sample, which may be due to high biological variability whereas, two 60 s treatment replicates were more closely associated with the control samples. For the 60 s treatment, the first two principal components explained 64% of the total variance (24% by PC1 and 24% by PC2) whereas for the 80 s treatment, 62% of the total variance was captured within the first two components (41% by PC1 and 21% by PC2). It is ideal to have most of the variance in the first PC vector, however, the data showed clear clusters between the samples and was used for further analysis. There was a total of 32,833 genes in 6 samples where 21,359 and 21,465 genes for 80 s and 60 s treatment respectively, passed the selected threshold which was having more than 2 reads per biological replicate (see Appendix).

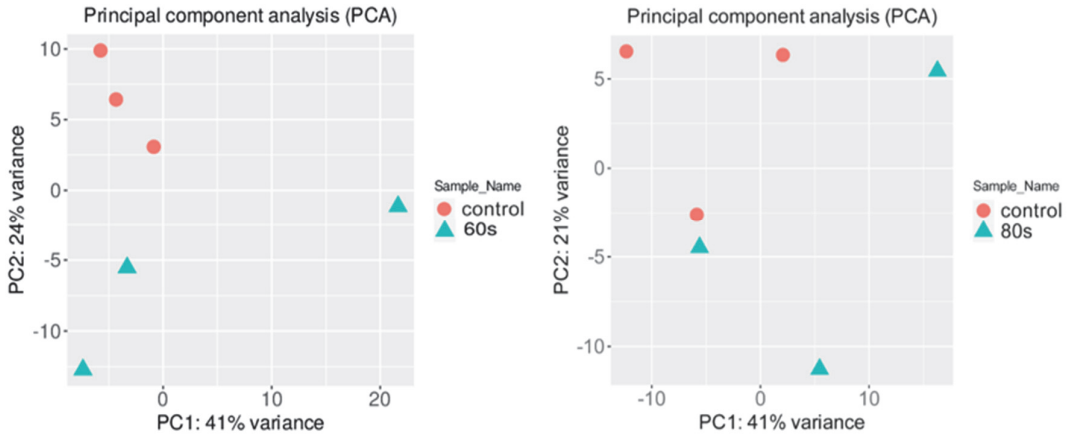


Figure 6.4. Principal Component Analysis (PCA) conducted on the normalized gene expression values of the 60 s (left) and 80 s (right) samples. X- and Y-axes show PC1 and PC2, respectively, with the amount of variance contained in each component, which is 41% and 24% for 60 s and 41% and 21% for 80 s, respectively. Each point in the plot represents a biological replicate, representing 30 seedlings, with a total of 6 biological replicates in the plot. Symbols of the same colors are replicates of the same experimental group where orange represents the control which are untreated *A. thaliana* seeds grown into seedlings and blue represents either 60 s or 80 s plasma-treated *A.thaliana* seeds grown into seedlings.

As shown in Figure 6.5, the hierarchical clustering demonstrates good agreement between the experimental groups where the control, untreated samples clustered together as well as the 60 s or the 80 s treated samples despite the variation seen in the upper axis (Hinneburg et al., 2009; Jin et al., 2011). The variation could be due to the inherent biological variability between seeds, handling of the extraction, or different seed responses to the plasma treatment. Despite this, the quality of the RNA and mapping was very high (Fig. S1, Table S1, Table S2). Compared to the 60 s treatment, the clustering was better in the 80 s treatment. Nevertheless, in both treatments, it is evident that there are more downregulated genes, shown in green, when treated with plasma compared to the control, which has more upregulated genes shown in red.

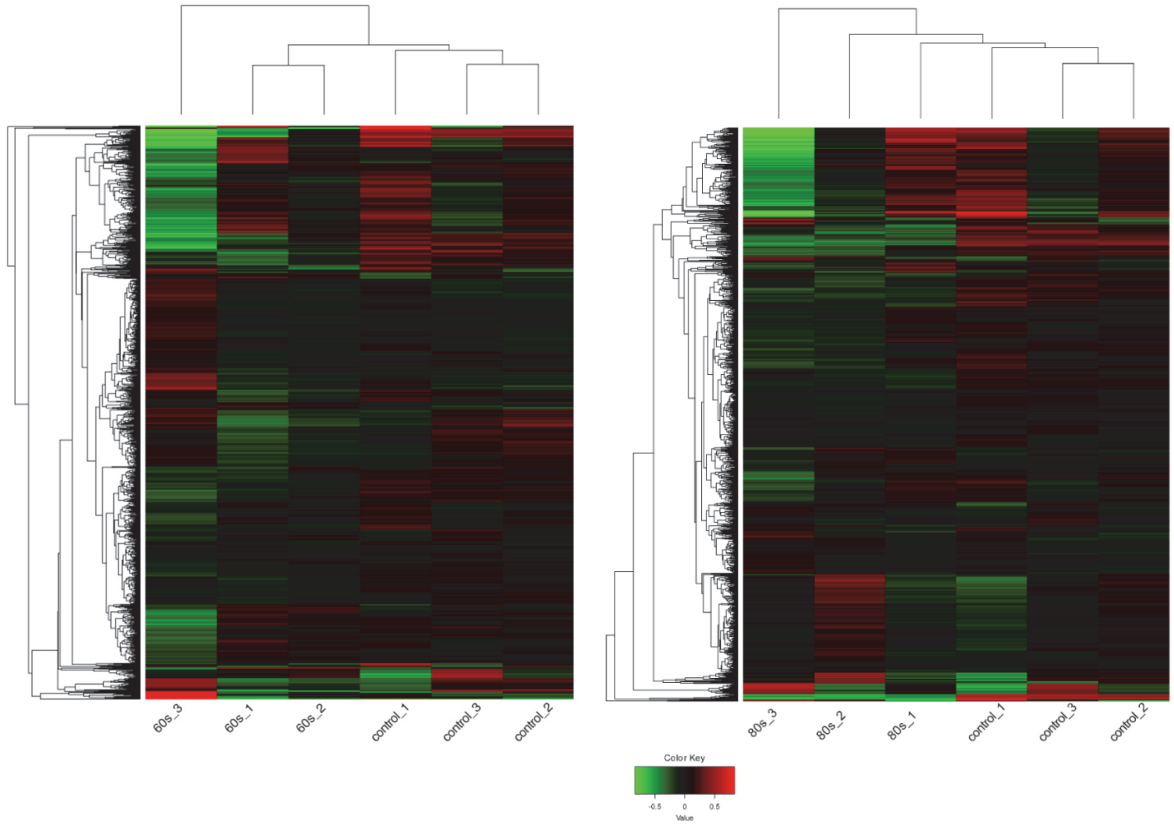


Figure 6.5. Heat map of the expression patterns (Z-scaled reads per kilobase of exon per million reads mapped (RPKM)) of the full transcriptome for 60 s (left) and 80 s (right). Hierarchical clustering of the relative expression profile of the top 2000 variable genes selected based on the lowest standard deviation using Euclidean distance. Individual samples are shown in columns, and genes in rows. The upper axis shows the clusters of samples, and the left vertical axis shows clusters of genes. The color scale represents the relative read count of genes: green indicates low relative read counts; red indicates high relative read counts; black indicates zero (no change). The overall trend shows that genes are mainly downregulated after plasma exposure compared to the control, untreated samples.

Differential expression analysis was conducted via DESEQ2 v1.30.1 across all conditions (Love et al., 2014). The MA plots in Fig. 6.6 showed the extent of differential gene expression in response to plasma treatment. With a log2foldchange (FC)>1 and false discovery rate (FDR) <0.15, a total of 269 differentially expressed genes (DEGs) were obtained for 60 s treatment, where 27 genes were upregulated and 242 were downregulated, whereas 422 DEGs were obtained for 80 s treatment, where 32 genes were upregulated and 390 were downregulated.

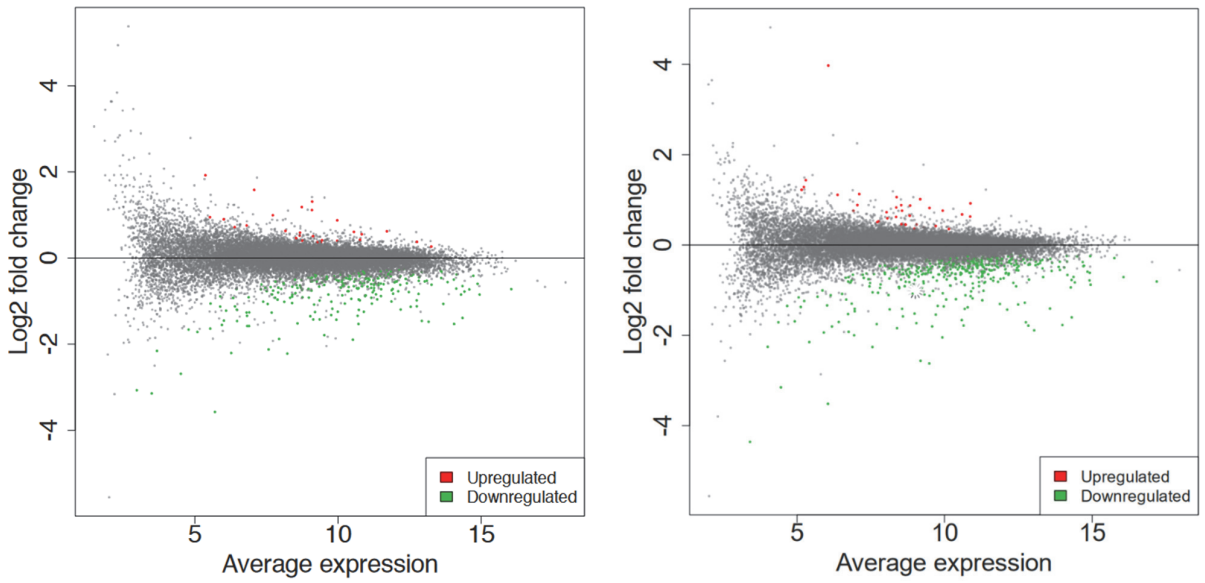
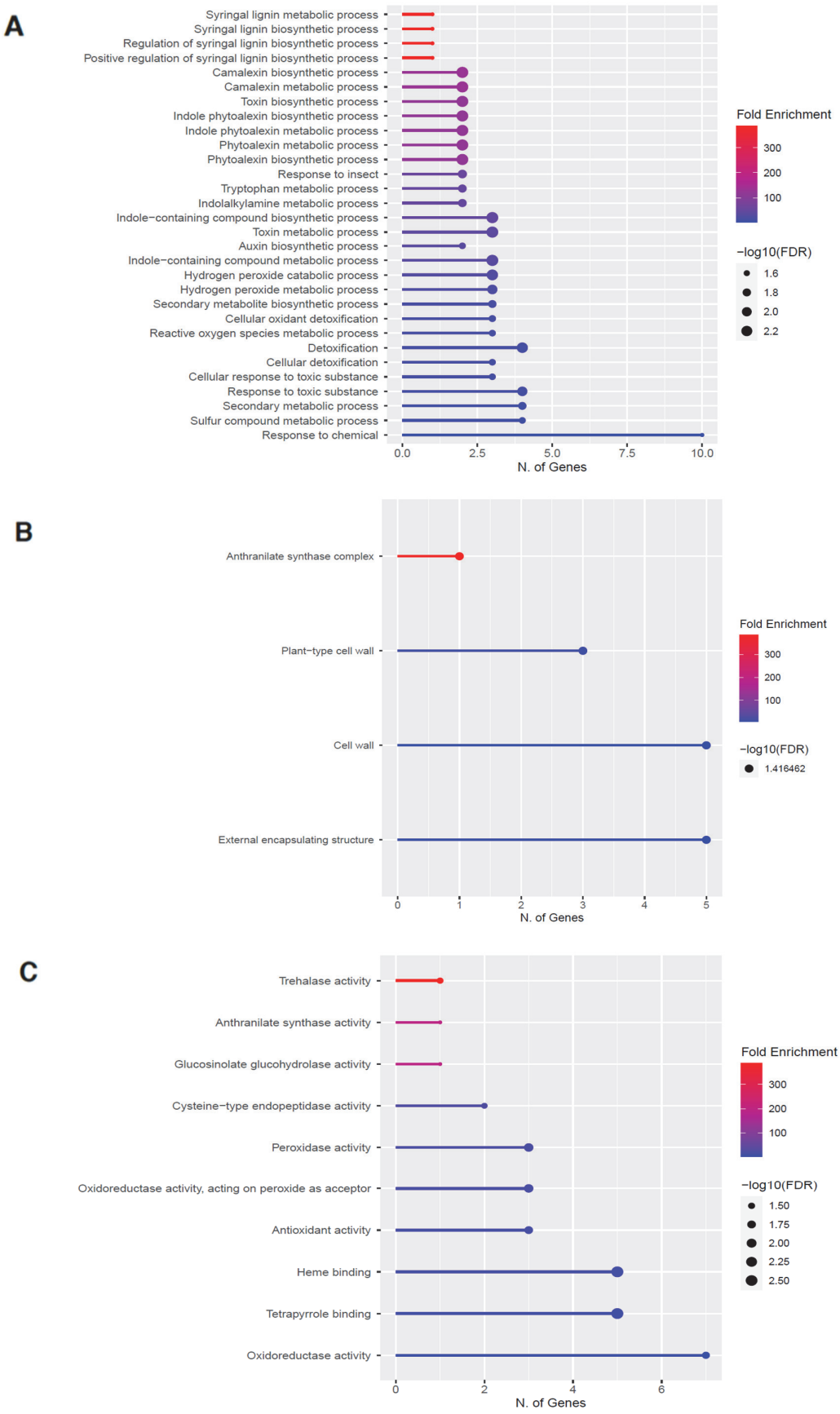


Figure 6.6. The MA plot shows the relationship between the average normalized expression on the x-axis and the significance of the differential expression test expressed as log2FC on the y-axis for each gene in the genome. It illustrates the number of DEGs for 60 s (left) and 80 s (right). Gray dots represent the genes that are not significantly differentially expressed, while red and green dots are the genes that are significantly up- and downregulated, respectively based on their p-values (not shown here).

To elucidate the potential biological functions of these DEGs, gene ontology (GO) analysis of specific groups of DEGs was performed using ShinyGO v0.66 software (Xijin et al., 2020) with the biological process, cellular component, molecular function, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways categories in Figures 6.7 and 6.8 for 60 s plasma treatment and Figures 6.9 and 6.10 for 80 s plasma treatment.

6.4.1 Upregulation of genes in the phenylpropanoid pathway

The GO categories for biological process, cellular component, molecular function, and KEGG pathway enrichment (overrepresented) are shown in Figure 6.7A, B, C, D respectively, for the upregulated genes after 60 s plasma treatment. The lollipop diagram shows the fold enrichment and number of genes in the pathway, and the network map in the supplemental section shows the relationship between the pathways (Fig. S4). Overall, there is increased expression of genes involved in secondary metabolic pathways, specifically the phenylpropanoid pathway and the metabolism of its precursor, tryptophan, as shown in Figure 6.7D. Specifically, lignin biosynthesis is highly upregulated as well as phytoalexin synthesis, whereas response to chemical has the highest number of genes enriched as shown in Figure 6.7A. Regarding the cellular component, anthrinilate synthase, an enzyme for tryptophan synthesis, as well as cell wall and related features were upregulated as shown in Figure 6.7B. The molecular functions which were upregulated in Figure 6.7C were enzymatic reactions related to trehalase activity and anthrinilate synthase.


A
Syringal lignin metabolic process
Syringal lignin biosynthetic process
Regulation of syringal lignin biosynthetic process
Positive regulation of syringal lignin biosynthetic process
Camalexin biosynthetic process
Camalexin metabolic process
Toxin biosynthetic process
Indole phytoalexin biosynthetic process
Indole phytoalexin metabolic process
Phytoalexin metabolic process
Phytoalexin biosynthetic process
Response to insect
Tryptophan metabolic process
Indolalkylamine metabolic process
Indole−containing compound biosynthetic process
Toxin metabolic process
Auxin biosynthetic process
Indole−containing compound metabolic process
Hydrogen peroxide catabolic process
Hydrogen peroxide metabolic process
Secondary metabolite biosynthetic process
Cellular oxidant detoxification
Reactive oxygen species metabolic process
Detoxification
Cellular detoxification
Cellular response to toxic substance
Response to toxic substance
Secondary metabolic process
Sulfur compound metabolic process
Response to chemical
0.0
2.5
5.0
7.5
10.0
N. of Genes
Fold Enrichment
300
200
100
−log10(FDR)
1.6
1.8
2.0
2.2
B
Anthranilate synthase complex
Plant−type cell wall
Cell wall
External encapsulating structure
0
1
2
3
4
5
N. of Genes
Fold Enrichment
300
200
100
−log10(FDR)
1.416462
C
Trehalase activity
Anthranilate synthase activity
Glucosinolate glucohydrolase activity
Cysteine−type endopeptidase activity
Peroxidase activity
Oxidoreductase activity, acting on peroxide as acceptor
Antioxidant activity
Heme binding
Tetrapyrrole binding
Oxidoreductase activity
0
2
4
6
N. of Genes
Fold Enrichment
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100
−log10(FDR)
1.50
1.75
2.00
2.25
2.50

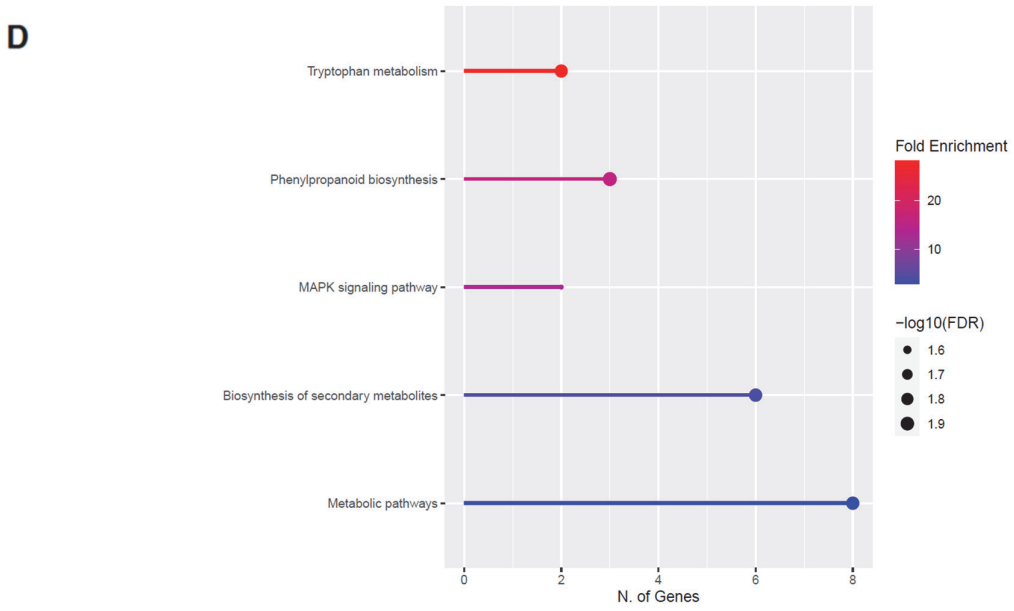
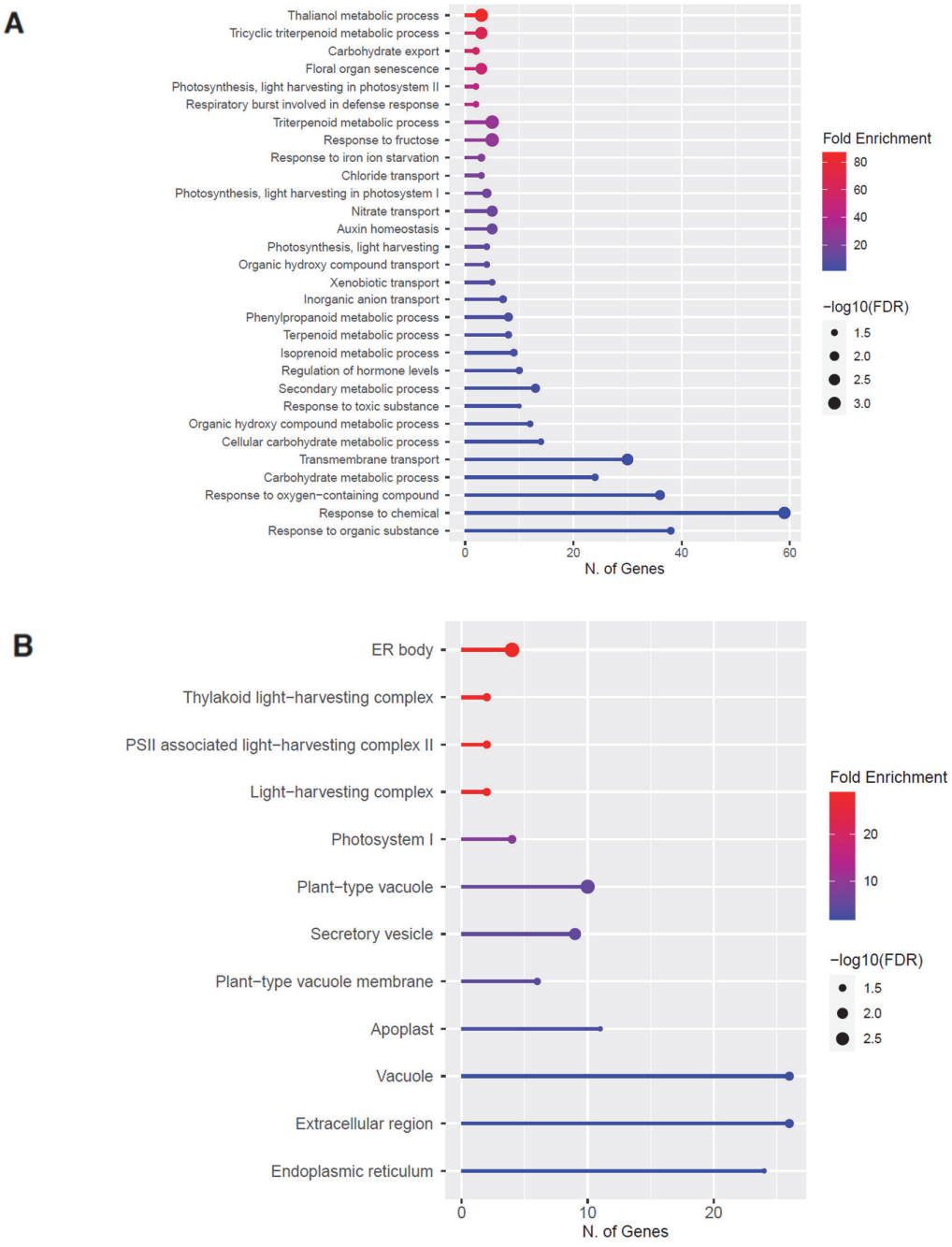


Figure 6.7. Gene Enrichment Analysis for upregulated genes after 60 s plasma treatment. Lollipop diagrams provide information about GO fold enrichment, significance (FDR in log10) and number of genes in each pathway. From top to bottom, GO categories are in the following order: (A) biological process, (B) cellular component, (C) molecular function, and (D) KEGG pathway.

6.4.2 Downregulation of other secondary metabolic pathways

The GO categories for biological process, cellular component, molecular function, and KEGG pathway enrichment, are shown in Figure 6.8A, B, C, D respectively, for the downregulated genes after 60 s plasma treatment. The lollipop diagram shows the fold enrichment and number of genes in the pathway, and the network map in the supplemental section shows the relationship between the pathways (Fig. S5). Overall, there is decreased expression of genes involved in generally other metabolic pathways, two of which are the phenylpropanoid pathway and nitrogen metabolism as shown in Figure 6.8D. In Figure 6.8A, the thalianol and triterpenoid pathways were the most downregulated, whereas the highest number of downregulated genes was observed in response to chemical or response to oxygen-containing compound. In Figure 6.8B, there was a more diverse response from organelles after plasma treatment where many organelles, especially the ER body and photosynthetic apparatus, were downregulated. In Figure 6.8C, auxin transmembrane transport activity was among the most enriched molecular functions, whereas the highest number of downregulated genes were involved in cation binding.


A
Thalianol metabolic process
Tricyclic triterpenoid metabolic process
Carbohydrate export
Floral organ senescence
Photosynthesis, light harvesting in photosystem II
Respiratory burst involved in defense response
Triterpenoid metabolic process
Response to fructose
Response to iron ion starvation
Chloride transport
Photosynthesis, light harvesting in photosystem I
Nitrate transport
Auxin homeostasis
Photosynthesis, light harvesting
Organic hydroxy compound transport
Xenobiotic transport
Inorganic anion transport
Phenylpropanoid metabolic process
Terpenoid metabolic process
Isoprenoid metabolic process
Regulation of hormone levels
Secondary metabolic process
Response to toxic substance
Organic hydroxy compound metabolic process
Cellular carbohydrate metabolic process
Transmembrane transport
Carbohydrate metabolic process
Response to oxygen−containing compound
Response to chemical
Response to organic substance
0
20
40
60
N. of Genes
Fold Enrichment
80
60
40
20
−log10(FDR)
1.5
2.0
2.5
3.0
B
ER body
Thylakoid light−harvesting complex
PSII associated light−harvesting complex II
Light−harvesting complex
Photosystem I
Plant−type vacuole
Secretory vesicle
Plant−type vacuole membrane
Apoplast
Vacuole
Extracellular region
Endoplasmic reticulum
0
10
20
N. of Genes
Fold Enrichment
20
10
−log10(FDR)
1.5
2.0
2.5

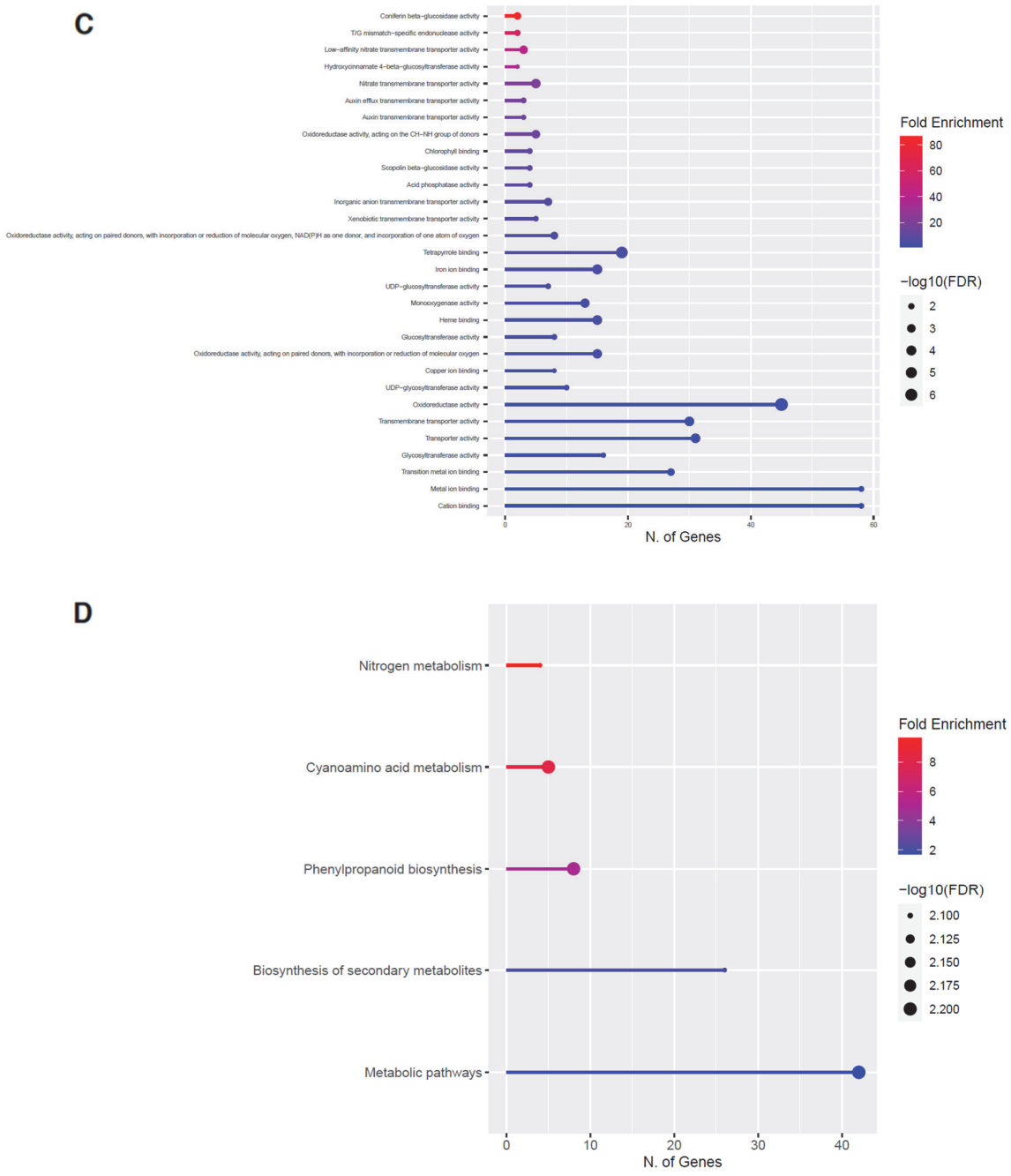
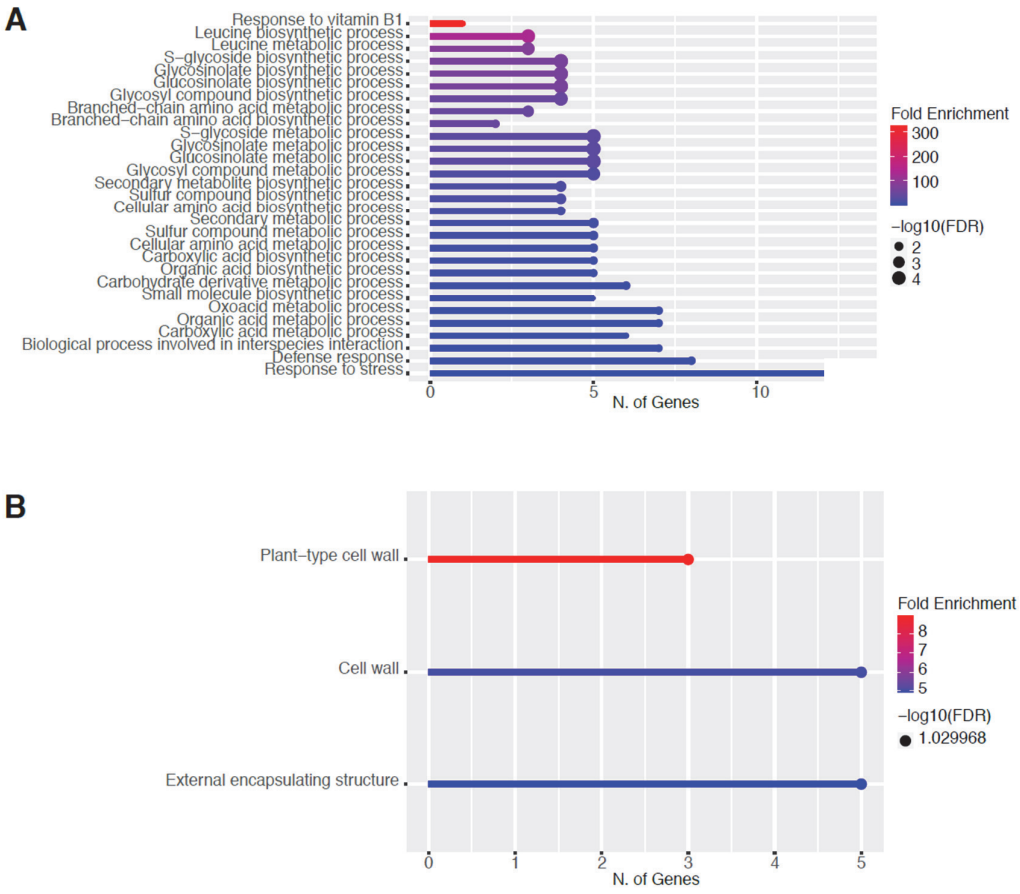


Figure 6.8. Gene Enrichment Analysis for downregulated genes after 60 s plasma treatment. Lollipop diagrams provide information about GO fold enrichment, significance (FDR in log10) and number of genes in each pathway. From top to bottom, GO categories are in the following order: (A) biological process, (B) cellular component, (C) molecular function, and (D) KEGG pathway.

6.4.3 Upregulation of genes in the glucosinolate pathway

The GO categories for biological process, cellular component, molecular function, and KEGG pathway enrichment (overrepresented) are shown in Figure 6.9A, B, C, D respectively, for the upregulated genes after 80 s plasma treatment. The lollipop diagram shows the fold enrichment and number of genes in the pathway, and the network map in the supplemental section shows the relationship between the pathways (Fig. S8). Overall, there is increased expression of genes involved in secondary metabolic pathways, specifically the glucosinolates pathway and biosynthesis of its precursors, valine, leucine, and tryptophan, as shown in Figure 6.9D. Specifically, the response of vitamin B1 is highly upregulated, whereas the defense and stress responses have the highest number of genes enriched, as shown in Figure 6.9A, all of which are interconnected. Regarding the cellular component, only the cell wall and related features were upregulated which included several genes as shown in Figure 6.9B. The molecular functions which were upregulated in Figure 6.9C were enzymatic reactions related to glucosinolates. All of these upregulated elements point to a stress and defense respond being upregulated after 80 s plasma treatment.



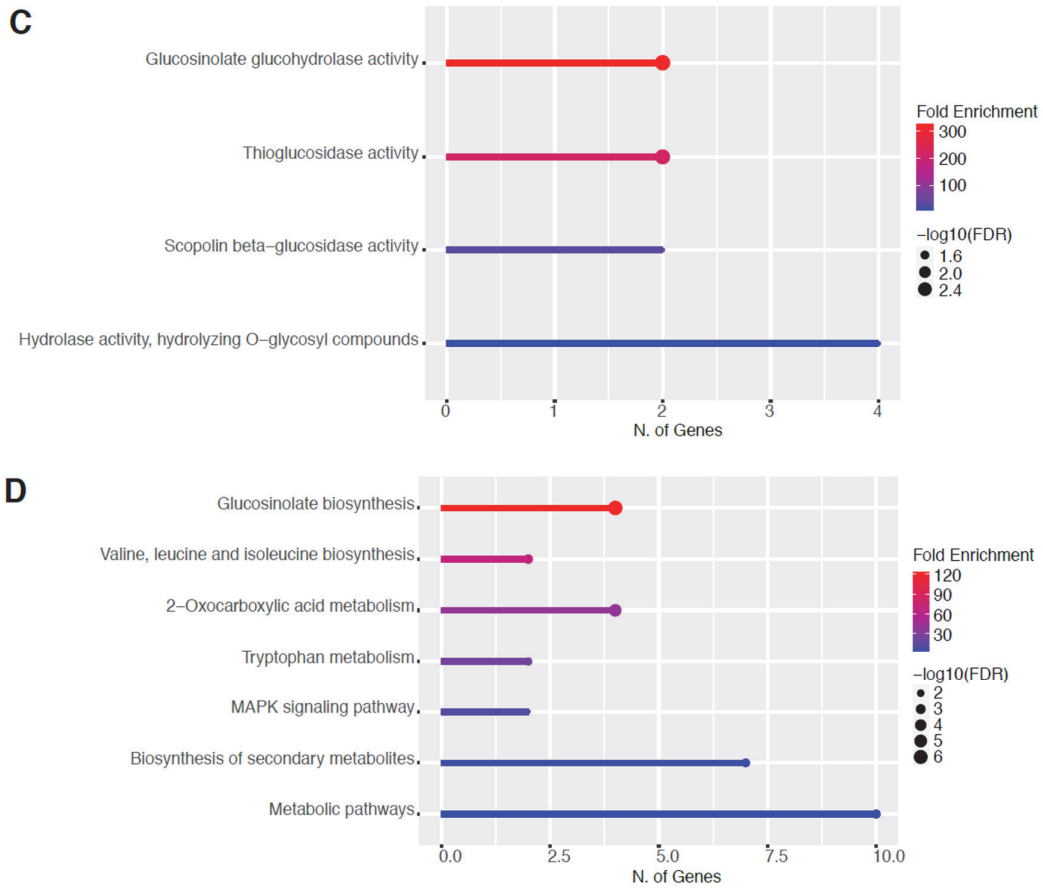


Figure 6.9. Gene Enrichment Analysis for upregulated genes after 80 s plasma treatment. Lollipop diagrams provide information about GO fold enrichment, significance (FDR in log10) and number of genes in each pathway. From top to bottom, GO categories are in the following order: (A) biological process, (B) cellular component, (C) molecular function, and (D) KEGG pathway.

6.4.4 Downregulation of other secondary metabolic pathways

The GO categories for biological process, cellular component, molecular function, and KEGG pathway enrichment, are shown in Figure 6.10A, B, C, D respectively, for the downregulated genes after 80 s plasma treatment. The lollipop diagram shows the fold enrichment and number of genes in the pathway, and the network map in the supplemental section shows the relationship between the pathways (Fig. S9). Overall, there is decreased expression of genes involved in secondary metabolic pathways, specifically phenylpropanoids, phenolic compounds like gingerol and stilbenoid, tyrosine as a precursor of the phenylpropanoid pathway, and glutathione metabolism as shown in Figure 6.10D. In Figure 6.10A, the thalianol and triterpenoid pathways were the most downregulated, whereas the highest number of downregulated genes were observed in catabolic processes. In Figure 6.10B, there was a more diverse response from organelles after plasma treatment where many organelles, especially the ER body, lysosome, and vacuoles, were downregulated. In Figure 6.10C, steroid and sterol binding were the most enriched, whereas the highest number of downregulated genes were involved in enzymatic processes.

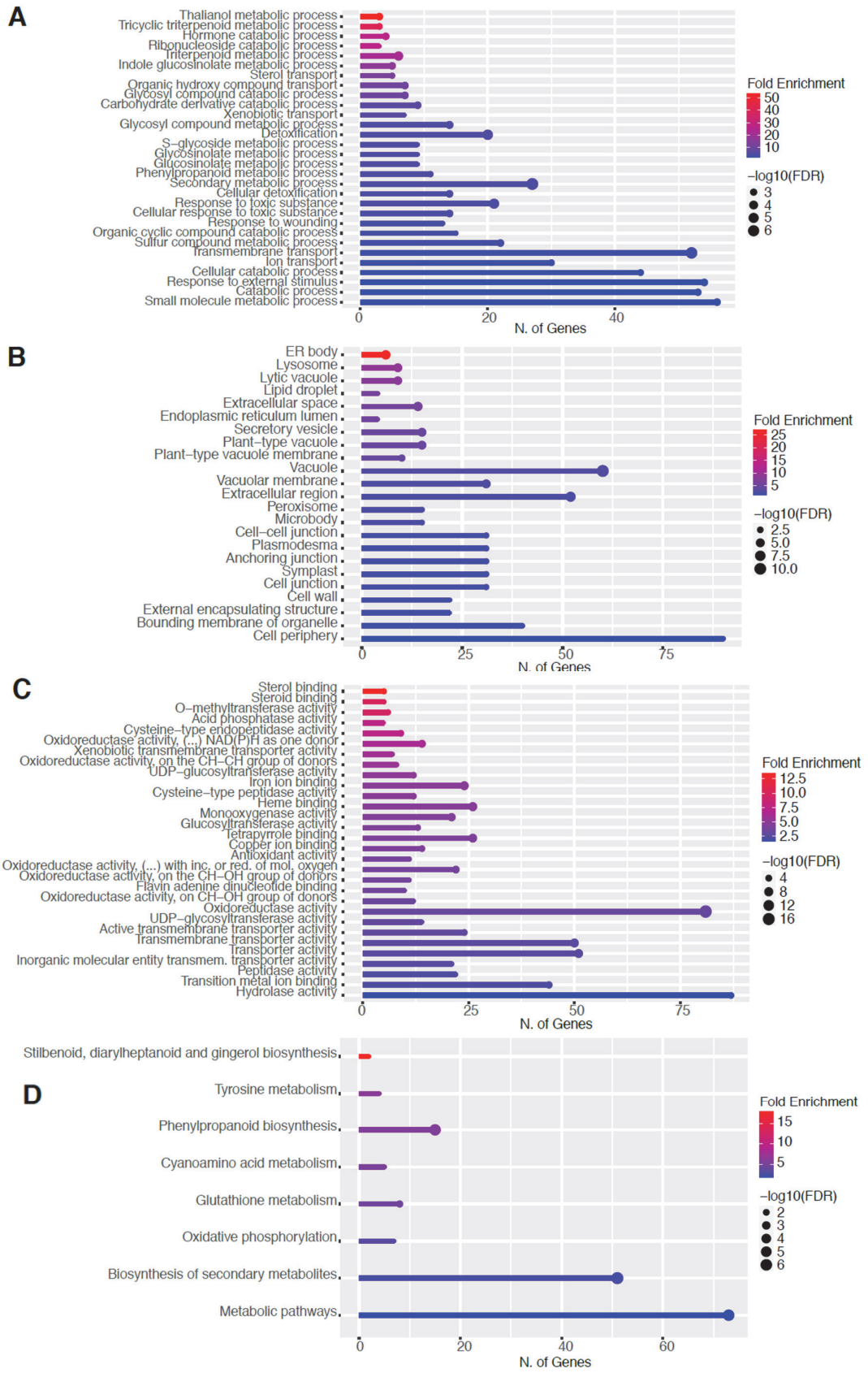


Figure 6.10. Gene Enrichment Analysis for downregulated genes after 80 s plasma treatment. Lollipop diagrams provide information about GO fold enrichment, significance (FDR in log10) and number of genes in each pathway. From top to bottom, GO categories are in the following order: (A) biological process, (B) cellular component, (C) molecular function, and (D) KEGG pathway.

6.5 Discussion and Conclusions

As stated previously, it is difficult to compare results across plasma-seed treatment studies due to the high number of variables and if molecular analysis is performed, often specific genes are selected based on economic or health-related importance. In previous plasma agriculture studies, the targeted genes were related to germination, primary or secondary metabolism such as starch degrading enzyme (Ji et al., 2018), drought related resistance genes (Guo et al., 2017), antioxidant genes (Rahman et al., 2018), pathogen resistance (PR) genes, and epigenetic regulation related genes (Adhikari et al., 2019; Adhikari et al., 2020), and plant specific secondary metabolites with pharmacological uses (Ghasempour et al., 2020; Iranbakhsh et al., 2020).

The aim of our study was to look at the long-term memory effect of the plasma treatment by using 6-day-old seedlings rather than 1 - 2 day old seeds because both the root and stem would have emerged from the seed and this would ensure that there would be transcriptional changes which could otherwise be absent if the extraction is done too soon; this was the case in the *Andrographis* study (Tong et al., 2020). Moreover, it is common practice to use young seedlings because they are more sensitive to stress and therefore, it would be possible to observe acute stress. A limited time treatment was chosen to avoid additional stresses, such as heat stress which induces a plethora of cellular effects (Zhao et al., 2021; Amen et al., 2021; Bourgine et al., 2021; Guihur et al., 2021). Early germination, an effect often observed in other studies, was the macroscopic parameter used as an indicator of changes in the molecular biology as shown in Fig. 6.3.

For this first exploratory study, RNA-seq was used since it is an unbiased method which provides a global overview of all genes. DESeq2 was used for this paper since it is common practice and it can balance stringency and flexibility (Wijesooriya et al., 2021). We analyzed approximately 21,000 *A. thaliana* genes out of approximately 33,000 genes using RNA-seq (NCBI project number PRJNA800224) on plasma-treated seeds grown until and including the 6th day. We found 422 DEGs, with 32 upregulated and 390 downregulated, and in good agreement between the biological replicates within the control and 80 s plasma-treated samples (Figs. 6.5 and 6.6). For the 60 s plasma-treated samples, we found 269 DEGs, with 27 upregulated and 242 downregulated (Figs. 6.5 and 6.6).

6.5.1 Gene enrichment as a result of short non-thermal air plasma treatment

As shown in Fig. 6.3, accelerated germination was observed which is in line with the growth enhancement effects observed in other studies (Koga et al., 2015; Guragain et al., 2021). Growth enhancement is not the only type of plant response to plasma and also includes seed surface functionalization, seed decontamination, and stress and defense response (see Chapter 2). Our findings in Figs. 6.7 and 6.8 for 60 s and Figs. 6.9 and 6.10 for 80 s suggest that on a molecular level, this increased germination rate is mainly an outward expression of a stimulated stress and defense response. However, this does not exclude other effects, such as cell wall modifications, ion homeostasis, and modified plant microbiome interactions as a result of plasma treatment. Therefore, these will be briefly mentioned followed by the main focus of our findings about the plant defense response.

Plasma is known to modify the seed surface directly and therefore, this could explain the upregulation in cell wall cellular components seen in Figures 6.7B and 6.9B. Bafoil and co-authors showed that that the total activity of peroxidases increased in plasma-treated seeds, which were also enriched here, and peroxidases can trigger internal changes which subsequently externally modify the cell wall (Bafoil et al., 2019). It is more

likely that this change has a chemical basis since the plasma treatment here was indirect with a 3.7 mm plasma-seed gap distance and thus it is unlikely, although not impossible, that there was much interaction between the seeds and electrons, ions, or electric fields confined close to the electrodes.

Through seed surface modifications, it is also possible to alter ion homeostasis, which was indicated by a downregulation in ion transport activity in Figure 6.10A. Previous studies have shown ion redistribution after plasma treatment in cations such as calcium, magnesium, or potassium migrating into the interior of the seed or being enriched on the surface (Ambrico et al., 2020, see Chapter 4). This, otherwise, could also be interpreted as seed damage and therefore, could be a symptom of a plant stress response.

It could be that through this stress response, plants modulate their own root activity thereby altering their microbiome interactions. Plasma is very commonly known for its decontamination application in order to remove bacteria (Butscher et al., 2015; Butscher et al., 2016), but recent studies have demonstrated that plants treated with plasma modulate their relationship with the microbiome; root activity is modified and nodulation is increased (Tamošiūnė et al., 2020; Tamošiūnė et al., 2020; Mildaziene et al., 2021). As shown in Figure 6.8A, our data revealed changes in the thalianol pathway, which could explain changes in plasma-treated plant root performance observed in other studies. This pathway is involved in root-specific metabolites to encourage plant-bacteria interactions and was shown to be important for shaping the *A. thaliana* root microbial community (Huang et al., 2019). Additionally, triterpenes were also downregulated (Figure 6.10A) and these compounds can influence metabolite exudation and therefore, indirectly modulate the rhizobiome and root bacteria to have either growth-promoting or inhibitory effects (Huang et al., 2019).

Based on our gene enrichment analysis in Fig. 6.9ACD, our main findings were an increased defense response, specifically of the glucosinolate pathway with an 80 s plasma treatment time several days after observing accelerated germination. The production of secondary metabolites involved in plant defense has been reported previously, as well as, changes in glutathione, an antioxidant involved in detoxification of reactive oxygen and nitrogen species during stress in living organisms (Tong et al., 2020; Cui et al., 2021; Wang et al., 2021). Our data in Figs. 6.7 – 6.10 suggests that the plant may still be responding to the stress even 6 days after the initial treatment, where a continuous cascade of programs has been triggered which affects both primary and secondary metabolisms.

Primary metabolism is used for growth, however, it has the precursors or building blocks for secondary metabolites. These precursor compounds are mainly from the pentose phosphate (PP) pathway for the synthesis of phenolic compounds within the phenylpropanoid pathway or glucosinolates, whereas other intermediates from glycolysis can be used for the mevalonic or methylerythritol 4-phosphate (MEP) pathway to produce terpenes and sterols (Sinha et al., 2019). Therefore, increased primary metabolism, such as the production of precursors PEP, acetyl CoA, and 3-phosphoglycerate, for secondary metabolites might result in the upregulation of organic acid pathways. Alternatively, branched chain amino acids like leucine or aromatic amino acids like tryptophan or tyrosine, as shown in Figures 6.9AD, 6.10D, S4, can be shuttled to produce these phenolic compounds or glucosinolates.

Glucosinolates are categorized either into tryptophan-derived indole, tyrosine or phenylalanine-derived aromatic, or aliphatic glucosinolates. Biosynthesis of aliphatic glucosinolates starts out from alanine, valine, or leucine with the most abundant group of aliphatic glucosinolates synthesized from methionine (Binder et al.,

2010). This would explain the upregulation of branched chain amino acids and increase in vitamin B1 (Figure 6.9A), which is involved in amino acid synthesis, pentose phosphate, and TCA cycle, and is being increasingly recognized and linked with plant defense (Ahn et al., 2005; Subki et al., 2018). Moreover, this complements the observed enrichment of oxoacid (Figure 6.9A, S6A) which is linked with generalist herbivory, possibly through glucosinolates (Hansen et al., 2008; Kittipol et al., 2019).

How this stress signal is transduced based on the DEGs is largely due to mitogen-activated protein kinases (MAPK) as seen in Figures 6.7D and 6.9D. There are multiple MAPK pathways that are involved in hormone signaling and they trigger various stress responses due to other abiotic and biotic factors (Jalmi et al., 2015; Jagodzik et al., 2018). After the signal is perceived, the organelles which are the most affected by the plasma treatment are related to the cell wall, which was upregulated, and ER body, which was downregulated (Figs. 6.7B, 6.8B, 6.9B, 6.10B).

It was unsurprising to find changes to the cell wall since it is the first point of contact between the seed and plasma. The effect on the cell wall could include reorganization to strengthen its defense against the stress. However, it was anticipated that the lysosomes and peroxisomes would also be upregulated due to oxidized, damaged macromolecule degradation from exposure to plasma-derived RONS. In Fig. 6.10B, the opposite was determined where lysosomes were downregulated. This might be because a perceived mild or moderate stress results in a transient upregulation, ranging from seconds to days. Since the mRNA was extracted at a later development stage, this could have been missed and or the cells could have adapted by then to the stress.

Many of the organelles were downregulated, especially the ER body, which was observed with both plasma treatment times (Figs. 6.8B and 6.10B). On the one hand, with the increase in glucosinolates, it could be expected that the ER body which houses these compounds would also be upregulated (Yamada et al., 2020). On the other hand, it could be that there are more than enough defense resources already available and stored in anticipation for the next attack and therefore, the ER body downregulated its activity as a means to not compromise resources still required for growth.

6.5.2 Comparison with other transcriptomic studies

To verify whether our interpretations are valid, the most relevant comparison can be made with the results available in another study using *A. thaliana* and where appropriate, other transcriptomic studies (Tong et al., 2020; Cui et al., 2021; Han et al., 2021; Wang et al., 2021). A detailed comparison between our experimental setup and that of Cui and co-authors is shown in Table S3 (see supplemental section).

One of the main differences was in the materials of the plasma device, as well as, in the plasma setup which used a continuous sinewave power supply with varying frequency, whereas ours used a 10% duty cycle. Their plasma chemistry may have differed where they measured atomic oxygen and NO with OES, whereas we used Fourier transform infrared spectroscopy (FTIR) and found that we operated mainly in ozone mode with lower concentration of NO_x, although NO was present within a few mm from the SDBD from preliminary laser-induced fluorescence (LIF) measurements (see Chapter 5). There were greater differences in the seed handling. Cui and co-authors worked with sterilized seeds, whereas our conscious choice was to keep the native microbiome even though we were aware that plasma can interact with microorganisms and change the plant growth upon their removal. However, avoiding seed rehydration with a sterilization protocol was a higher

priority for this study than to sterilize the seed since this can change the metabolic activity, add additional stress to the plant and thereby possibly change the final results. It is understandable that Cui et al. placed the seedlings in water to avoid dehydrating and stressing the seedlings, although it should be borne in mind that the presence of water can change the chemistry of the plasma considerably. Therefore, not only is the substrate different, seedling instead of seed, but the chemistry could also be different. Nevertheless, it is encouraging to see significant overlap between these studies because this promises some robustness in the experimental results and this might be explained by using the same seed type, similar operating parameters for the plasma-seed treatment, and same extraction time point (see Chapter 7). The RNA extraction was performed 48 hours after plasma treatment on 4-day-old seedlings so therefore, both studies extracted on 6-day-old seedlings and the main difference was that plasma treatment was done on either the seed or seedling. Both studies achieved changes in germination using a similar treatment time interval of around 1 minute, however, stronger effects were observed in our study using 80 s. Heat was monitored and controlled in our study to better delineate the mechanisms by limiting additional stresses other than plasma. It has also been demonstrated that shorter treatment times, such as 1 minute, are more effective than longer treatment times like 3, 5, or 10 min.

Three studies, including ours, show a similar general pattern of predominantly downregulated genes (Cui et al., 2021; Wang et al., 2021). However, this is not the case for other studies (Tong et al., 2020; Han et al., 2021). These differences may be due to physiological differences between different organisms, but also to the plasma conditions or the selected RNA-seq approach. Cui and co-authors found enrichment in glutathione metabolism, MAPK signaling pathway, indole alkaloid biosynthesis, and plant-pathogen interaction pathway, which confirms the trends observed in our study. A study using sunflowers showed a shift towards the phenylpropanoid pathway rather than the glucosinolate and judging by the short treatment time, it could be more similar to our results after 60 s treatment. Since we included two plasma treatment times, it is possible to propose a model based on the findings in this study. It seems that increasing the treatment time shifts the plant response from the phenylpropanoid pathway, which reinforces the cell wall with lignin and rapid response with phytoalexins or camalexin at 60 s (Figs. 6.7 and 6.8), towards glucosinolate production at 80 s (Fig. 6.9 and 6.10) as if the plant is protecting itself from an insect or herbivore attack. Therefore, depending on the plasma intensity, varying biological responses can be elicited from the plant.

Regarding secondary metabolism, Cui and co-authors found mostly a decrease in amino acids, except for an increase in tyrosine and tryptophan after plasma treatment. In our study, it is clear that leucine, valine, and tyrosine were the key amino acid precursors for subsequent secondary metabolite production, such as glucosinolates and phenylpropanoids, and therefore it is in agreement with their findings. Although they had a high enrichment of glutathione-related processes, it was not as pronounced in our dataset as glucosinolates or phenylpropanoids. Glutathione is involved in the biosynthesis of glucosinolates, specifically specialized glutathione-S-transferases (and for phytoalexins) (Kittipol et al., 2019). One explanation could be that glucosinolate production is supported and since the treatment was done on the seed surface, the RONS, whether plasma- or plant-derived, may have dissipated shortly after germination. At the point of extraction, detoxification was no longer necessary and therefore was downregulated; perhaps even reset to a new threshold with the activated stress response.

Triterponoids are also defense compounds, however, they were downregulated. To attempt to explain this, it may be a question of how to best use resources where the plant prefers to use glycolysis products towards the synthesis of specific phenylpropanoid compounds or glucosinolates, especially with a common precursor like tryptophan (Figs. 6.9D and 6.10D). For example, stilbenoids were downregulated and are classified as phytoalexins and phenolic compounds (Akinwumi et al., 2018), therefore resources might have been shuttled towards glucosinolates instead; it has been shown that the glucosinolate pathway can limit phenylpropanoid production (Kim et al., 2020). Alternatively, perhaps it is also faster to produce phenylpropanoids than glucosinolates or it is launched as an earlier line of defense.

Although all other transcriptomic studies reference plant hormones as an important reason for the macroscopic changes, the only plant hormone detected here was auxin (Tong et al., 2020; Wang et al., 2021). It showed downregulation of auxin homeostasis and catabolic process in Figs. 6.8A, 6.8C, S7, which would make sense owing to its role in growth and the accelerated root emergence during germination. Interestingly, glucosinolate metabolism is a modulator of auxin homeostasis. This could be another explanation for changes particularly with auxin (Figs. 6.9A, 6.9D, S2A, S3A, S7A) (Malka et al., 2017), which could still be indirectly in line with the hypothesis of hormone modulation as a possible mechanism of action.

6.5.3 Transcriptomic plant response to plasma treatment and its limitations

In this study, we demonstrated that stress and defense pathways are upregulated after plasma treatment. This seems to depend on the plasma treatment time exposure and therefore, this interpretation is schematically proposed in Figure 6.11. The plasma treatment could be perceived as a wounding, perhaps from ion bombardment, even though this is not likely during indirect plasma treatment.

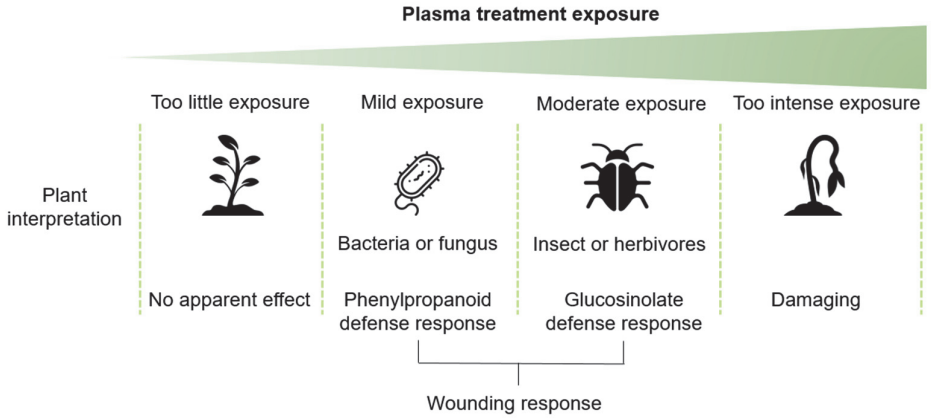


Figure 6.11. A tentative hypothesis summarizing the findings in this study, where mild 60 s plasma treatment and moderate 80 s plasma exposure result in phenylpropanoid or glucosinolate biosynthesis, respectively.

Plasma is a type of stress that the plant has not been previously exposed to, let alone, developed a specific stress or defense response and could thus be considered xenobiotic. Moreover, plants and pathogens continuously co-evolve, where the plant will recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Plasma might not exactly mimic a bacterial or fungal infection or insect wounding, however, there might be sufficient overlap to elicit these responses, partly due to the individual plasma components which are already familiar to the plant.

More transcriptomic studies are required to validate this hypothesis, however, results are highly dependent on the conditions i.e. the extraction day, or tissue type. As an example, a study plasma treating

Andrographis included three time points with the highest number of DEGs at 28 hours, followed by 48 hours and 0 hours. Future studies will include multiple time points closer to the plasma treatment, as well as, weeks after plasma exposure on hydrated seeds, dehydrated seeds, and seedlings. Additionally, a time series experiment targeting specific genes in the phenylpropanoid and glucosinolate pathways using qPCR will be explored. The more transcriptomic studies become available due to increased affordability, the more possibility there will be to compare the type of response that can be activated, regardless of the plasma treatment setup, as a first proof-of-concept. The next task would be understanding the operating parameters and which plasma-seed treatment is needed to trigger specific plant responses while proving that the results are reproducible, reliable, and robust for practical application in industry.

Different responses may be elicited when using a volume DBD direct treatment compared to an indirect treatment, since the seeds are in contact with electric fields and electron/ion bombardment. Additionally, depending on the seed type, it might not be possible to understand what is happening if the entire genome is not available as it is for *A.thaliana*. Although *A.thaliana* belongs to the *Brassica* family which includes several crop species, it would be more useful to check this on crop species to understand the applicability of this technology. Furthermore, germination rate is often selected as a parameter to judge the positive effect of a plasma treatment, but it is not necessarily the standard that all should go by since effects can be observed later in time even if absent earlier on. Koga and others found that harvest mass showed greater improvement compared to germination rate (Koga et al., 2015). Another author found that initial negative effects on germination were, in fact, positive for long-term growth (Pauzaite et al., 2018). Therefore, other phenotypic changes should be explored, and both positive and negative results should be considered to help us better understand how gene expression profiles align with macroscopic changes. As mentioned in other papers, results in the plasma agriculture studies are more safely interpreted using multiple diagnostics in a single study. As an example, in the case of transcriptomics, it is generally known that mRNA levels do not necessarily correlate with protein levels (Guihur et al., 2021). Moreover, for transcript downregulation, it is not clear whether the transcripts are degraded and proteins are not replenished, or whether they are actively degraded by the cell. Therefore, it would be useful to complement these studies with proteomics and metabolomics.

Additionally, having a molecular marker gene set for plasma exposure would be useful to know whether a plasma treatment was effective; it is difficult to discern whether the plasma or plasma-seed treatment needs to be optimized, or whether the seed is simply unresponsive. This may be difficult to pinpoint since there is great diversity in plasma chemistry with the myriad of plasma sources. Using Genevestigator, both upregulated and downregulated gene signature profiles for 80 s were compared against other transcriptomic studies using *A. thaliana*, however, there were no clear matches between plasma and other perturbation studies (see supplemental section, Fig. S10). Therefore, it remains difficult to assign a plasma response to an already known stress with our findings in this study as it seems that multiple stresses can be activated. The concept of a plasma treatment gene signature would be interesting to follow up on as an alternative to monitoring macroscopic changes. Perhaps, it would be possible to detect effects which are not expressed phenotypically.

Finally, it is always a question for living organisms of how to best use their resources since there is a trade-off between growth and defense. It needs to be stated that the application of plasma will largely depend on the context. In well-controlled environments, one might want to use plasma to harvest secondary metabolites for pharmaceutical applications and therefore, there is little risk of abiotic and biotic stresses. Care

should be taken to not jump ahead with applications since most results are in the context of a lab and are done on a short timescale. It is unknown whether this would be advantageous for the plant over the long-term and in a more complex environment. The best way to evaluate this would be in a field study with stresses related to weather change, microbes, various soil conditions, and pests. Therefore, work still needs to be done to test the feasibility of upscaling these results, although others have shown that these effects can persist for several years within the same generation on non-thermal plasma-responsive seeds (Sirgedaitė-Šėžienė et al., 2021; Hayashi et al., 2022).

6.5.4 Conclusions

This study is among the first to perform RNA-seq on plasma-treated *A. thaliana* seeds. Here, we demonstrate that a brief (60 or 80 s) plasma treatment of dry seeds causes modifications in primary and secondary metabolisms measured after 6 days, which is evidence of a long-term memory effect. Specifically, a 60 s plasma treatment time upregulates the phenylpropanoid pathway where the seedling reinforces its cell wall with lignin and launches antimicrobial compounds like phytoalexins, a defense response to bacteria or fungal plant pathogens. A longer plasma treatment of 80 s upregulates the glucosinolate pathway, a defense response to insects and herbivores to deter feeding. In both cases, it appears that plasma clearly acts on the plant to change the redox state and also seems to elicit a wound response. It should not be mistaken that plasma is recognized exactly as these stressors since seeds have never previously been exposed to plasma in their natural environment and therefore, plasma is still likely recognized as a foreign and abnormal stress. Indeed, accelerated germination and increased stress and defense response were all observed, although, it should be underlined that this needs to be considered carefully for future applications since there is often a trade-off between growth and stress/disease resistance. Future studies should perform a time series of RNA-seq analyses after plasma treatment, explore the possibility of a gene signature profile specific to plasma, and include field studies where abiotic and biotic stresses are tested to check the survival of these plants under realistic conditions.

6.6 Introduction to RNA sequencing study with 24-hour delayed extraction time point

Next, a follow-up study was done using RNA sequencing again. Previously, 6-day-old seedlings from plasma-treated seeds with two different plasma treatment times of 60 s and 80 s at 8 kV were analyzed. Here, an exploratory, preliminary study was done on 7-day-old-seedlings treated under similar plasma conditions, a 60 s treatment time at 7.5 kV, with the main difference being a different extraction time point.

Specifically, a comparison between 6-day-old and 7-day-old untreated, control seedlings was performed, followed by a comparison between the untreated, control seedlings at days 6 and 7 with the corresponding plasma-treated seedlings to determine whether the changes in gene expression were due to the plant age or plasma treatment. Finally, we conclude with the differentially expressed genes and pathways of 7-day-old plasma-treated seedlings

6.7 Global RNA-seq analysis of young seedlings after 60 s non-thermal plasma-seed treatment

Principal component analysis (PCA) was performed on the normalized gene expression values which did not initially show a similar clustering among three replicates (data not shown). However, duplicates did show clustering among the control samples and among the plasma-treated samples so one control and one plasma replicate sample were removed due to the variability (Fig. S12). The first two principal components explained 75% of the total variance (59% by PC1 and 26% by PC2). There was a total of 32,833 genes in 4 samples where 21,168 genes passed the selected threshold, which was having more than 2 reads per biological replicate (see Appendix).

With a log2foldchange (FC)>1 and false discovery rate (FDR) <0.15, 56 differentially expressed genes (DEGs) were obtained, where 27 genes were upregulated, and 29 were downregulated (data not shown). The main focus of this study was to highlight the similarities and differences between 6-day-old and 7-day-old seedlings, treated with a similar plasma. Although 8 kV was measured in the previous time series study, we assumed that 7.5 kV produces a similar plasma. These two voltages are different by only 6%, which is within experimental error of the voltage supply to the SDBD electrodes. Furthermore, the same plasma treatment time of 60 s was used again.

6.7.1 Comparison of gene expression between 6-day-old and 7-day-old untreated, control seedlings

To ensure that the observed changes in secondary metabolism were caused by the plasma treatment and not plant age, we cross-referenced our data and analyzed the gene expression profiles for 6-day-old untreated, control seedlings (data taken from our previous study) and 7-day-old untreated seedlings (data obtained during this study). PCA analysis and hierarchical heat map clustering revealed significant differences between 6-day-old and 7-day-old seedlings (Figs. 6.12A, B), however, the main differences were linked to plant development according to the pathway enrichment analysis (Figs. 6.12C, D).

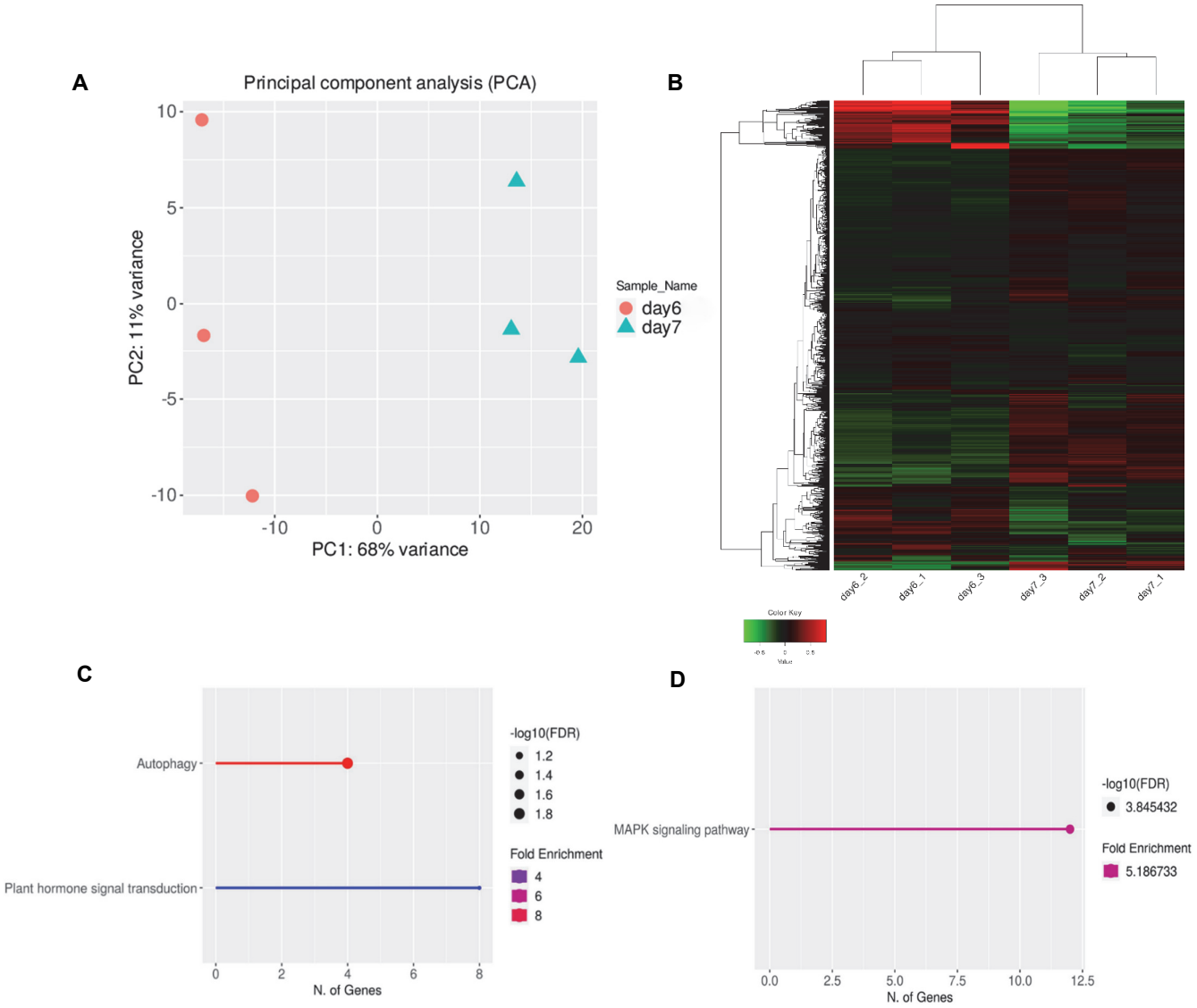


Figure 6.12 A) Principal Component Analysis (PCA) conducted on the normalized gene expression values of the 6 and 7-day-old samples. X- and Y-axes show PC1 and PC2, respectively, with the amount of variance contained in each component, which 68% and 11%. Each point in the plot represents a biological replicate, representing 30 seedlings, with a total of 6 biological replicates in the plot. Symbols of the same colors are replicates of the same condition where orange represents 6-day-old untreated *A.thaliana* seeds grown into seedlings and blue represents the same except 7-day-old seedlings. B) Heat map of the expression patterns (Z-scaled reads per kilobase of exon per million reads mapped (RPKM)) of the full transcriptome for 6-day-old and 7-day-old untreated seedlings. Hierarchical clustering of the relative expression profile of the top 2000 variable genes selected based on the lowest standard deviation using Euclidean distance. Individual samples are shown in columns, and genes in rows. The left vertical axis shows clusters of genes. The color scale represents the relative read count of genes: green indicates low relative read counts; red indicates high relative read counts; black indicates zero (no change). C) Pathway enrichment analysis of upregulated genes using KEGG category. D) Pathway enrichment analysis of downregulated genes using KEGG category. Significant differences between untreated 6-day-old and 7-day-old untreated, control seedlings are due to plant development.

6.7.2 Comparison of DEGs between 6-day-old and 7-day-old untreated, control seedlings to DEGs in plasma-treated seedlings

We then compared the DEGs of untreated, control seedlings and seedlings grown from plasma-treated seeds to check the similarities and differences in gene expression profiles. To make it possible to produce a Venn diagram, DEGs from 6-day-old and 7-day-old, untreated seedlings were first identified and then compared to the DEGs of plasma-treated seedlings. In both Figure 6.13A and 6.13C, there is a minor overlap of significantly DEGs for both days 6 and 7. The untreated and 60 s plasma-treated seedlings (6 s plasma ON time) showed 88 genes in common between the two conditions, however, there were 5,869 DEGs and 181 DEGs in untreated and plasma-treated samples, respectively. A similar pattern was observed with 80 s plasma-treated seedlings. There were 5,838 DEGs and 303 DEGs for untreated and plasma-treated samples, respectively, of which 119 genes overlapped between the two conditions. In both instances, the overlapping genes were related to primary metabolism (Figs. 6.13B, D), which is involved in growth and development. These genes were found in pathways related to photosynthesis and oxidative phosphorylation, which are known to produce energy. This provided more confidence to ascribe the changes in secondary metabolism to the plasma treatment.

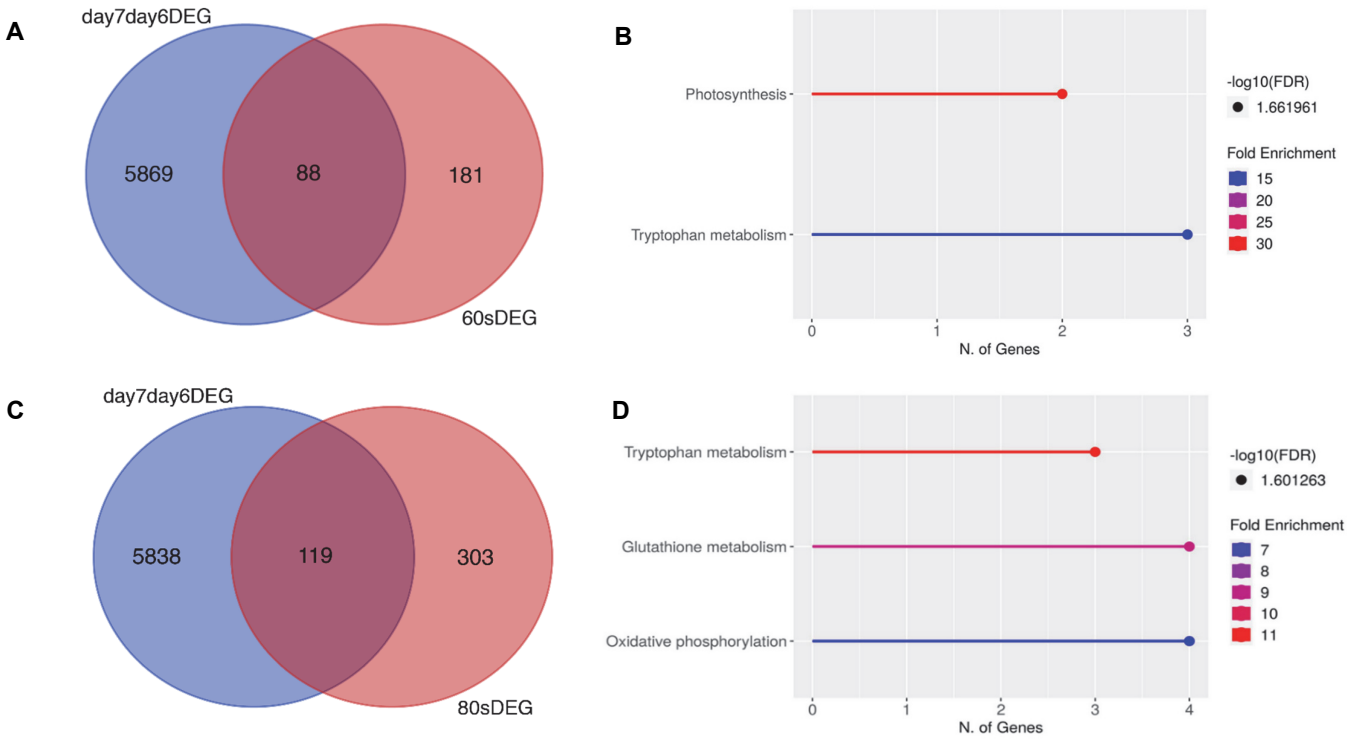


Figure 6.13. A) A Venn diagram with the number of DEGs that overlap or differ between DEGs in 60 s plasma treatment (red) and DEGs shared between 6-day-old and 7-day-old untreated seedlings (blue) B) Pathway enrichment analysis using KEGG for DEGs in A). C) A Venn diagram with the number of DEGs that overlap or differ between 80 s plasma (red) compared to DEGs shared between 6-day-old and 7-day-old untreated seedlings (blue). D) Pathway enrichment analysis using KEGG for genes in C). Very few genes overlap between untreated and plasma-treated seeds grown into seedlings. Related genes are involved in primary metabolism and growth.

6.7.3 Gene expression of plasma-treated seeds grown into 7-day-old seedlings

The pathway enrichment was done again using gene ontology (GO) analysis of specific groups of DEGs using ShinyGO v0.76 software. Biological process, cellular component, and molecular function categories are shown in Figure 6.14A, B, C respectively, for the upregulated genes after 60 s plasma treatment (6 s plasma ON time) at 7.5 kV. The lollipop diagrams show the fold enrichment and number of genes in the pathway and the hierarchical tree clustering is shown in the supplemental section (Fig. S13). Overall, gene expression increased in secondary metabolic pathways, mainly products from glucosinolate metabolism. Specifically, nitrile biosynthesis and metabolism were highly upregulated, as shown in Figure 6.14A. Regarding the cellular component, components concerning the cell periphery are upregulated and have equally the highest number of genes upregulated, as shown in Figure 6.14B. The upregulated molecular functions in Figure 6.14C were enzymatic reactions related to glucosinolate glucohydrolase activity and other enzymes involved in glucosinolate production.

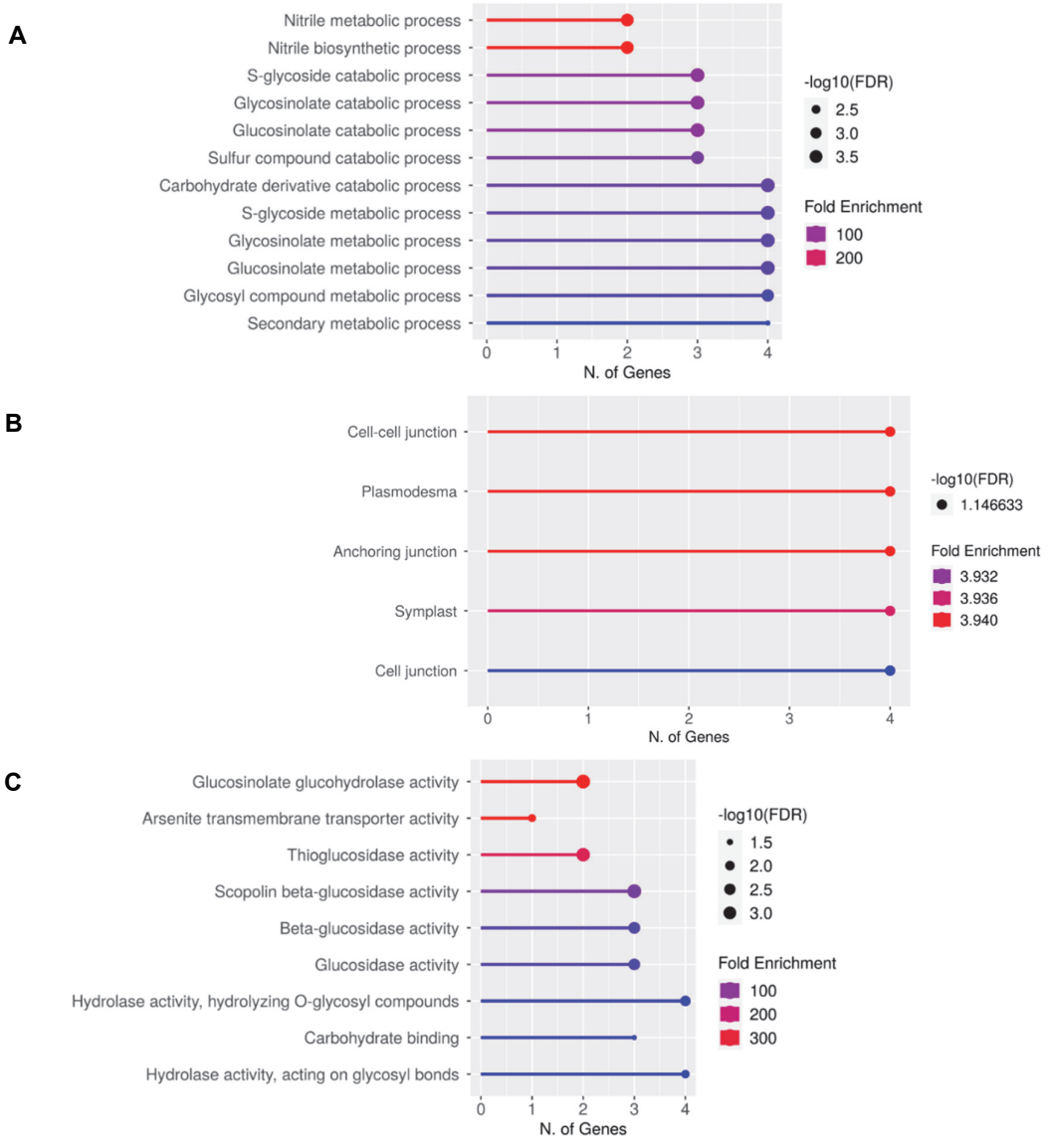


Figure 6.14. Gene Enrichment Analysis for upregulated genes after 60 s at 7.5 kV plasma treatment. Lollipop diagrams provide information about GO fold enrichment, significance (FDR in log10) and number of genes in each pathway. From top to bottom, GO categories are in the following order: (A) biological process, (B) cellular component, and (C) molecular function.

The lollipop diagrams for the downregulated genes are shown in Fig. 6.15 and trees are in Fig. S14. Overall, gene expression decreased in diverse pathways related to stress or chemical stimulus response. Specifically, cellular response to iron ion starvation and reactive oxygen species were highly downregulated whereas response to oxygen-containing compound had the highest number of genes enriched, as shown in Figure 6.15A. Regarding the cellular component, lysosome and lytic vacuole were the most downregulated and extracellular region had the highest number of genes downregulated, as shown in Figure 6.15B. The downregulated molecular functions in Figure 6.15C were enzymatic reactions related to nitrate transmembrane transporter activity or oxidative response.

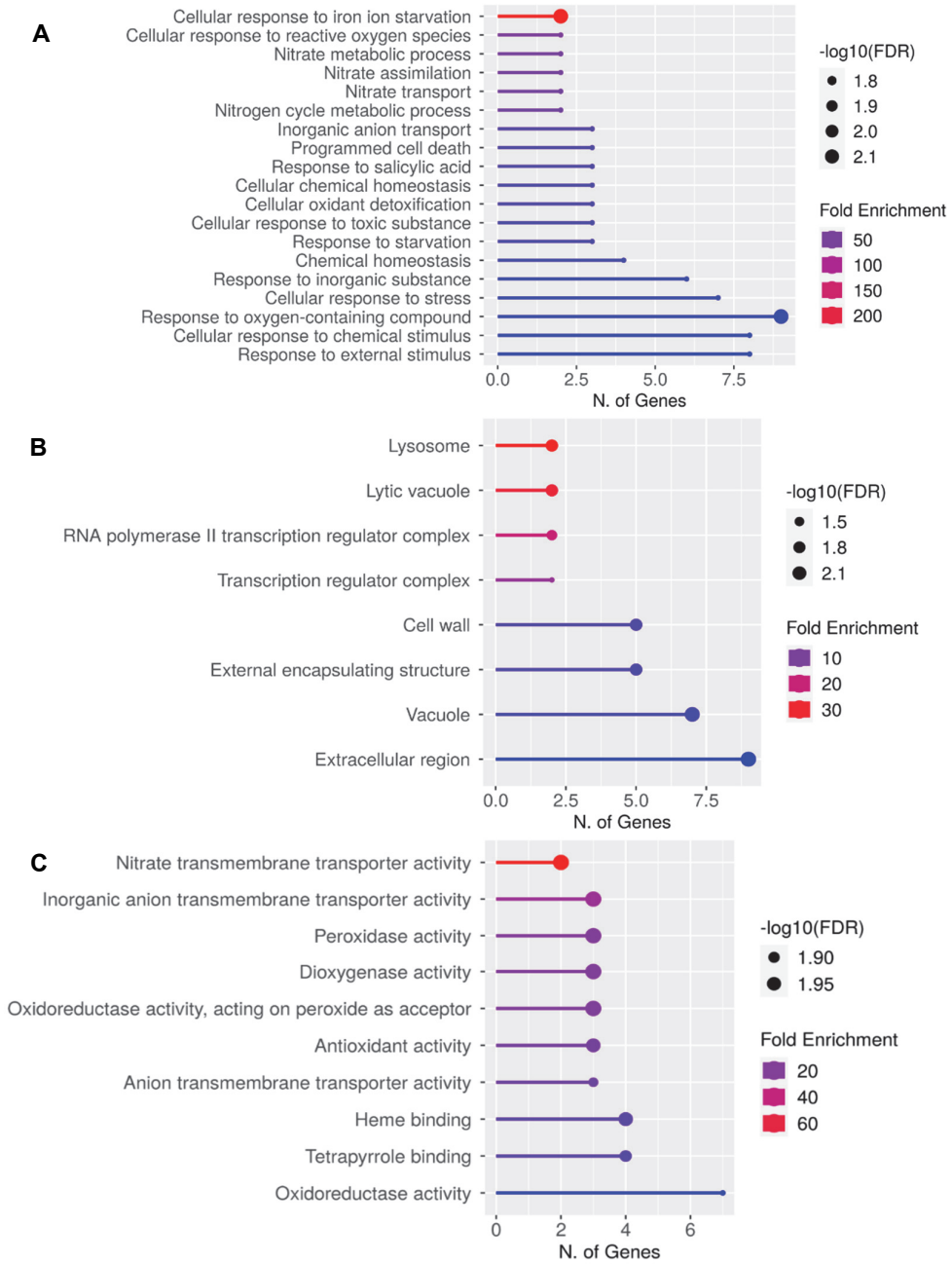


Figure 6.15. Gene Enrichment Analysis for downregulated genes after 60 s at 7.5 kV plasma treatment. Lollipop diagrams provide information about GO fold enrichment, significance (FDR in log10) and number of genes in each pathway. From top to bottom, GO categories are in the following order: (A) biological process, (B) cellular component, and (C) molecular function.

6.8 Discussion and Conclusions

6.8.1 Comparison between our studies

The aim of our study was to look at the long-term memory effect of the plasma treatment by using approximately one-week-old seedlings after root and shoot emergence to ensure transcriptional changes and increased sensitivity to stress. Limited treatment times and voltages were used to minimize additional stresses such as heat. Moreover, only the two formerly mentioned parameters among five (voltage, time, gas flow rate, plasma-seed gap distance and frequency) resulted in accelerated germination, which increased our confidence that there would be molecular changes (see Chapter 5). Our previous findings are supported by a study done by Sera et al. (2021), which showed how the pools of hormones change depending on a short or long plasma treatment time. However, this was not yet shown using transcriptomics and therefore, this was done by us using different plasma treatment times, and now in this study, we are comparing different extraction time points.

The time series study used 8 kV for two plasma treatment times of 60 and 80 s and the RNA was extracted 6 days after plasma-seed treatment whereas here, 7.5 kV with a single plasma treatment time of 60 s was used with the RNA extracted 24 hours later or 7 days after plasma-seed treatment. Previously, we demonstrated that a brief plasma treatment of dry seeds caused modifications in the primary and secondary metabolisms of 6-day-old seedlings, evidence of a long-term memory effect. The 60 s plasma treatment time upregulated the phenylpropanoid pathway and showed cell wall reinforcement with lignin and production of antimicrobial compounds like phytoalexins. This could be interpreted as a defense response to bacteria or fungal plant pathogens. The 80 s plasma treatment upregulated the glucosinolate pathway, a defense response to insects and herbivores to deter feeding. In both cases, it seemed that plasma behaves as a wounding and oxidative stress.

To attempt to explain the lack of clear clusters in the triplicates between the untreated and plasma-treated samples, it could be the inherent seed variability, plasma-seed treatment variability, or the delayed extraction time. Fewer DEGs were identified in this study, likely due to the response dampening over time. The latter is possible because this was demonstrated in another study where the latest time point had fewer DEGs (Tong et al., 2020). Nevertheless, the results of the data analysis were coherent with previous observations and still provided novel insights.

To ensure that the gene expression changes were, in fact, due to the plasma treatment and not plant physiology, the gene profiles were compared and indeed, mainly genes in developmental processes were detected (Figs. 6.12, 6.13). The list of genes specifically induced after plasma treatment are listed in Table S6.

When comparing our two studies, the gene expression trend in the first study showed few upregulated genes and vastly more downregulated genes, whereas in the second, it is an equal ratio of up- and downregulated genes. It was initially expected that the phenylpropanoid upregulation would be observed again when comparing 60 s at 8 kV to 60 s at 7.5 kV despite the plant age difference. However, the 60 s at 7.5 kV mimics more closely the 80 s at 8 kV because there is an increase in glucosinolate related products and enzymatic activity (Fig. 6.14). Upon further thought, it would be reasonable to observe this because within those additional 24 hours prior to extraction, the phenylpropanoid response could have shifted towards a glucosinolate response. It could be that over time, the plant runs through a sequence of pathways and the

same events can be observed with a less intense plasma, given a longer sampling time. In other words, each of these plasma-treatments could have underwent phenylpropanoid biosynthesis, followed by glucosinolate biosynthesis, and then nitrile biosynthesis. Depending on the plasma intensity and elapsed time, a different response can be observed, especially since it is entirely plausible that gene transcription could have changed within 24 hours; it is the case for some heat shock proteins to change within only 30 minutes (Guihur et al., 2022).

In terms of downregulated genes, there are differences in the hormones detected where auxin catabolism was previously observed, but salicylic acid is observed here for the first time (Fig. 6.15A). Auxin was reasonable to observe since aldoxime is a precursor to indole glucosinolates, camalexin, and auxin (Zhao et al., 2015). It is known that indole glucosinolates are blocked by high levels of auxin and it is likely the same inversely. However, SA is involved in systemic acquired resistance, which would be complementary to the upregulation of secondary metabolism.

Similarities between the two studies remain with the organelles, especially with lysosomes being the most downregulated. The same rationale applies where the oxidized proteins could have been cleared in the meantime. Also, oxidation plays a role again in eliciting a response since many functions related to oxidation, detoxification, or chemical stress are observed (Fig. 6.15C).

6.8.2 Data supports hypothesis about wounding and oxidative stress as a plant response to plasma

If the upregulated genes are analyzed closely, the data supports the previously proposed hypothesis where plasma could be interpreted as a wounding from an insect, a penetrating fungus or bacterium. We observed here an increase in pathway enrichment for cell wall biogenesis. It seems that the plant is repairing damage or reinforcing the cell wall, although it remains unknown which plasma components caused the inflictions. However, changes to extensin for cell wall protection (AT1G26240) and chitinase family protein (AT2G43610) were also upregulated, suggesting cell wall reinforcement and protection against invasion. This could be because mechanical stimulus is detected, which triggers plant defense against wounding since mechanosensitive channel of small conductance-like 9 (AT5G19520) was upregulated. This might be triggered through mechanical erosion by ions and electrons, however, it may have been through RONS since it was an indirect plasma treatment. Based on the DEGs, the plasma component could have travelled through an aquaporin, which is known to be involved in hydrogen peroxide transport (AT4G19030). Alternatively, it may be due to a few UV photons since a gene involved in DNA repair and toleration (AT3G12610) was upregulated and this would be a typical response to UV.

From this mechanical stimulus, the plant seems to respond with cell wall loosening using expansin, which has been mentioned before in other studies and one of these genes (AT5G02260) was upregulated in our dataset. It is interesting to see the listed effects persist for 7 days, even though they would be expected to occur shortly after the plasma-seed treatment and with the onset of germination, within the first 48 hours in this case. It is often mentioned that abscisic acid decreases and gibberelic acid increases prior to germination and in our dataset, a gene (AT5G15230) which promotes gibberelic acid and exhibits redox activity was upregulated. Since the extraction took place 6 or 7 days after the plasma treatment, the response to the initial stimulus seems to have evolved into a glucosinolate response which has now been shown twice by us with the upregulation of two genes encoding myrosinases (AT1G51470, AT1G47600). The novel aspect was that

these glucosinolates were further broken down into nitriles, as indicated by the increased expression of a nitrile specifier protein (AT3G16390), which seems to promote simple nitrile but not thiocyanate formation.

6.8.3 Plasma defense activated with increased nitrile synthesis

There are several breakdown products when glucosinolates are in contact with myrosinases, enzymes which are typically stored in different compartments. Once in contact, a defense response is released with the production of: thiocyanate, isocyanate, or nitriles (Zhao et al. 2015, Fig. 1). It was shown in another study that plasma-treated microgreens had an increase or even doubling of isothiocyanates (Saengha et al., 2021; Luang-In et al., 2021), which is coherent with the activation of the glucosinolate pathway here.

These processes are regulated by MYB transcription factors, which have not been observed in our list, but there is a strong presence of nitriles. It is not yet clear why nitriles are favored over other forms. Ultimately, nitriles can be broken down further into cyanogenic glucosides so it appears that a very potent response is elicited from the plant after plasma treatment. In some instances, nitriles are less toxic than isothiocyanates (Ting et al., 2021). However, certain organisms have evolved to consume breakdown products through coevolution (Eckardt, 2001). In other instances, it can be more toxic to some herbivores or particularly to insects so it seems to depend on what is attacking the plant (Eckardt, 2001). “The complexity of variation in glucosinolate concentration, glucosinolate type, and type of hydrolysis products generated may reflect the complex interplay between numerous types of insects, herbivores, and pathogens and the costs associated with mounting an adequate defense system” (Eckardt, 2001). The variation in glucosinolate biosynthesis leads to the production of more than 120 different glucosinolates from only a few amino acids (Halkier and Gershenzon, 2006). This variation is amplified during glucosinolate hydrolysis, as a single glucosinolate can be hydrolyzed to different products with diverse physicochemical and biological properties and therefore, it is difficult to reach a conclusion about the nitrile biosynthesis after plasma treatment without understanding the fundamental biology (Burow et al., 2009).

6.8.4 Conclusions

Overall, our findings here support the previously proposed hypothesis, which was that the plant interprets plasma as an oxidative stress as well as a wounding, seemingly tuned to the plasma intensity. Yet this time, another dimension has been added by varying a biological parameter, instead of a physical parameter. Plasma indeed can elicit a plant defense response. Although this is possible and the biosynthesis of particular compounds can be increased and beneficial, it is important to understand at what cost and under which context it would remain so. For example, the biosynthesis of sweeteners increased at the expense of other secondary metabolites (Judickaitė et al., 2022). In our study, nitriles are not as poisonous as other glucosinolate breakdown products to certain biota so it may not be problematic but it might be better tolerated than other glucosinolate forms. Therefore, it is very difficult to estimate what effect this would have on plant-biota interactions without multiple bioassays reflecting a more natural environment. Furthermore, care should be taken to not make the plant more susceptible to attacks.

So far, it seems that different pathways can be activated when using different combinations of plants and plasma treatments. For example, plants belonging to the *Brassica* family, like our studies with *A.thaliana*, can activate the glucosinolate pathway after plasma treatment. However, this is absent in other plant families so other plants, such as basil and pea, might activate the phenylpropanoid pathway to increase essential oil

production or increase lignification, simply due to the plant characteristics rather than the plasma. It thus would be valuable to understand how these changes occur between different plants. In the event that similar pathways are activated, there might be a preference of one over the other. Therefore, one of the next aims should be to understand under which conditions a pathway is activated (Jasim et al., 2021) and follow the genetic changes in parallel (Marzban et al., 2022).

Furthermore, it would be important to identify the limit of plasma treatment before it becomes deleterious or activates apoptosis, programmed cell death. In certain contexts, the biosynthesis of particular compounds could be desired and therefore, it might not matter that the plants would die a few days later, given that they are harvested beforehand. However, this would be critical to understand if the plants are grown over the long-term. Therefore, experiments monitoring changes over time after sowing at multiple time points would expatiate our understanding. As an example, an extended version of a study done by Holubova et al. (2021), where the authors monitored and observed increased heat shock proteins in the first two days after sowing in plasma-treated corn, should certainly be considered when designing experiments.

Lastly, to echo what was stated previously, a multi-omics approach will significantly advance the pace of this research field. Overall, plasma duration was studied before, and now, extraction time, which are both among the first transcriptomics studies performed using *Arabidopsis* and plasma and hopefully, more will be done in future. More layers of our understanding would be unraveled with epigenomics by looking at the modification of wrapped DNA since changes in methylation are presently observed but are not yet correlated with the phenotypic changes induced by plasma treatment (Pérez-Pizá et al., 2021).

In summary, it appears that regardless of minor changes in plasma-seed experiment, a similar sequence of events might be observed where phenylpropanoid biosynthesis is triggered first, followed by glucosinolate biosynthesis, and then glucosinolate catabolism. Future studies will include multiple extraction time points, as well as, variables in the plasma treatment to determine whether the same response is observed regardless of minor plasma treatment changes or whether minor changes in plasma can trigger exclusively different responses.

Chapter 7

Reproducibility and standardization of plasma-seed treatments

Abstract

Plasma treatments are currently being assessed as a seed processing technology. There is sufficient information as a proof-of-concept, but a lack of standardization in the methodology prevents a convincing evaluation of the true interest of plasma treatment on seeds. It would be helpful to co-ordinate research efforts to make the entry for newcomers into this interdisciplinary field less overwhelming, and to aid in transferring this technology into industry by establishing a common protocol. This chapter proposes a checklist which captures many of the parameters to be used as a template for recording an experimental procedure. It is divided into four main parts, namely plasma device, seed preparation, seed plasma treatment, and seed post-treatment. Each parameter is presented in a dedicated subsection where the literature is briefly discussed, with the importance of the parameter and its practical implications. This summary of the plasma and biological parameters is intended to raise awareness and motivate others to diligently record protocol details in order to reproduce the same results.

This chapter is largely based on the review article cited below. I performed the germination experiments and measurements, designed and performed the sterilization study, had a minor contribution towards the bioassays, carried out the ozone measurements, observed the problems with the SDBD, performed the plasma priming experiments, and analyzed the EDX data. I contributed towards conceptualization of the review, designed and illustrated the guidelines (Figs. 7.1 and 7.2) based on personal experience and literature, performed the literature search independently, developed the structure for the paper by citing relevant literature for each subsection, wrote the manuscript, selected the journal, and handled the submission process.

Plasma Processes and Polymers

Entering the plasma agriculture field: An attempt to standardize protocols for plasma treatment of seeds

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Keywords: protocol, plasma, seed, plasma agriculture, parameters, reproducibility

7.1 Introduction to designing plasma-seed treatment guidelines

Both plasma and biological parameters need to be carefully recorded and selected in plasma-seed treatments, yet the challenge in this interdisciplinary field is often the lack of awareness of each variable and/or how the variable affects the outcome. The importance cannot be stressed highly enough in this cross-disciplinary field, where clear scientific communication requires more effort due to differences in terminology and working styles, and variations in protocols between fields or even within the same field. There are issues with overlooking minor but important details, and working with implicit assumptions which should be challenged rather than passively accepted. By entering the plant agriculture field from the plasma decontamination field and having to build this project and lab from the ground up, it provided the right conditions to discover each variable organically and observe the impact on the outcome. The evolution of a research topic with high quality reporting and documentation is a gradual process that develops over years of research, as seen in plasma microbial inactivation which started in the mid-1990s and expanded to eukaryotic cells for medical applications in the early 2000s (Laroussi, 2018). Although the trial-and-error approach in this thesis was not the most efficient, it at least succeeded in gathering the necessary information to eventually develop a structured, systematic approach which will hopefully accelerate the research progress in plasma agriculture. Throughout the text, specific experiments which inspired these guidelines are included. Now with this structured approach, it should facilitate the disentanglement of plasma-seed treatment variables in future studies.

7.1.1 Parameters to consider during plasma treatment of seeds

Seeds are the focus of this thesis, because they are more robust substrates (Seol et al., 2017; Pawlat et al., 2018; Kobayashi et al., 2020). It does not include seed decontamination of seedborne pathogens by plasma, because of existing reviews (Butscher et al., 2020). Of course, plasma decontamination and intrinsic plasma effects could both improve seed development (Perez-Piza et al., 2019) but a summary of the parameters in seed plasma treatment and how plasma may be affecting the seed and its development will be discussed instead.

A plethora of variables comes into play for both the plasma physics and the seed biology. The parameters to be considered are shown pictorially in Fig. 7.1, and are listed in the checklist of Fig. 7.2 in four main parts as follows: (i) The first step is to describe the plasma device. In this checklist, it is arbitrarily assumed that the components of the plasma reactor and its diagnostics are part of the laboratory infrastructure which do not change during an experimental campaign. Naturally, any of these parameters will change in experiments specifically designed to investigate their particular influence. (ii) All steps which include preparing the seed for the plasma treatment are listed. (iii) The parameters of the plasma treatment itself are noted. For the purposes of these plasma agriculture experiments, the control seeds undergo exactly the same overall procedure, except for the steps in this section. (iv) The seed post-treatment, such as seed handling, growth, and seed measurements, are covered. In the protocol checklist (Fig. 7.2), each category is broken down into subsections with the corresponding names in the text. Each term is briefly discussed by comparing the methodologies in the literature to give an idea of current trends that lead to effective seed-plasma treatments. Some pitfalls are also highlighted with recommendations that can be adopted by current researchers and newcomers. This is not a straightforward task because most parameters are interdependent, making it difficult to pinpoint and isolate the cause(s) for the effects. However, it remains a worthwhile task to pursue for clarity. This preliminary protocol may help to ensure that all the relevant experimental parameters are fully recorded.

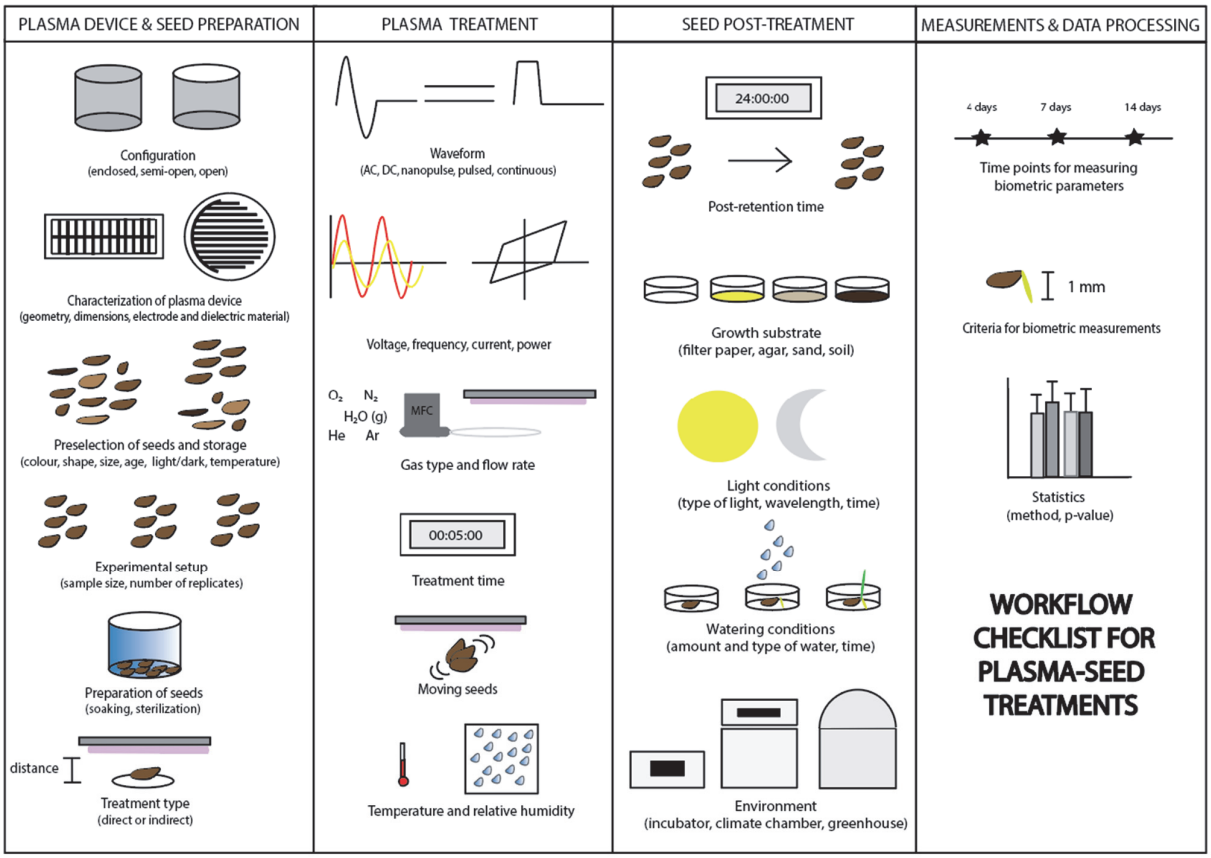

Figure 7.1. Pictorial template of a preliminary protocol for plasma treatment of seeds.

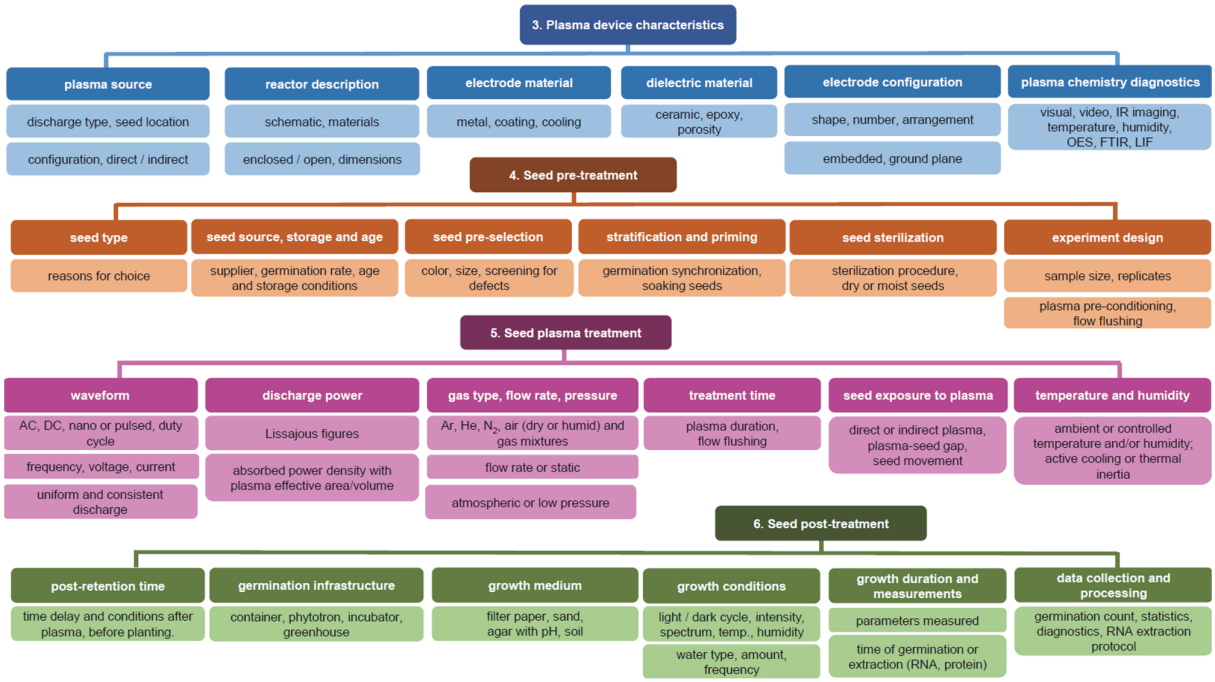

Figure 7.2. Checklist of a proposed protocol for the plasma treatment of seeds. Example parameters are discussed in the corresponding subsections.

7.2. Plasma device

Plasma devices are described in detail in Chapter 3. Nevertheless, it should be pointed out here that different types of plasma devices can be expected to yield different effects for seed treatment in plasma agriculture, yet only a few direct comparisons have been made. For example, different results for the germination and measurements of sprouts when comparing gliding arc, a corona array, a downstream microwave plasma (DMP), and a diffuse coplanar SDBD, were evaluated in Sera et al. (2012). They found that the SDBD required shorter exposure times due to its high plasma density relative to other devices, likely because it was a direct treatment, as opposed to the indirect/remote treatment with the DMP where the seeds were 10 cm away from the plasma. Similarly, another study was carried out comparing a gliding arc to a downstream microwave plasma, finding that the gliding arc had a positive or neutral effect, whereas the downstream microwave plasma had an inhibiting effect on the seedling length, accretion, and weight (Sera et al., 2017). VDBD plasma required much longer treatment times than RF plasma for similar germination effects, even though both were direct treatments (Gomez-Ramirez et al., 2017). Regardless, plasma treatment almost invariably uses DBDs because these sources are well suited to the atmospheric air environment of seeds, and they cover a wider surface area than plasma jets. Although it is possible to have an array of multiple jets, it remains challenging to maintain treatment uniformity. One example of a custom-built SDBD discharge is shown in Figure 7.3.

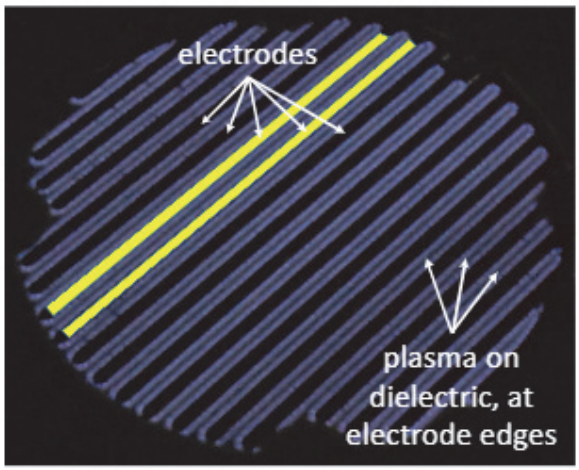

Figure 7.3. Photograph of a plasma generated by a custom-built Surface Dielectric Barrier Discharge (SDBD). Two electrodes are shown in yellow to aid the eye. The plasma is the most intense on the Kapton dielectric, around all the edges of the electrode fingers, where the electric field is strongest. The photograph shows the difficulty of uniform plasma treatment of seeds in this DBD source.

The air environment is in stark contrast to the low pressure, hazardous gas plasmas commonly used in the semiconductor industry, where vacuum chambers are universally applied to isolate the plasma from atmospheric contamination (Lieberman and Lichtenberg, 2005). Low pressure plasmas are easier to ignite with a lower voltage, and reactive radicals have a longer mean free path to reach the seeds, but the inconvenience and expense of scaling up a vacuum chamber would disfavour low pressure plasma treatments on an industrial scale. For air plasmas, a vacuum chamber is not strictly necessary, but a closed reactor provides a controlled environment. Nevertheless, there are many instances where plasma treatments are performed in open, or semi-open environments with DBDs (Stolarik et al., 2015; Zahoranova et al., 2016; Bafoil et al., 2018; Stepanova et al., 2018), plasma jets (Kim et al., 2017; Pawlat et al., 2018; Sidik et al., 2019; Lofty et al., 2019), or gliding arcs (Pawlat et al., 2018).

DBDs have several major advantages which, at the same time, can bring problems: DBDs are simple and inexpensive to make, they can be adapted to arbitrary geometries, and their operation is usually reliable and robust. On the other hand, this convenience and flexibility has led to many individual innovative designs, and hence a serious problem of comparison of experimental results between different groups. This contributes to the dilemma of reproducibility in plasma agriculture: the field has no accepted standard for DBD plasma sources. To the authors' knowledge, norms exist for plasma jets (COST jet, Neoplas) but not for the many types of DBDs, except for the DCSBD of Roplass (Roplass, Stolarik et al., 2015; Zahoranova et al., 2016; Stepanova et al., 2018). Note that commercial SDBD ozone generators are also used (Sihon, Kang et al., 2020).

Seed location: There are many possible configurations for the seed positions relative to the electrodes, so it is recommended to indicate the seed location in a schematic of the plasma source. Furthermore, as shown in Fig. 7.4, it is important to identify where exactly plasma ignition takes place because this will clarify the type of treatment style that is being experienced by the seed. Moreover, having clear definitions of what constitutes a direct, indirect, and remote treatment would be helpful. With the first SDBD prototype, it was clear that the seeds would only be in direct contact with the plasma if they are lined up along the electrode edge. However, since there is ion flow and seeds can move due to electrostatic charging, each seed within the same treatment may experience different plasma exposure.

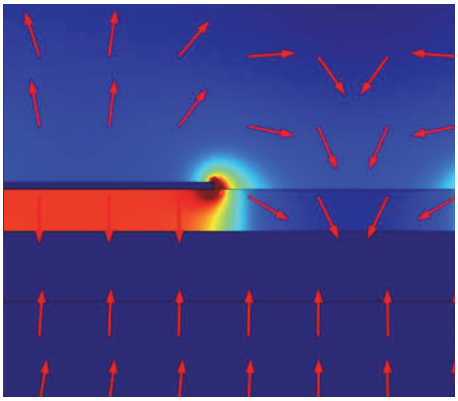

Figure 7.4. COMSOL simulations demonstrate that electric field and plasma are strongest at the electrode edge.

7.2.1 Electrode and dielectric materials

The most commonly listed electrode materials are stainless steel, copper, or aluminium, and it is important to consider their thermal properties, chemical resistance to corrosion, and biocompatibility. The electrodes could be thin films deposited on dielectric, nickel paste tracks, or metal structures, such as grids or wire meshes. Electrodes can be water cooled (Chen et al., 2019), or the thermal capacity of bulk metals could be used to absorb heat and minimize the temperature rise of the seed substrate during plasma treatment, depending on the duration of the experiment. Stainless steel is not as easily oxidized in the presence of ozone and therefore extends the lifetime of the electrodes and the reactor. On the other hand, copper electrodes are quickly oxidized and have to be replaced frequently. It should also be noted that copper has antimicrobial properties and therefore one should consider whether this changes the results of the plasma treatment and whether a biologically-inert metal should be used instead (Grass et al., 2011). Electrodes susceptible to corrosion (such as copper) can be protected, for example, by a thin gold coating. However, the possibility of

metallic nanoparticle contamination of the seed coat should be borne in mind (see Chapter 4). Electrodes can also be protected from the plasma by a type of lacquer, or by embedding them in a dielectric, as in the DCSBD of Roplass. Higher voltages are required for embedded electrodes because the plasma is further from the electric field concentrated near the electrode edges, although the plasma is more uniform for the same reason. The most commonly listed dielectrics are glass, ceramic, alumina (of various qualities regarding purity), Kapton, quartz, fibreglass, or FR4 (epoxy resin for printed circuit boards). The importance of the dielectric is to ensure reliable plasma ignition and maintenance of the discharge, especially under humid conditions, and to extend the lifetime of the plasma device. The dielectric can have an effect due to differences in dielectric properties, or plasma-surface interactions (Butscher et al., 2016). To test the influence of dielectrics in seed plasma treatments, two different dielectrics called Thernofase and Pertinax with Mylar in combination with either oxygen or nitrogen were used in Perez-Piza et al. (2018) and Perez-Piza et al. (2019) to treat infected soybean seeds. There were no significant differences in the germination rate and vigour index when using the two dielectric materials, although disinfection seemed to be more effective with nitrogen or oxygen using Pertinax with Mylar in comparison to Thernofase. Concerning damage from plasma exposure, the dielectric may be more in need of protection than the metallic electrodes. For example, a Kapton and F4R dielectric surface were activated by plasma exposure in the SDBD configuration, becoming strongly hydrophilic. Moreover, the Kapton and F4R irreversibly degraded over time and became porous, as witnessed by a gradual whitening of the dielectric surface, accompanied by a shrinkage of the plasma discharge area (Avino et al., 2022; see Chapter 3). These deteriorating effects are shown in Fig. 7.5 after one-hour operation at intermediate humidity level of 50% and show how the plasma became “patchy”.

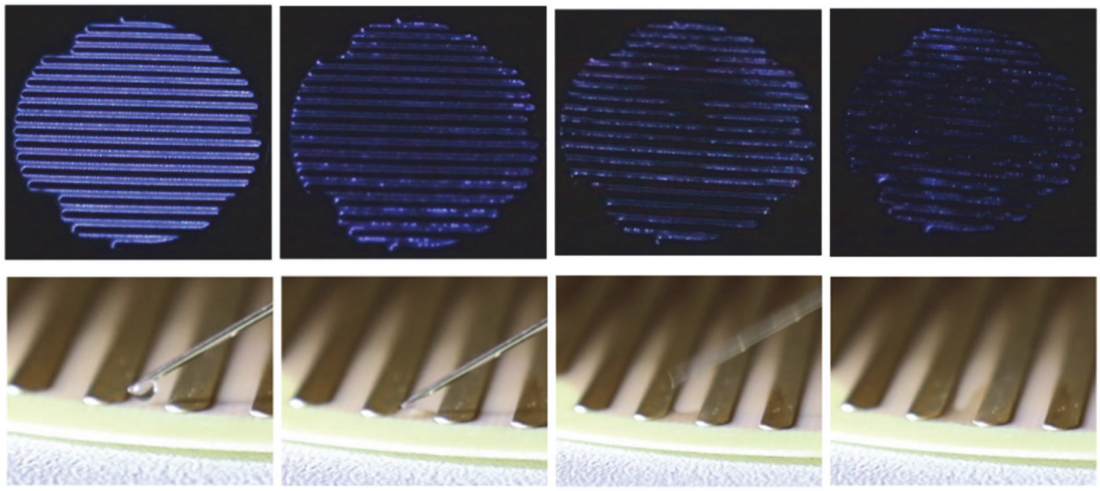

Figure 7.5. (Top row) From left to right, patchy plasma is visible within and around the plasma area after exposure to higher levels of humidity. (Bottom row) Flat droplet of water indicates that plasma activates the F4R dielectric and increases its hydrophilicity. White oxidation from overuse is visible on the electrodes.

This non-uniformity can possibly affect the plasma gas chemistry and then in turn, the effect on biological substrates. For example, with the striped SDBD, the ozone concentration in parts per million (ppm) varied between independent experiments, as shown in Table 7.1. It is not yet clear whether it is due to the diagnostic, subtle fluctuations in relative humidity, or prolonged SDBD use.

Table 7.1. Measurements of ozone concentration in ppm generated from AC powered stripes SDBD. Measurements are obtained from two different SDBDs by the same manufacturer.

	Rep. 1	Rep. 2	Rep. 3	Avg.	Dev.	Rep. 4	Rep. 5	Rep. 6	Avg.	Dev.
20 s	93 ppm	165	31	**96.33**	67.06	13	22.2	35.7	**23.63**	11.42
60 s	335	418	224	**325.67**	97.34	122	104	105	**110.33**	10.12
80 s	410	471	311	**397.33**	80.75	158	136	179	**157.67**	21.50
7 kHz	418	456	353	**409**	52.09	253	212	188	**217.67**	32.87
10 kHz	335	418	224	**325.67**	97.34	122	104	105	**110.33**	10.12
2 L/min	335	418	224	**325.67**	97.34	122	104	105	**110.33**	10.12
4 L/min	252	233	223	**236**	14.73	74.3	65.1	67.1	**68.83**	4.84
5 L/min	200	229	219	**216**	14.73	67.9	75.4	78.4	**73.9**	5.41
6.5 kV	227	272	199	**232.67**	36.83	135	126	124	**128.33**	5.86
7.5 kV	366	333	214	**304.33**	79.95	131	131	157	**139.67**	15.01
8.5 kV	355	284	242	**293.67**	57.11	125	131	147	**134.33**	11.37

If, for example, it was affecting the plasma gas chemistry, it would then explain the inconsistent reproduction of the bioassay experiments done with the indirect SDBD4 treatment with Kapton dielectric. The purpose of the bioassay was to measure the feeding of *Spodoptera* caterpillars by taking the weight of the caterpillars; if the caterpillars weighed less on average, it would have been a first indication of activated plant defense. To do this experiment, seeds treated with plasma for 2 and 5 min at 3.5 kV were grown into mature, 3-week-old plants. The results showed that the treated plants were able to fend off the caterpillars in the first experiment, however, the effect could not be reproduced in the latter experiments, as shown in Figure 7.6.

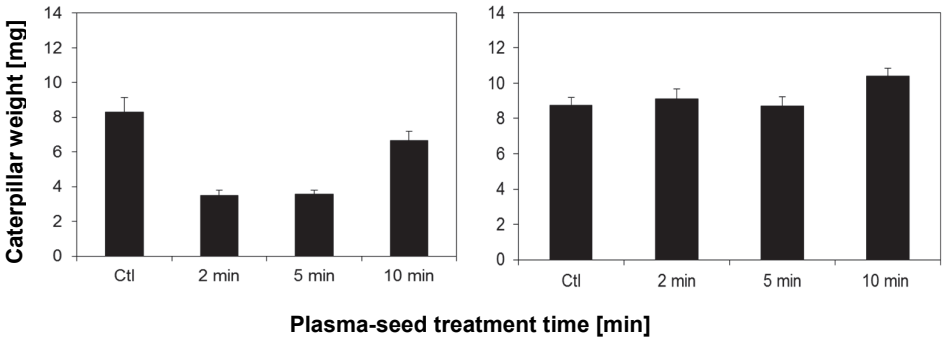

Figure 7.6. Bioassay experiments with *Spodoptera* used to assess the feeding success of caterpillars on plasma-treated seeds grown into plants. From left to right, there is a decrease in effect over time between the first and second experiment. This was later correlated to poor plasma performance (courtesy of Dr. Elia Stahl).

As described in Chapters 3 and 4, empirical observations of the patchy plasma raised suspicions and questions about whether the plasma was the same between experiments. Therefore, it is important to monitor and record all values before, during, and after the plasma treatment since these fluctuations can occur and changes in the biology can be ascribed appropriately. It may not always be feasible to do this during operation so using two different diagnostics to cross-check the values would help remove ambiguity. Finally, this also stresses the importance of a visual check on the uniformity of the plasma visible emission before and during experiments, and especially during long campaigns, to avoid false conclusions and ensure reproducibility. This is a necessary first step to assess the correct functioning of the studied devices before moving to more quantitative characterizations.

7.2.2 Electrode configuration

The shape, size, number, and arrangement of the electrodes determine the performance of a DBD. The electrodes can be arranged in various patterns such as fingers, stripes, or honeycomb etc., on one side of the dielectric, with a similar pattern or a full ground plane on the other side. A highly non-uniform SDBD plasma forms around the electrode edges where the electric field is strongest. Hence, seeds placed randomly over the surface of a SDBD (Park et al., 2018; see Chapter 3) will generally experience a wide range of plasma conditions, possibly exacerbating the variance of seed plasma treatment results. Furthermore, seeds may move during the plasma treatment due to ion wind, or electrostatic forces, thereby adding to the uncertainty of the effective plasma exposure. All of these factors should ideally be checked, for example, by fast imaging, and reported in an experimental description.

Seed positioning was also later discovered to be important not only during the plasma treatment but during seed sowing. Technologies, like seed sowing robots, are more readily available and are being used now to assess the effects of seed positioning on germination and growth (Tereza from Labdeers, private communication). If seeds are placed too closely together, the plants might feel the need to compete for resources. Evidently, this effect should be avoided when trying to understand how plasmas affect seeds and plants. Moreover, seeds should be dispersed at similar distances in each replicate to ensure reproducibility.

7.2.3 Plasma chemistry diagnostics

Apart from the electrical diagnostics of the plasma itself, many other in situ seed plasma measurements are considered in the references cited here, with the plasma reactor sometimes being designed around the diagnostics to facilitate optical access. In order of increasing complexity, one can imagine a visual or video check of uniformity via appropriate windows (Kadowaki and Kurisaka, 2014); a photograph of the discharge can be particularly helpful in a publication (Ono et al., 2011; Sakiyama et al., 2012; Kadowaki and Kurisaka, 2014; Li et al., 2014; Butscher et al., 2015ab; Khamsen et al., 2016; Gomez-Ramirez et al., 2017; Meng et al., 2017; Park et al., 2018; Matra, 2018; Pawlat et al., 2018ab; Cui et al., 2019). Thermocouples (Gomez-Ramirez et al., 2017; Pawlat et al., 2018), fiber-optic probes (Butscher et al., 2016ab; Stepanova et al., 2018), and/or infrared imaging cameras can be used for surface and seed temperature measurements (Kitazaki et al., 2014; Khamsen et al., 2016; Stepanova et al., 2018; Lofty, 2019). The gas composition of the plasma can be monitored by a relative humidity probe (Butscher et al., 2016); gas sensors (Kitazaki et al., 2014; Pawlat et al., 2018; Pawlat et al., 2018; Kang et al., 2020) for NO_x and ozone; optical emission spectroscopy (OES) of the plasma for qualitative or quantitative measurements of electronically-excited

species (Ono et al., 2011; Butscher et al., 2016; Seol et al., 2017; Wang et al., 2017; Cui et al., 2019; Lofty et al., 2019; Homola et al., 2019; Kang et al., 2020; Tomekova et al., 2020; Ambrico et al., 2020); Fourier transform infrared (FTIR) absorption spectroscopy (Sivachandiran and Khacef, 2017; Wang et al., 2017; Abdelaziz et al., 2019; Chen et al., 2019; Tomekova et al., 2020) measured directly within the FTIR sample compartment if possible (Sakiyama et al., 2012; Yoon et al., 2017; Dascalu et al., 2021) for measurements of non-homopolar molecules; UV absorption spectroscopy (Ono et al., 2011; Moiseev et al., 2014; Kitazaki et al., 2014; Perez-Piza et al., 2021), and/or laser induced fluorescence (LIF) measurement of specific radicals (Ono et al., 2011; Dilecce et al., 2012; Ries et al., 2014; Gao et al., 2017). It is recommended to provide a diagram in published works, indicating where and how readings are taken. The more measurements made (Lu et al., 2016), the more potential there is for comprehension and comparison with other experiments.

7.3 Seed preparation

Plasma treatments were done on seeds because preliminary experiments indicated that seedlings can be too sensitive and die shortly after plasma exposure (unless the plasma treatment is at a distance and the seedlings are kept in a liquid medium to prevent dehydration stress). A priming experiment, performed in the beginning of this thesis with the first SDBD prototype, was done to determine whether plasma could rescue seedlings from heat stress through cross-tolerance. It did not rescue the plant from heat shock and it was evident that indirect short plasma treatments of 5 or 15 s were sufficient to trigger leaf senescence, seen as the whitened leaves in Figure 7.7. It may be possible to perform experiments on seedlings after optimizing the plasma treatment, however, working with seeds proved to be less complicated.

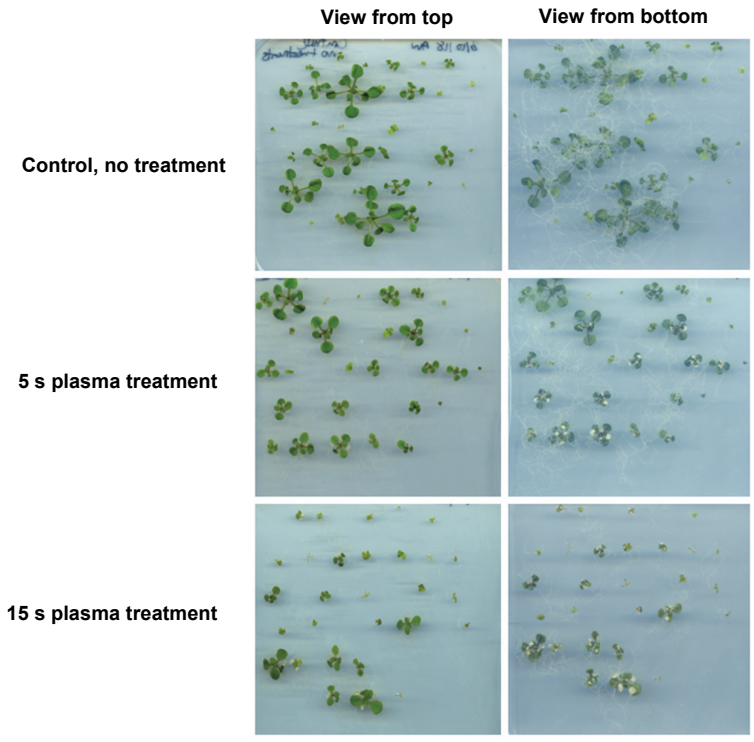

Figure 7.7. Plasma priming experiment used to assess seedling tolerance to heat stress. From top to bottom, seedlings were treated indirectly with 5 s and 15 s plasma. The images show that seedlings are sensitive to plasma treatment, indicated by the whitening and yellowing leaves.

7.3.1 Seed type

It seems reasonable that the effect of plasma on seed germination would depend on the individual plant species and its natural germination capacity, seed size, seed coat hardness, thickness of endosperm, and surface morphology, etc. (Zahoranova et al., 2016). Differences between dicots and monocots have also been mentioned by Zhang et al. (2018), where dicots were more sensitive to plasma than monocots. However, with another cultivar, the same effect was not observed (Iranbakhsh et al., 2017), even within the same wheat species. When using the same plasma device across three separate studies, broccoli and radish behaved similarly with a 2 min plasma treatment being optimal for inactivation of microbes and plant growth, whereas more than 1 min of plasma exposure had a negative effect on rapeseed (Kim et al., 2017; Puligundla et al., 2017; Puligundla et al., 2017).

Likewise, Saessal and Saechal barley seeds were compared with respect to the differences in their GABA content; the optimal plasma treatment condition varied depending on the seed type (Park et al., 2018). Also, plasma treatment on *Arabidopsis* Columbia (Col) and Landsberg erectra (Ler) seedlings was investigated by Kobayashi et al. (2020). Although the growth was negatively affected in both, Col was more sensitive and lost chlorophyll, whereas Ler remained the same, suggesting that the genetic background leads to a different response; this variation in response due to ecotype has been previously pointed out by Lo Porto et al. (2019). In Liu et al. (2019), seeds were soaked in water and treated with plasma, generating plasma-treated water in situ, or the PTW was prepared first and then the seeds were soaked. Among wheat, sticky bean, lettuce, dianthus, tomato, mustard, radish, and mung bean, only the last three showed statistically significant results. Although PTW is different from a dry plasma treatment, this simply showed that different seed types require different plasma treatment conditions. Additionally, differences in sensitivity to plasma treatment among three hemp cultivars (Carmagnola, Bialobzeskie, and Finola) were explored and the cultivar Finola had better growth (Sera et al., 2017).

The differences between seeds might be due to the surface structure. For example, coffee seeds, which have thick and tough teguments, needed a longer treatment time of 120 s for a better effect on germination, whereas shorter treatment times of 30 - 60 s were sufficient for grape seeds (Tounekti et al., 2018). Corn and eggplant were treated in Sidik et al. (2019), who found optimal plasma treatment times of 3 and 5 min, respectively. A similar line of thought is included in Sera et al. (2019), since the authors provided a list of plant species which germinate well after plasma treatment, such as *Chenopodium album agg.*, *Papaver somniferum*, *Zea mays*, *Pisum sativum*, *Brassica napus*, *Morus nigra*, *Raphanus sativus*, and those that do not, such as *Avena sativa* and *Rhododendron smirnowii*. Moreover, significant differences were found in *Fagopyrum esculentum* and *Cannabis sativa* when different apparatus was used. The reason remains unclear since the type of response may be due to genetics, seed coat structure, or chemistry, such as pigments in the seed coat acting as antioxidants and contributing to seed coat hardening (Koga et al., 2020). Nevertheless, it is clear that treatments need to be tailored to each seed type or groups of similar seed types, and so optimization will be required for each setup to ensure reproducibility.

7.3.2 Seed source, storage, age at application, and pre-selection

The source of the seeds should be stated to know whether all of the seeds will be similar, or if there will be a higher natural variation within or across seed batches. For example, there will be fewer differences

between seeds grown in the laboratory under controlled conditions with limited contamination (Cui et al., 2019), compared to those bought at a local market (Lofty, 2019) or retailer (Los et al., 2019), which may not have been sterilized or pre-treated, or those that are sold commercially which could be pre-treated. Seeds can also be collected in the wild, with conditions and climate carefully noted (Lo Porto et al., 2019). Another source option are seed banks where seeds are preserved for an extended amount of time, which could limit the variation between generations. If grown by the experimenters themselves, the seed source, treatment, and seed age are determined by their individual choices. On the other hand, it is possible, in principle, for others to purchase the same seeds if commercial seeds are used, thus facilitating cross-comparison between studies. Regardless of the source, information concerning germination rate, pre-treatments like sterilization, along with the specific seed type and, if applicable, cultivar (Sera et al., 2017; Cui et al., 2019), should also be stated as this too can affect the results of the seed-plasma treatment. For example, if a particular commercial pre-treatment already ensures a high germination rate of the source seeds, it is clear that a plasma treatment experiment has little chance of demonstrating a statistically significant improvement.

Storage: Not all studies state the seed storage conditions, although this is important to avoid variability of the seeds, their interaction with the plasma, and hence, the final results. Typically, dry seeds are stored in the dark between 4 - 10°C regardless of the seed type. For example, Norway spruce seeds (Pauzaite et al., 2018) and purple cornflower seeds (Milaziene et al., 2016) were stored in the dark for 6 months at 10°C, while soybeans were stored in the dark at 5°C (Perez-Piza et al., 2019). Wheat grains were stored between 0 - 4°C (Meng et al., 2017; Li et al., 2017) or at 10°C in the dark (Zahoranova et al., 2016). Peas were stored in the dark at 10°C (Stolarik et al., 2015), while rice at 5°C for 9 months (Khamsen et al., 2016) and at 4°C (Kang et al., 2020). According to Pradhan et al. (2012), who did a cross comparison of different storage conditions, the optimum temperature was 4°C. There are experiments where seeds are stored in dry air at room temperature for 1 or 10 months (Cui et al., 2019), or at room temperature at 50% relative humidity (Lotfy, 2019). It could also very well be that some studies unknowingly stratify the seeds by storing them in a cool, dark environment given that enough moisture is present. Therefore, experimental descriptions should carefully distinguish between “storage” and “stratification”.

Age of seeds: Deterioration also occurs as the seed ages, and the overall germination probability decreases. The seed age should therefore be taken into account because it may be that no statistically significant improvements can be made by plasma if young seeds already have high initial germination rates. Therefore, experimenters could consider working with seeds having a lower intrinsic germination rate, to investigate if there is any plasma effect, at least, on germination. General storage guidelines are available for many seed types. The optimal storage conditions depend on the seed type, but generally, most parameters do not affect seedling growth, except for high relative humidity, which deteriorates the seed. As a rule, seeds should be stored carefully without excess humidity (Wang et al., 2018; Wawrzyniak et al., 2020).

The importance of seed storage was realized during the parametric study in Chapter 5. The striped SDBD powered by AC was selected because the germination rate was the most consistent across the untreated seed replicates. Despite using seeds from the same harvest for all of the experiments, the germination rate at 48 hours was noticeably different in an unopened Eppendorf tube relative to a tube that was opened repeatedly over months for long, experimental plasma-seed campaigns. It was expected that the germination rate would decrease over time since seeds produce more ROS with age, however, differences in

the germination rate of the untreated seeds were very noticeable (Figure 7.8). The seeds in the first tube dropped to 40% over time, whereas the seeds in an unopened tube were at 80%. The decreasing germination rate in the first tube, from 75% to 40%, was attributed to the repetitive tube opening throughout the experimental campaign, hence the constant exposure to humidity and perhaps light. This aspect makes it difficult to compare datasets that have different germination rates for the untreated, control seeds and determine whether there was, in fact, an effect. Furthermore, statistically significant results are more easily obtained with a lower germination rate, so how does one decipher whether there is an effect, if it is observed with low germination rate seeds but not with high germination rate seeds?

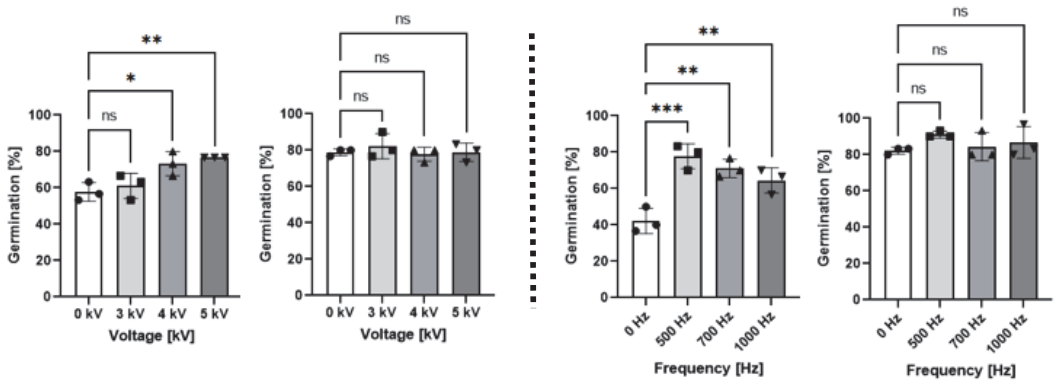

Figure 7.8. Germination rate at 48 hours of plasma-treated *Arabidopsis thaliana* seeds. (Left set) Voltage scan of nanopulse powered stripes SDBD (Right set) Frequency scan of nanopulse powered stripes SDBD. Each scan includes two independent experiments. Each set used seeds from a previously opened tube and seeds from an unopened tube, but all seeds were from the same harvest. The differences in the bar graphs show how the initial germination rate can determine whether the plasma-seed effects are significant.

To drive this point further, two experiments with varying voltage values were performed with seeds from two different tubes (again, same harvest and age) in Figure 7.9. The individual experiments were statistically significant, but these effects were cancelled when the replicates were pooled together. Therefore, there could have been more plasma-seed combinations which had an effect on germination, but a conclusion could not be inferred. Therefore, it is important to plan ahead and prepare a large enough seed batch so that all of the seeds are undergoing the same changes and thus, are uniform during long plasma-seed experimental campaigns. This, yet again, emphasizes the relevance of these seemingly minor details, such as seed handling, because they clearly can affect the interpretation of the results tremendously.

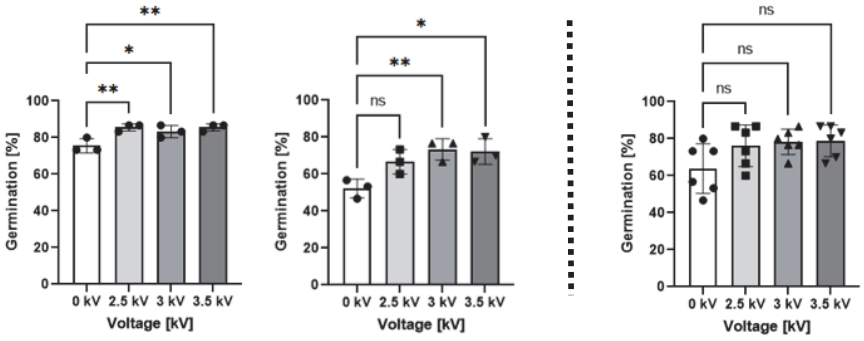

Figure 7.9. Voltage scan of nanopulse powered mesh SDBD treatment. (Left set) First two bar graphs represent two independent experiments (Right) The bar graph is the average of the two independent bar graphs on the left. If the experiments are considered individually, they are statistically significant but if they are pooled together and averaged, the variation between experiments cancels the statistical significance.

7.3.3 Seed pre-selection

Pre-selection of seeds based on their colour, size, weight, and shape, prior to plasma treatment can be found in some studies, for example: “Only healthy seeds without visible defects or signs of infection were selected for the studies” (Los et al., 2019) or “...seeds that showed molds were removed”, and “Only intact seeds without visible defects were selected” (Porto et al., 2019). Seed colour is an important factor because it indicates different polyphenol contents in the seed coat, which are linked to germination (see Chapter 2). Therefore, it is important to know if poor quality seeds are eliminated and according to which criteria, or whether every seed is accepted irrespective of its condition out of the packet, because this could influence the variance in the results. Pre-selection should be used advisedly: Is it relevant to work with batches of seeds sorted, for example, for consistent colour, size, mass, and/or shape? In industry, seeds are also sorted, but it is likely that the diversity is greater than in the laboratory. The use of uniform, pre-selected seeds may be well justified in research, in order to find real underlying trends in absence of spurious variations, but any improvements discovered in the laboratory must be sufficiently significant to still provide some industrial advantage in the presence of natural variation. This remains a moot point for plasma agriculture today.

7.3.4 Seed sterilization

The majority of studies do not mention whether the seeds have or have not been sterilized, and unknowingly neglect this aspect. Seeds may be pre-sterilized by the supplier, for example, using sodium hypochlorite or hot water, and/or sterilized by the experimenter to eliminate seedborne pathogens, which would otherwise influence the results. Depending on the method, the sterilization may act only on the surface, or throughout the seed volume. Interestingly, seed sterilization seems to be more common in recent studies, mostly by authors who have a plant biology background (Cui et al., 2019; Ghasempour et al., 2020). The original investigation of plasma treatments for microbial inactivation applications could explain the lack of seed sterilization; plasma decontamination would have been part of the treatment without requiring additional decontamination procedures. However, if seeds are sterilized by other methods prior to the plasma treatment, then any observed effects, compared with the sterilized, control seeds, will be intrinsic to the plasma and not attributable to the inactivation of seedborne pathogens that could be hampering seedling development and growth. This does not exclude that sterilization may affect the seeds by hydrating or otherwise modifying them before plasma treatment, which adds another unspecified variable to the seed plasma treatment protocol.

A preliminary experiment was designed to compare the effects of ethanol seed sterilization to plasma treatment in order to determine whether seed sterilization should be included as a pre-treatment. In Figure 7.10, the experiment included: unsterilized seeds, traditional which was seeds submerged in an ethanol-water mixture for several minutes (see Appendix), spray 5 which was seeds sprayed 5 times with only ethanol, spray full which was spraying until the paper holding the seeds was uniformly covered in ethanol, 10 min SDBD plasma, and combinations of the options listed. The root lengths were measured with FIJI simple neurite tracer using the number of pixels in the primary roots per treatment replicate on days 3 and 7. There were no obvious changes in root length qualitatively nor quantitatively across all treatments.

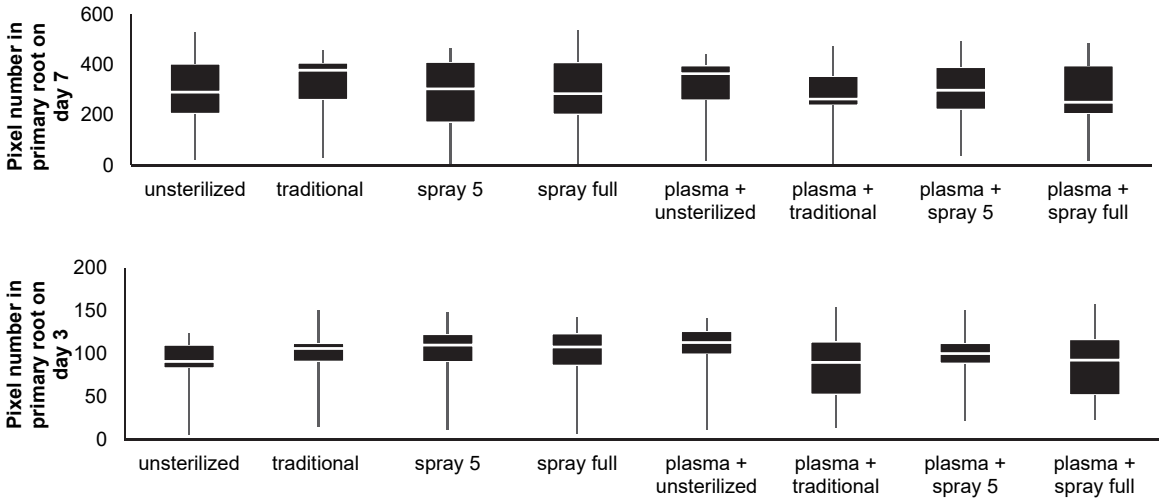

Figure 7.10. Evaluation of seed sterilization techniques using root length. There are no obvious differences in root length between different treatments.

Despite observing no changes in root length, there were minor differences in leaf area in 3-week-old plants. The total leaf area per treatment replicate was measured using Easy Leaf software, again based on the number of pixels. In Figure 7.11, the combination of plasma with ethanol sterilization was the most effective, followed by only ethanol sterilization

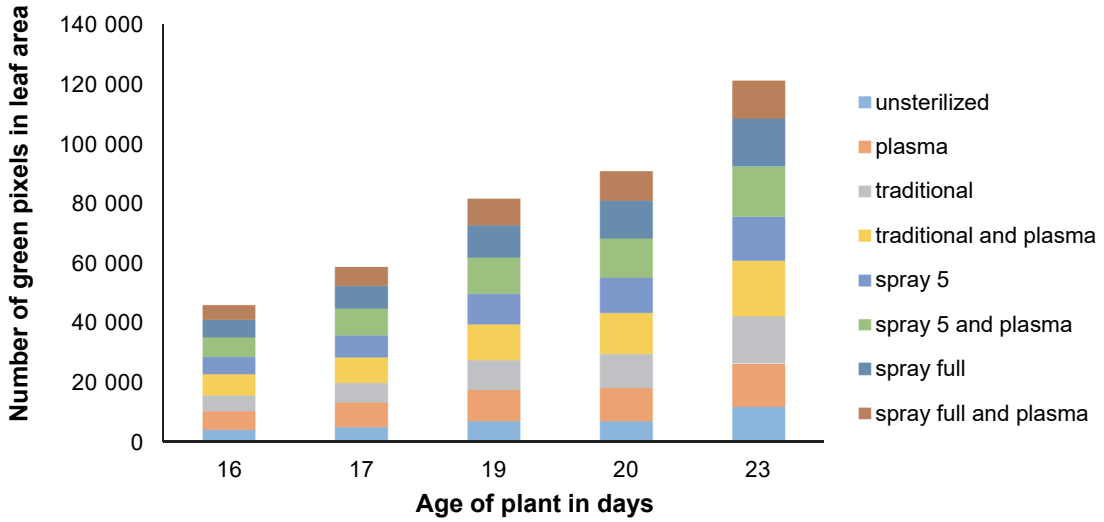

Figure 7.11. Evaluation of seed sterilization techniques using total leaf area. The largest total leaf areas are found in plants grown from seeds treated with plasma and traditional (ethanol), followed by only traditional.

Based on the obtained data, it was concluded that separating the sterilizing effect of plasma and/or the pre-treatment from the germination result would be difficult. Moreover, sterilizing seeds with liquids, such as ethanol diluted in water, is a form of soaking that can activate the metabolism of the seed, in comparison to gas sterilization using chlorine. For this reason, the decision was consciously taken to work strictly with dry, unsterilized seeds for all experiments.

This does not mean that this approach should be adopted universally; both wet and dry seeds, as well as, untreated and pre-treated should be carefully compared. Seeds sterilized prior to plasma treatment have

been used in these studies (Meng et al., 2017; Guo et al., 2017; Li et al., 2017; Iranbakhsh et al., 2018ab; Babajani et al., 2019; Moghanloo et al., 2019; Seddighinia et al., 2019; Cui et al., 2019; Sera et al., 2019; Ghasempour et al., 2020; Kobayashi et al., 2020), with the protocol details given in most cases. Within these protocols, sodium hypochlorite, benomyl, ethanol, bleach, tween20 and H_2O_2, are used as single or combinatorial treatments in a step-by-step workflow. It would be helpful if every publication would explicitly state if seed sterilization was performed and by which method. Furthermore, it is known that sterilization can hasten germination, for example H_2O_2, so this aspect must be carefully controlled if sterilization is included in the preparation protocol (Masse, 1913; Barampuram et al., 2014). Moreover, for cross-comparison, it remains difficult to have an agreement on the sterilization method due to the number and combinations of sterilization agents available. No method seems to be better than the others, but the procedure needs to be taken into account. For these reasons, full details should be described each time, as given by Butscher at al. (2016ab), Iranbakhsh et al. (2018), and Cui et al. (2019).

Soaking: Dry or moist seeds. There are conflicting results concerning seed moisture content. On the one hand, it is suggested that results are best using soaked seeds, but on the other hand, it has been observed that moisture content makes no difference. Moreover, this question is often overlooked and remains understudied. If working with wet seeds, moisture might be released from the seeds during the plasma treatment and affect the plasma chemistry (Butscher et al., 2016ab), the plasma uniformity, and the plasma device itself. Radish, tomato, and sweet pepper seeds were studied using combinations of plasma and PTW on dry or soaked seeds prior to treatment, and the combinations had to be optimized for each seed type (Sivachandiran and Khacef, 2017). Plasma treatment on soaked seeds had a negative effect on germination and seedling growth, but had a positive effect on plasma-treated dry seeds and PTW. However, pea and zucchini seeds soaked for 1 hour prior to plasma treatment resulted in a faster germination (Khatami and Ahmadinia, 2018). In contrast, fresh watermelon seeds were kept in storage in the dark at room temperature until they were dry in Lotfy (2017). It was shown that the germination rate increased after plasma treatment. Likewise, dry *Arabidopsis* seeds were treated and afterwards soaked to monitor their germination; either stimulation or inhibition were observed depending on the energy input of the plasma discharge (Kadowaki and Kurisaka, 2014). One argument for soaking seeds prior to plasma treatment is that the activation of the seed metabolism may improve the receptiveness and efficacy of plasma-generated species, such as reactive oxygen and nitrogen species (RONS) or UV (Babajani et al., 2019). However, when dry and imbibed seeds were compared specifically for changes in lipid peroxidation, no differences in malondialdehyde (MDA) content were found (Perez-Piza et al., 2018). This may partly depend on the seed type since each seed may require different soaking intervals. For example, different results were observed for dry or soaked seeds before priming (Ghasempour et al., 2020). The timing of water uptake, metabolic respiration, hormonal changes, and transcription after water absorption varied between plant species, as explained in Iranbakhsh et al. (2018) and Moghanloo et al. (2019). To list several examples: *C. roseus* seeds were soaked for 24 hours in water in Ghasempour et al. (2020); Moghanloo et al. (2019) did a germination pre-test where germination occurred 24 h after soaking, so *Astragalus fridae* seeds were soaked for only 12 h before plasma treatment; *Melissa officinalis* seeds were plasma treated 48 h after soaking (Babajani et al., 2019); and more water was absorbed in wheat seedlings with plasma treatment after 2 h imbibition than 8 h imbibition (Zahoranova et al., 2016). Instead, Park et al. (2018) controlled the water uptake of barley seeds by soaking them after plasma treatment for 24 hours to synchronize them to the same germination phase. The authors took the reverse approach by

taking dry, plasma-treated seeds and then separating them into two groups for soaking and non-soaking. Plasma treatment was observed to have some effect, whether or not the seeds were soaked afterwards.

Generally, it may be simpler to work with dry seeds since there are seeds which secrete mucilage, such as *Arabidopsis* and basil seeds but since it is not clear whether soaking seeds prior to plasma treatment is beneficial, both dry and wet seeds should ideally be considered in the experimental design. In both instances, it is important to record the moisture content of the seeds by direct heating in a drying oven according to the AOAC standard method (Kim et al., 2017), or by using a thermogravimetric analysis to measure wet-based or dry-based moisture content (Butscher et al., 2016).

7.3.5 Seed pre-conditioning

In nature, dormant seeds require certain conditions to germinate. Stratification is the name of the artificial process whereby seed embryonic dormancy is broken by mimicking the same conditions required for natural germination. The term originally refers to when seeds were layered (stratified) between layers of moist soil which were then exposed to winter conditions. The conditions depend on the seed type, so they may be cold and moist, or warm and moist, depending on the natural environment of the seed. There are existing recommendations for the stratification procedure of each seed type. The objective for using this method is to synchronize germination (Tounekti et al., 2018). Some examples of stratification before plasma treatment are wild asparagus seeds in moist sand for 56 days at 25°C in the dark (Lo Porto et al., 2019), *Arabidopsis* seeds at 4°C for 2 days (Cui et al., 2019), or 4 days (Koga et al., 2016). As mentioned previously, it could be that some studies unwittingly stratify the seeds by storing them in a cool, dark environment if enough moisture is present. Lo Porto et al. (2019) found that seeds stratified at 15°C had improved germinability compared with fresh seeds. Moreover, 1 min treatment of stratified seeds by RF low pressure air plasma shortened the germination time by 5 days, and the observed 15% higher germinability was attributed to a strong reduction of mold growth due to the disinfectant action of the plasma (Lo Porto et al., 2019). This attention to the initial seed conditions is an important step in designing the experiment, so that changes in the germination can be correlated to plasma treatment instead of the preparation conditions.

Stratification, vernalization, and priming are all methods to prepare the seed with a specific objective in mind. As stated previously, stratification is used to synchronize the germination, whereas vernalization is used for earlier flowering. It uses cold conditions, whereas stratification can be warm or cold. The objective of priming is to improve plant resilience to stress, by using water or other means (wet or dry) to enhance these properties. All of these, in one form or another, can use water and temperature to influence the seed. It is important to be clear on the differences in these terminologies to avoid confusion in the literature and to recognize that the seed will behave differently if subjected to one of these processes prior to plasma treatment. It is noteworthy to mention that plasma can be used either as a standalone alternative to replace these methods or work in conjunction with others.

7.4 Experiment design

Sample size and replicates: Experiment design includes the setup itself and the definition of the procedure to be followed. For example, the sample size can be predetermined by selecting a fixed number of seeds for treatment and planting. In other cases, a batch of seeds, usually measured out by weight, are treated and a certain number of seeds are then randomly chosen for planting. If dealing with a low number of seeds, it is

possible to be precise and go by seed number, but if a large number of seeds are needed or if they are very small in size, it is more practical and less tedious to use seed mass and then select the seeds to be planted. In terms of replicates, the convention is to use triplicates, and it is important to be able to reproduce the experiment independently - ideally with time in between experiments - to ensure that the results obtained for the combination of plasma and seed are sufficiently robust to resist minor changes in the environment or storage, yet yield the same conclusions.

Parameter space: Experiment design also involves defining the parameter space to be investigated: a central message of this paper is that plasma treatment of seeds is a multi-variable problem. Faced with so many parameters, the plasma effect on seeds should be sufficiently robust to yield a significant positive result for wide ranges of plasma treatment parameters, so that almost any plasma setup would be effective. Experience shows that this is not always the case, and scans of individual plasma parameters should be compared with controls without plasma to find the optimal value of each parameter. A proposal for a typical experiment design could be as follows and is shown in Figure 7.12 pictorially:

1. First, the seed preparation and seed post-treatment methods are defined and fixed for all seeds, including the control replicates. This is because the interest here is focused only on plasma effects. Nevertheless, it should be borne in mind that any one of those choices may influence the susceptibility of seeds to plasma, which would require further separate investigations.
2. A single set of all the plasma parameters is now arbitrarily defined as the “standard plasma conditions”. This is the initial guess at the optimal plasma conditions for obtaining the ultimate desired properties of the plant.
3. Whilst maintaining every other parameter constant, one plasma parameter is changed step-by-step on consecutive experiments for a range of values straddling its “standard” value (one factor at a time - OFAT approach), using a group of replicate seeds for each of the values. The range is chosen with the aim of bracketing the optimal value (i.e. the outcome would ideally be best when the parameter is between its extreme values).
4. This process is repeated for the available plasma parameters, although it is not practical to vary every parameter. The most convenient variables to be scanned, namely plasma treatment time, voltage or power, flow rate, or gas type and mixture. More rarely studied are the excitation frequency, gas pressure, temperature, humidity, the seed exposure, or the plasma device itself.

5. Finally, if the optimal parameter values turn out to be far from the previous “standard” plasma conditions”, then these values should be substituted as the new standard set, and the whole procedure iterated until, ideally, the set of standard values coincides with the optimal set.

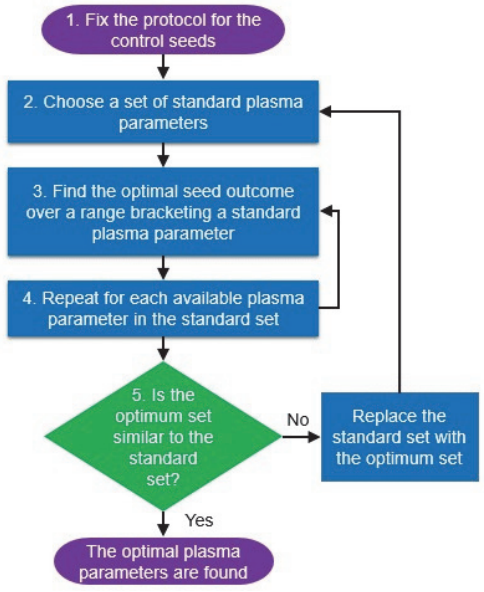

Figure 7.12: Experiment design for a parametric study to optimize the plasma-seed treatment.

The best result that can be obtained is the cumulative effect of individual-parameter improvements. Reporting in the literature tends not to mention the necessary fine-tuning of the plasma parameters, which hides the difficulty of entering the plasma agricultural field from newcomers. Therefore, it is recommended to perform a parametric study and report these findings. This may not avoid each experimenter having to perform this tedious task for their own particular seed-plasma treatment, but it would reduce the trial-and-error time needed if there were general guidelines to follow (which type of plasma device, plasma power and duration, whether to use gas flow, the approximate distance range, etc.). However, the OFAT procedure could be extremely time-consuming, if not unrealistic, as the number of input variables becomes large. In this framework where a high number of variables can affect the outcome of complex systems like seeds, the design of experiments (DOE) concept could find a wide application. This branch of applied statistics is used for planning and conducting experiments where multiple input variables can affect a measured response variable. DOE was first applied in agriculture by Fischer in the 1920s (Fisher, 1971). In the response surface methodology (Box and Wilson, 1951; Myers, 2009), a sequence of experiments is designed to obtain an optimal response, exploring the interactions between several process variables and one or more response variables based on a regression analysis. In the fractional factorial design, higher order interactions are neglected to reduce the number of input variables to be explored, finding applications in both biology (Younes et al., 2014) and physics (Vizzari et al., 2020). A DOE approach could both reduce the time of the experiments, as well as allow for an easier identification of the key variables leading to the first order effects on seeds.

Plasma pre-conditioning: Finally, to ensure that the conditions for the plasma are the same prior to each experiment, plasma pre-conditioning should be carried out, without seeds, to desorb humidity or other gases by heating the electrodes and reactor surfaces, and to decontaminate the treatment space (Butscher et al., 2016). This also gives the opportunity to perform a visual check for plasma uniformity and reliable ignition, and to check that all seeds have the same plasma exposure to reduce variation (Kadowaki and Kurisaka,

2014). If the plasma setup uses a gas flow, flow flushing is recommended for a sufficient time to establish the nominal gas conditions (Butscher et al., 2016; Pawlat et al., 2018; Hosseini et al., 2018) before beginning the plasma treatment of the seeds, and to avoid the accumulation of reactive oxygen and nitrogen species between replicates. Additionally, flow flushing helps to reduce the effect of humidity on DBD electrodes.

7.5 Seed plasma treatment

7.5.1 Waveform

The design of the plasma reactor and the electrodes was described in Chapter 3. The electrical properties and the gas environment are now described to define the plasma parameters specific to each plasma treatment, in terms of frequency, voltage, and current.

Frequency: DBD operation requires a time-varying voltage to cause electrical breakdown of the gas (Kogelschatz, 2003; Brandenburg, 2017). Individual volume or surface streamers occur between different micro-areas on the electrodes, and are extinguished as the local surface of the dielectric barrier charges up, hence suppressing the local electric field. A wide range of frequencies can be used for the alternating voltage. DBDs generally work in the kHz range, although experiments can operate from 50 Hz (Moiseev et al., 2014; Dobrin et al., 2015; Meng et al., 2017; Li et al., 2017; Guo et al., 2017; Perez-Piza et al., 2018; Magureanu et al., 2018; Perez-Piza, 2019; Los et al., 2020) to 30 kHz (Park et al., 2018) and up to nanopulse frequencies (Laroussi et al., 2004; Ono et al., 2011; Kadowaki and Kurisaka, 2014; Butscher et al., 2016ab). Plasma jets have been operated from 500 Hz in AC bipolar square waveform (Liu et al., 2019), to 58 kHz (Kim et al., 2017; Puligundla et al., 2017). The continuous wave AC mode is preferentially implemented rather than the pulsed mode. This may be due to minimizing heating, or producing more reactive species. On the other hand, nanopulses (Seol et al., 2017) allow for a higher control on triggering the plasma bursts, with a higher instantaneous power. Low pressure RF plasmas, heated by a continuous oscillating electron current (Lieberman and Lichtenberg, 2005), feature standard excitation frequencies of, for example, 5.28 MHz (Mildaziene et al., 2017; Pauzaite et al., 2018; Mildaziene et al., 2019; Filatova et al., 2020), or the common industrial, scientific, and medical (ISM) standard frequency of 13.56 MHz (Volin et al., 2000; Ono et al., 2015; Butscher et al., 2015; Li et al., 2016; Gholami et al., 2016; Hayashi et al., 2016; Gomez-Ramirez et al., 2017; Zhang et al., 2018; Jiang et al., 2018; Singh et al., 2019; Lo Porto et al., 2019; Dawood, 2020). A microwave plasma torch used a 2.45 GHz power supply (Ji et al., 2015), and gliding arc discharges have been explored at 50 Hz (Sera et al., 2017; Pawlat et al., 2018a).

Voltage and current: The discharge peak voltage values in DBDs depends on the gas mixture, pressure, and discharge gap. It ranges from 0.6 kV (Ji et al., 2015) to 24 kV in a needle-to-plane VDBD configuration (Perez-Piza et al. 2018), but most values are close to 10 kV (Stolarik et al., 2015; Zahoranova et al., 2016; Junior et al., 2016; Sarinont et al., 2016; Gomez-Ramirez et al., 2017; Zhang et al., 2017; Bafoil et al., 2018; Iranbakhsh et al., 2020). In the DCSBD (Roplass) where the electrodes are embedded in the dielectric, a higher voltage than standard SDBDs is necessary to maintain the plasma, but a more homogeneous plasma is generally produced (Stolarik et al., 2015; Zahoranova et al., 2016; Stepanova et al., 2018; Sera et al., 2019). Other examples are cold atmospheric plasma jets (CAPJ) at 2.6 kV (Lotfy, 2019), gliding arcs at 3.8 kV (Pawlat et al., 2018) and 15 kV (Khatami and Ahmadinia, 2018), corona discharges at 20 kV (Kim et al., 2017), and RF discharges from 101 −102 V (Lieberman and Lichtenberg, 2005). Current

peak values are generally in the range of tens of mA, with 30 mA in CAPJ (Lotfy, 2019) and gliding arcs (Pawlat et al., 2018). In DBDs, current values span over three orders of magnitude depending on the applied voltage, from 7 mA (Gomez-Ramirez et al., 2017) to 1.5 A (Park et al., 2018). Because of the wide variety of DBD excitation, it is necessary to show a time trace of the voltage and current to distinguish between different modes of plasma (homogenous glow discharge, (multi)filamentary, etc.).

7.5.2 Discharge power

The power dissipated per unit area in the plasma is probably the most relevant parameter for comparison between different plasma experiments, because it results from the combination of applied frequency, voltage, current, and scale, which are not sufficiently representative of the experiments when considered individually. It is often measured directly, using current and voltage probes, by time integrating the current-voltage product (Kadowaki and Kurisaka, 2014; Ji et al., 2015; Li et al., 2017; Rahman et al., 2018; Park et al., 2018; Perez-Piza et al., 2018; Sera et al., 2019; Islam et al., 2019; Billah et al., 2020). However, because a precise time-dependent measurement of the fast current pulses in DBDs is generally difficult, the dissipated discharge power is more conveniently calculated from the DBD voltage and the cycle-averaged DBD current, using the charge measured on a series capacitor. The enclosed area of the charge-voltage diagram, known as a Lissajous figure whose locus repeats for each cycle (Manley, 1943), gives the energy per AC period. Examples of Lissajous figure calculations can be found in many papers (Manley, 1943; Kogelschatz et al., 2003; Moiseev et al., 2014; Dobrin et al., 2015; da Silva et al., 2017; Zhang et al., 2017; Magureanu et al., 2018; Kobayashi et al., 2020; Kang et al., 2020; Dascalu et al., 2021; Perez-Piza et al., 2021). For a given electrode configuration and peak voltage, the power is proportional to the frequency if the energy-per-cycle remains constant. If the AC voltage is modulated by a lower-frequency on/off waveform (Ambrico et al., 2020), then the DBD power is proportional to the number of AC cycles per modulation period, which is the duty cycle of the AC power. In the literature, the explored applied power for the seed treatment ranges from as low as 0.41 W up to 1000 W, although the majority fall between 10-100 W for discharges of a few cm^2 area. Generally speaking, diffuse glow-corona discharges require low power with small gap sizes. Gliding arcs or coronas instead require higher power which could lead to a temperature increase in the electrodes, so then an indirect seed treatment might be preferred to avoid overheating the seeds. Looking at the optimal power doses reported in the literature to enhance the seed features, there is a large range of reported values, such as 140 W for radish to obtain higher mass (Matra, 2016), 50 W for Ajwain (Gholami et al., 2016), 120 W for peanut (Li et al., 2016), and 0.48 W for chili (Thisawech et al., 2020). However, as underlined in Song et al. (2020), power alone is not a sufficient parameter to characterize a seed treatment. Treatment time should also be considered to determine the energy dose. This shows that there is a window of operating parameters where germination and growth improve and an optimal value of process energy can be identified. For excessive energy values, seed features worsen compared to the control, reference seeds. To fully characterize a seed treatment, the amount of energy transferred per seed has been proposed in Park et al. (2018) as the reference parameter for a comparison between different plasma devices, power densities, and treatment times. In that work, the implementation of a SDBD allows for an estimation of 0.42 J/seed. A power density of 0.025 W/cm^3 (9 W total) on 50 seeds for a duration of 2 and 7 minutes was applied in Filatova et al. (2020), whereas 1.5 W on 50 seeds from 1 to 13 minutes was used in Li et al. (2017) and Guo et al. (2017). In most experiments, however, plasma energy-per-seed cannot be easily obtained if there is no direct plasma-seed contact, mainly for geometrical reasons. With SDBDs, seeds can be either separated from the

plasma by a gap, or arranged directly on the dielectric where the discharge occurs. Moreover, the plasma is rather inhomogeneous because of the presence of surface streamers; the plasma energy is not completely transferred to the seeds, especially in SDBDs where the plasma is highly non-uniform across the electrode surface. Hence, it is important to have a visual check of the seed positions with respect to the plasma. It is likely that SDBD experimental results will correspond to an undefined mix of seeds in direct and indirect contact with the plasma, thereby confusing interpretation of the seed plasma treatment effects. Lastly, as a general remark, all probes should be calibrated prior to any long campaigns to ensure no fluctuations in values.

7.5.3 Gas type, flow rate, and pressure

Gas type: Most plasma devices use air as process gas, although helium, argon, oxygen, and nitrogen are also used depending on the aim of the study. Gas type determines the radical and ion chemistry in the plasma and hence, the seed's biological response. Changes in germination or plant growth parameters have been observed with all the gases listed above, such as helium (Li et al., 2016; Junior et al., 2016; Iranbakhsh et al., 2017; Tounekti et al., 2018; Bafoil et al., 2018; Zhang et al., 2018; Jiang et al., 2018.; Sidik et al., 2019), argon (Khamsen et al., 2016; Zhang et al., 2017; Iranbakhsh et al., 2018; Seddihhinia et al., 2019; Babajani et al., 2019; Ghasempour et al., 2020; Dawood 2020; Iranbakhsh et al., 2020; Thisawech et al., 2020), air (Mildaziene et al., 2017; Wang et al., 2017; Khatami and Ahmadinia, 2018; Pauzaite et al., 2018; Bafoil et al., 2018; Sera et al., 2019; Cui et al., 2019; Kobayashi et al., 2020; Billah et al., 2020; Kang et al., 2020), nitrogen (Lofty et al., 2017; Iranbakhsh et al., 2017; Wang et al., 2017; Pawlat et al., 2018; Pawlat et al., 2018; Hosseini et al., 2018; Lofty, 2019), or various gases (Sarinont et al., 2016; Zhou et al., 2016). Additionally, mixtures of gases are used in Matra et al. (2018), Chen et al. (2019), Lo Porto et al. (2019), and Singh et al. (2019), while nitrogen or oxygen (Ono et al., 2015; Hayashi et al., 2016) is used as a carrier gas in Perez-Piza et al. (2019), and Perez-Piza et al. (2021). Evidently, the reactive species composition differs for different ratios of oxygen and nitrogen, as shown by FTIR in Tomekova et al. (2020); the least amount of DNA damage in pea seedlings was seen when using ambient air. Seed treatment results differ with various gases that can either accelerate or delay germination: carbon tetrafluoride and octadecafluorodecalin delayed germination, whereas aniline accelerated germination in two different seeds (Volin et al., 2000). Interestingly, hydrazine and cyclohexane could accelerate germination, however, not in both seed types (soybean and corn). By treating *A. thaliana* with either helium or air in the plasma device, both the testa and endosperm ruptured with an air plasma but only the testa ruptured with a helium plasma, suggesting that the type of gas has a distinct effect on the seed (Bafoil et al., 2018). Argon was found to be the most effective gas on wheat seeds compared to nitrogen (Meng et al., 2017). In that work, pictures of the DBD plasma discharge using argon, nitrogen, air, and oxygen are shown, where oxygen had the weakest discharge and this correlated with the germination results; the germination index was best with argon and the worst with oxygen. However, these results are in contrast to other experiments where oxygen plasma was best with *Brassicaeceae* (Ono et al., 2015), even if the plasma setups were different. Argon plasma damaged the seed coat the most and it is known that argon ion bombardment damages bacterial endospore coats. Argon ions can penetrate a thick seed coat more easily than other gases but it could be too damaging for seeds with thin seed coats (Butscher et al., 2016). The combination of gases can also have varying effects on the plant: Ar/O_2 is more efficient than Ar/air in wheat seed growth (Kabir et al., 2019), while different combinations of nitrogen and oxygen in low pressure RF plasma can have opposing effects on the germinability and germination time for asparagus seeds (Lo Porto et al., 2019). Along similar lines, a microplasma array in air yielded the best results on mung beans when they were in solution (Zhou et

al., 2016). The results may differ in solution because the mechanism under this circumstance may provide the highest concentration of exogenous H_2O_2 and nitrates, as opposed to triggering internal H_2O_2 production when using a gaseous plasma treatment.

Flow rate: Another parameter relevant for gas is the flow rate. Pressure multiplied by the reactor volume, divided by the flow rate, determines the gas residence time, which is the time to replace the gas in the reactor due to the flow (Ambrico et al., 2020). The flow rate influences the substrate/seed temperature by convection, but also directly affects the plasma chemistry by diminishing the concentration of radicals produced by the plasma in the reactor by partially flushing them away. The highest density of reactive species will be obtained in a static gas (enclosed reactor; no gas flow) by accumulation and secondary chemical reactions between radicals during the plasma, whereas a gas flow will result in a steady-state composition at a lower radical concentration, if the plasma duration is longer than the gas residence time in the reactor. One important example is the influence of gas flow on the relative humidity; lower humidity due to gas flushing can facilitate more uniform plasma and reduce degradation of the DBD materials (see Chapter 3). A few studies have demonstrated how germination rate changes according to flow rate (Ji et al., 2015). The authors kept nitrogen constant but altered the oxygen flow rates from 50 - 300 sccm and found that 200 sccm was best for early germination rate, root, and shoot length. The reason for an optimal flow rate may be related to cooling, as mentioned in Lotfy et al. (2019), but it may also be due to RONS species compositions. Air, oxygen, and NO plasmas are observed to have an effect on radish seeds, but not He, Ar, nor N_2. Notably, O_2 fumigation had an effect on plant development (Sarinont et al., 2016) and therefore, it is important that the gas flow without plasma is included as a control.

Pressure: Atmospheric pressure is more commonly used than low pressure for seed plasma treatment, although both can be successfully applied. Pressure determines the reaction rates and mean free path of species in the plasma, in turn influencing the plasma chemistry and the seed response. Therefore, if the pressure used is different from atmospheric, the pressure effect should be tested using a control (Sera et al., 2010). In Hayashi et al. (2016), different results over a range of pressure values are reported. For example, at 40 Pa, growth measured by plant length was suppressed whereas at < 20 Pa or > 60 Pa, the plant was longer. They suggested that it is due to an appropriate concentration of reactive species, so variables such as pressure are important when considering the plasma chemistry. Moreover, the reaction kinetics are altered and this may affect the biochemical reaction rates of enzymes. In the case of Zhang et al. (2017), this may partially explain the increased methylation and more active methylase.

7.5.4 Treatment time

Treatment time should be carefully noted in studies since the treatment time often includes both the plasma ON and OFF times together if a bursting technology is used and it would be helpful to explicitly mention the plasma ON time. Generally speaking, most report the total treatment time and most of these plasma treatments that yield positive results are in the seconds to minutes range (Pawlat et al., 2018ab), mostly less than 15 min, but this depends on the plasma setup, applied power, and corresponding plasma density. For example, in Magureanu et al. (2018), tomato seeds were treated for up to 45 min. However, a high flow rate of 15 L/min was used in a fluidized bed concept where the seeds were circulating within a low power coaxial VDBD discharge, probably requiring a longer treatment time to interact with the plasma sufficiently. In contrast, cucumber and pepper were treated for less than 1 min with DCSBD with optimal treatment times of 20 s and

4 s, respectively, where the seeds were gently moved and were mainly within the plasma (Stepanova et al., 2018). In another study, wheat seeds were treated for 30 s using the same DCSBD as in the cucumber and pepper study (Zahoranova et al., 2016) so in both cases, the treatment times were similar with the same plasma device and different seed types. However, if studies working with a single common seed type such as wheat are considered, there is a range of times which yield an effect with different plasma devices. In Filatova et al. (2020), a plasma treatment of 5 min was found to be the most effective, whereas in Dobrin et al. (2015), 15 min yielded the most substantial increase in length. Finally, in Li et al. (2017), increases in germination potential, rate, index, and vigor index were observed with 7 min treatment. The corresponding power of each study was 9 W, 2.7 W, and 1.5 W respectively. Nevertheless, it remains difficult to compare without knowing the effective power density across the different styles. It should be stated that a longer treatment time is not necessarily beneficial to seeds (Filatova et al., 2020). In Wang et al. (2017), a needle-to-plane DBD was used to treat cotton seeds up to 27 min, and it was shown that it was not necessarily superior to the 3 min treatment time in terms of water uptake. It seems that the time and conditions need to be optimized for each seed and it is not worth surpassing this treatment time. As mentioned above, the flow rate influences the average radical density by flushing away the plasma products; this is equivalent to reduced power and/or reduced treatment time. The reactor volume, flow rate, gas composition, gas pressure, gas temperature, plasma power, and treatment time all influence the dose of energy and radicals to which the seeds are exposed, and therefore should all be reported to allow meaningful comparison between experiments.

7.5.5 Seed exposure to plasma

The effect of the plasma treatment depends on whether the seed is in direct or indirect contact with the plasma. As a reminder, direct contact means that the seeds are immersed in the plasma; exposed to electric fields and ion bombardment, which are both absent in indirect treatments. Seeds are also exposed to UV radiation (Sarinont et al., 2016; Tomekova et al., 2020) and neutral reactive radicals in both direct and indirect contact. The latter species have different lifetimes and mean free paths in the gas, hence their composition and flux reaching the seed substrate depends on the plasma-seed separation (Kitazaki et al., 2014). Their effect is expected to diminish strongly with distance from the plasma source. As a rule of thumb, seeds placed on an electrode are nominally in direct contact with the plasma, as in a VDBD, a SDBD (Kadowaki and Kurisaka, 2014; Dobrin et al., 2015; Stolarik et al., 2015; Zahoranova et al., 2016; Stepanova et al., 2018; Park et al., 2018; Sera et al., 2019), or a RF capacitively-coupled reactor (Li et al., 2016; Gholami et al., 2016; Hayashi et al., 2016). This proximity to the plasma brings the most heating, the strongest etching via ion bombardment, and the highest concentration of reactive radicals and UV radiation. If the seed substrate is separate but placed closely parallel to a SDBD so that the seeds are approximately within the plasma glow, this results in a weaker form of direct contact. For such millimetric gaps, the thickness of the seed layer itself reduces the effective seed-plasma gap (Ambrico et al., 2020).

Plasma-seed gap: By definition, experiments designed for indirect plasma treatment feature a wider gap between the seed substrate and the plasma (Guo et al., 2017; Kang et al., 2020), or the seeds are entirely separated from the plasma and placed downstream of the plasma region (Los et al., 2018; Los et al., 2019), or are screened behind a grid or a plate (Kitazaki et al., 2012). The configuration and distance play a determining role in terms of heating and radical diffusion onto the seeds. In gliding arcs, gaps of 10 mm (Khatami and Ahmadinia, 2018), 45 mm (Pawlat et al., 2018), and up to 250 mm (Sera et al., 2017) have been

investigated. CDPJ have also been used for seed treatment with a 25 mm gap (Kim et al., 2017; Puligundla et al., 2017). Generally, stronger effects were achieved for direct treatment as compared to indirect (Los et al., 2018), however, indirect treatment could be more effective than direct depending on which parameters are observed, and on various combinations of the plasma treatment time and post-retention time (Los et al., 2019). Also, the results are different if the plasma treatment is applied in multiple, short intervals or as a single treatment, where first indications suggest that repetitive treatments are more harmful than a single exposure (Iranbakhsh et al., 2017). Alternatively, treatments can be performed in aqueous form with PTW, where new variables, such as low pH and aqueous species, come into play (Liu et al., 2019). PTW may have the advantage of accessing seed pathogens more efficiently because absorbed water penetrates the seed coat (Kang et al., 2020) and therefore, PTW treatments are not easily comparable to gaseous plasma treatments. Even for this single parameter of seed exposure to plasma, there is a large array of possibilities, making comparisons difficult.

Seed movement: To ensure uniform plasma treatment over the whole seed surface, some plasma treatment methods move the seeds mechanically (Perez-Piza et al., 2018; Sera et al., 2019), for example, by shaking the electrodes on a vibrating table (Stolarik et al., 2015; Zahoranova et al., 2016; Butscher et al., 2016ab; Sera et al., 2019; Tomekova et al., 2020), or by rotating the seed container (Volin et al., 2000; Singh et al., 2019; Billah et al., 2020). Turbulent gas flow (Pawlat et al., 2018ab; Kang et al., 2020), a cyclone, or suspension such as in a circulating fluidized bed reactors (Butscher et al., 2015; Magureanu et al., 2018) can be used to stir and mix the seeds. Alternatively, it may be possible to install moving brushes to displace the seeds (Stepanova et al., 2018). Seeds were observed to move across the honeycomb pattern of a SDBD electrodes, possibly due to electrostatic forces or ion wind created by the plasma near the electrode edges (Li et al., 2014); starting with a random distribution of seeds over the electrode surface, the seeds became grouped in the centre of each hexagon (see Chapters 3 and 4). The non-uniformity of SDBD discharges, combined with the observed movement of small seeds due to gas flow, ion wind, or electrostatic forces, causes unintentional variation in the plasma exposure from one seed to another, mixing direct and indirect plasma effects in the same experimental batch of seeds. Clearly, these random phenomena will exacerbate the inherent biological variance of seeds, confusing any interpretation in terms of plasma effects. Therefore, authors should mention how and where they position the seeds, whether they arbitrarily place the seeds across the DBD without agitation (Park et al., 2018), or whether they intentionally align the seeds in the plasma along the electrodes (Dobrin et al., 2015), and be aware of how the seeds can move from their initial positions if the treatment is done under nominally static conditions. In fact, the importance of uniformity is questionable because it is not known if it is necessary to treat the entire seed surface with direct plasma contact to yield a biological effect. To take one example, seed coat erosion is a simple and tangible observation to see how the seed interacted with the plasma treatment; Mildaziene et al. (2016) saw erosion only on the seed surface in direct contact with the plasma treatment, whereas others observed biological effects with or without seed coat erosion (see Chapters 2 and 4). Therefore, it does not seem that treatment uniformity is absolutely necessary to obtain a biological response from the seed. However, for the sake of reproducibility and consistency, it would be useful to ensure that all seeds are treated in the same manner to discern whether there is a difference between direct and indirect treatments, and under which circumstances each method should be used.

7.5.6 Temperature and humidity

Temperature can be difficult to define since there are multiple locations and time points to take measurements. It could be on the surface of the dielectric or electrode, the gas flow, the surface of the treated seeds, during the plasma treatment, or shortly after the plasma treatment, although it should be noted that the temperature of the electrode's surface and the plasma microfilaments will differ significantly. In the end, these measurements will partly depend on what is technically feasible. Often, the temperature is not recorded because it is difficult to measure during operation in a closed reactor with an infrared camera. Fiber-optical temperature probes (Butscher et al., 2016ab; Stepanova et al., 2018) are immune to electrical interference. Thermocouples can be monitored shortly before or after the plasma if electrical interference perturbs the measurement (Butscher et al., 2015). Few papers report the seed or plant temperature, although authors are mindful of having to avoid excessive heating during plasma treatment (Mittler et al., 2012; Butscher et al., 2016ab; Pawlat et al., 2018; Kobayashi et al., 2020). Seed temperature was measured in Butscher et al. (2015), Lofty (2019), and Tomekova et al. (2020), while a temperature of 121°C was measured around seeds in Kang et al. (2020). Kang et al. (2020) suggested that heat and the reactive species might have contributed to the fluctuation in the seed germination, which suggests the importance of at least controlling the temperature during treatment. Thermal damage to seeds was considered in Stepanova et al. (2018), although no control for heat was provided. This may or may not have an effect depending on the seed type and its corresponding sensitivity, since they found that pepper seeds were more sensitive than cucumber. A possibility to minimize heating would be to alter the flow rate (Tounekti et al., 2018), and reduce the time-averaged power, for example by adjusting the duty cycle of power modulation to restrict the temperature rise to less than 20°C above ambient temperature (Ambrico et al., 2020). Temperature control is necessary to avoid triggering a heat response of the seeds, which may incur unintentional biological effects independently of the plasma (Mittler et al., 2012). This temperature control is meant in two ways where on one hand, it is measured and recorded and on the other hand, a separate heat experiment at the same temperature as the plasma treatment is used as a control to ensure that the results are due to plasma, and not heat.

A short experiment explored the effect of temperatures over 40ºC on the germination of radish seeds, as shown in Figures 7.13 and 7.14. By measuring the temperatures during the plasma treatment (30 s, 1 min, 2 min, and 5 min) and establishing the same temperature profile as a separate heat treatment using a hot plate, it was possible to observe whether the effect was due to heat. Indeed, with high temperatures above 40ºC, the germination rate can be influenced. For this reason, low temperature plasmas close to room temperature should be used to eliminate the need for a heat control, but if higher temperatures are reached, a separate heat control should be done in tandem to avoid misinterpretation of results.

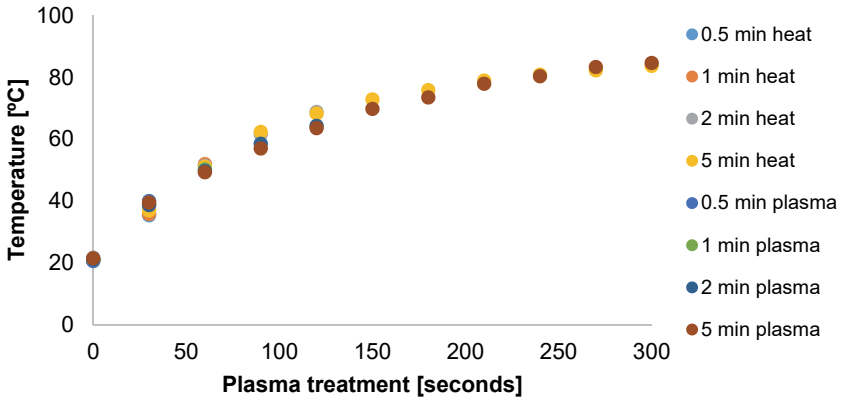

Figure 7.13. Temperature profile of SDBD plasma-seed treatment with two technical replicates where temperature in degrees Celsius is a function of plasma treatment time in seconds.

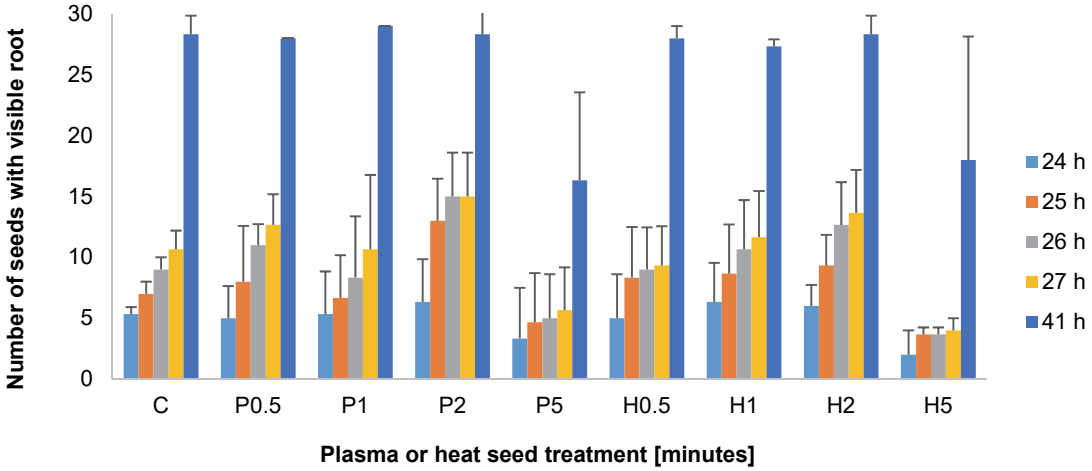

Figure 7.14. Number of seeds germinated as a function of plasma or heat treatment time in minutes, where C is for the untreated control, P is for plasma, and H is for heat. The increase in germination observed in radish seeds correlates with the increase in temperature.

Moreover, the gas temperature not only affects the biological substrate but also affects the relative humidity in the reactor and plasma chemistry (Chen et al., 2019). Depending on the plasma duration, the temperature rise can be limited by thermal inertia of the electrodes on a heat sink, or can be controlled by a cooling circuit or a Peltier junction, to give three examples. Humidity has multiple effects: it enriches the plasma chemistry (Sakiyama et al., 2012; Moiseev et al., 2014; Butscher et al., 2016; Sarinont et al., 2016; Abdelaziz et al., 2019; Chen et al., 2019; Tomekova et al., 2020; Dascalu et al., 2021); it can deteriorate seeds, but it can also break seed dormancy.

The humidity level depends on whether seeds are dry or moist. Ideally, the humidity should be measured inside the reactor during the plasma treatment (Cui et al., 2019). The humidity in ambient atmosphere is not controlled compared to operating from a gas bottle and adding a well-defined percentage of humidity with a bubbler. It is difficult to discern from the literature whether ambient conditions are at 40% relative humidity (RH), or whether there are variations that may contribute to irreproducibility of the results. On the one hand, 40% - 90% RH was optimal for radish seed growth (Sarinont et al., 2016) and likewise, 40% RH (ambient humidity) was used with basil seeds (Ambrico et al., 2020). On the other hand, it is known that high

levels of humidity are liable to partially extinguish a SDBD plasma and this is where, again, gas flow may be relevant. Humidity influences plasma decontamination efficiency (Butscher et al., 2016), perhaps because it influences ozone generation in DBDs. Ozone is quenched in the presence of water, whereas increased formation of NO, NO_2, and other products can increase the rate of bacterial reduction for relatively long periods in the post-discharge and at higher rates than ozone alone (Moiseev et al., 2014).

7.6 Seed Post-treatment

7.6.1 Post retention time

The post retention time is the seed storage interval between the end of the plasma treatment and the beginning of the growth conditions. The longer the post retention time, the more biochemical reactions could occur. Obviously, the storage environment (dark/light, temperature, substrate medium) should be stated. For example, if the seed container is sealed or open, this will determine the degree of humidity or condensation surrounding the seeds. It was shown that short-lived plasma species cause a long-term effect even after months of seed storage (Sarinont et al., 2016). A 24 h post-treatment time in a contained reactor was found to yield the best germination results (Los et al., 2020). However, it is not always clearly written if seed sowing/planting is done immediately after the plasma treatment unless authors explicitly mention it, for example if they soak the seeds for 48 hours and plant them on filter paper or agar. It may be commonly assumed that the planting is done shortly after the treatment, however, there may be subtle differences if it is directly after the plasma treatment or 4 hours later.

7.6.2 Germination infrastructure

The options that exist for germination or plant growth range from small, artificial environments to large, natural environments: Petri dish, sprouting chamber (Kim et al., 2017), light incubator, climate chamber/phytotron, greenhouse, and field experiments. To validate results and limit variations in the environment, artificial environments work well until the point where the technology needs to be transferred into industry and more variables are introduced, requiring the technique, in this case seed-plasma treatment, to be robust. If plants are grown artificially in a phytotron, which provides complete control over the seed environment using pre-programmed control of the light/dark cycle, temperature and humidity, it is simpler to decipher and understand the mechanisms with the reduced variation. However, in a natural environment like the field (Koga et al., 2016), it is possible to evaluate whether the technology is applicable since there are disturbances, such as nutrient deficiencies, diseases, and stressors.

Although a phytotron is a well-controlled system, even here it is difficult in practice to ensure a reproducible illumination between different experimenters. For example, the effective light intensity incident on the seeds depends on the number, type, power, age, and spectrum of the fluorescent tubes, as well as the distance of the seed container from the light source, its relative orientation, shadowing effects, transparency of the container walls, and the degree of condensation inside the container (fogging). Concerning root length, seedlings are typically in soil and hence not exposed to light, so to be precise, plastic Petri dishes plates can be covered or painted in black where the roots are growing (Silva-Navas et al., 2015). It goes without saying that the plasma-treated samples should be grown in close proximity to the control to minimize differences in their illumination.

7.6.3 Growth medium

The choice of growth substrate medium can affect the overall results by influencing the growth, thereby potentially promoting or masking a plasma effect. There are numerous options for growing seeds and these include artificial environments, such as Petri dishes with water, or filter paper, which may or may not be supplemented with agar, such as water or MS agar, or additional nutrients, like Hoagland solution or sucrose. It can also be done by mimicking natural environments by using sterilized sand, peat, perlite, or a combination of all in different ratios. This can be performed statically in a Petri dish, using hydroponics, or in the field. The majority of studies use Petri dishes with agar or filter paper (Bafoil et al., 2019; Babajani et al., 2019; Los et al., 2019; Lo Porto et al., 2019; Moghanloo et al., 2019; Kabir et al., 2019) or hydroponics (Rahman et al., 2018), and there are a few reports of field studies that use both plates and field (Koga et al., 2016; Li et al., 2016; Zhang et al., 2018; Zhang et al., 2018; Sidik et al., 2019; Filatova et al., 2020). In Pauzaite et al. (2018), Norway spruce seeds were grown in filter paper in vitro and in cassettes containing peat; the rationale was to use soil to be able to observe longer term effects with plasma. Seeds germinated faster in vitro than in soil, showing how important it is to choose the appropriate medium according to the study duration. Moreover, media should be interchanged to verify plasma effects, or lack of them, because additional nutrients may mask changes due to plasma treatment. Moreover, there is an optimal pH range for plant growth so it is important to note the media pH because this can influence root length, as shown in *Arabidopsis* by Gujas et al. (2012). Therefore, one might see more obvious results at one particular pH but not necessarily at another.

Although the preparation of fresh media is often considered a mundane task, the thickness, pH, age, and positioning of the plates are all important details. The thickness is important because a sufficient amount of water needs to be available to ensure the plants will be sufficiently hydrated. The water evaporates over time and concentrates the salts and thus, this should be taken into consideration for longer term studies over weeks or months. The salt concentration will particularly increase around the edges and therefore, the placement of the seeds needs to be done away from the plate corners to avoid salt stress. Among the first plasma-seed treatments, the effects of the salt stress were initially mistaken as plasma effects. However, since the results were not reproducible with fresh plates, the initial effects were associated with the increased salt concentration in the plates (Figure 7.15).

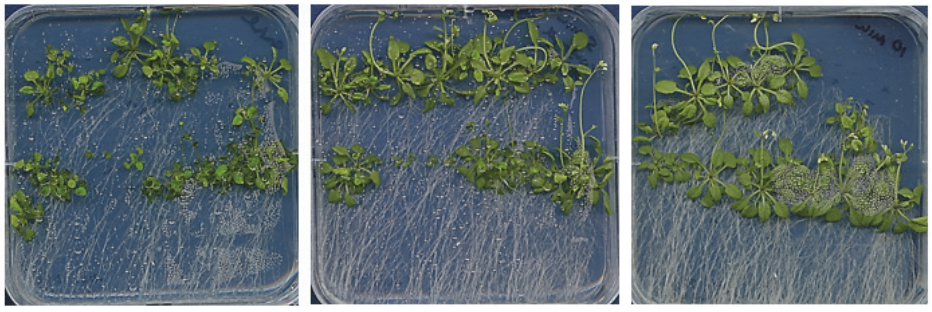

Figure 7.15. Plasma-treated *Arabidopsis thaliana* seeds are shown as 3-week-old plants. From left to right, plantlets were grown from untreated, 5 min plasma-treated or 10 min plasma-treated seeds. The 10 min treatment has the largest plantlets, but this enlargement was later linked to salt stress.

7.6.4 Growth conditions

Parameters for growth conditions include the light cycle, its intensity and spectrum, temperature, humidity, water type, amount, and frequency of watering. Concerning the light cycle, studies predominately use the 16 h/8 h cycle to grow seeds into plants (Bafoil et al., 2019; Babajani et al., 2019; Cui et al., 2019; Singh et al., 2019; Liu et al., 2019; Seddighinia et al., 2019; Moghanloo et al., 2019; Mildaziene et al., 2019; Kang et al., 2020; Kobayashi et al., 2020; Ghasempour et al., 2020), with variations in the light and/or dark cycle from 8 h to 16 h (Koga et al., 2016; Khamsen et al., 2016; Pawlat et al., 2018ab; Tounekti et al., 2018; Perez-Piza et al., 2019; Islam et al., 2019; Kabir et al., 2019).

The growth rate will depend on whether the specific plant requires no, low, medium, or high light intensity. Plant exposure to too high intensity negatively affects the growth results (Bayat et al., 2018). Studies with Moringa (Dawood, 2020), black gram (Billah et al., 2020), and wheat (Lotfy, 2019; Los et al., 2020) seeds used dark conditions and this again may be due to the seed type. Almost all other studies use short or long day cycles, which are less stressful for the plant than continuous light but the results are obtained later. The light intensity is often not quantified except by certain authors (Iranbakhsh et al., 2017; Meng et al., 2017; Tounekti et al., 2018; Zahoranova et al., 2018; Pawlat et al., 2018; Babajani et al., 2019; Liu et al., 2019; Monghanloo et al., 2019) who specify a range from 18 - 260 µmol m^{-2} s^{-1} (the units of light intensity are described, for example, in Thimijan and Heins, 1983). Others state the intensity according to seed type: for *Arabidopsis*, Cui et al. (2019) used 140 µmol m^{-2} s^{-1}, the Arabidopsis Biological Resource Center at Ohio State University recommends 130 - 150 µmol m^{-2} s^{-1} for 16 hours per day, and Nottingham Arabidopsis Stock Centre uses 122 µmol m^{-2} s^{-1} for 24 hours per day in greenhouses. These are guidelines to aid consistency.

Last but not least, the light source should be carefully chosen because its spectrum affects plant metabolism (Monostori et al., 2018; Liu et al., 2019). The correct lighting conditions are important, because additional stress could otherwise mask any beneficial plasma effect. We note in passing that the temperature and humidity for seed post-treatment are most likely not the same as for the plasma treatment.

Finally, the water type, amount, and frequency should also be reported (Kim et al., 2017). Plasma-treated water may also be part of the investigation (Bafoil et al., 2018). The growth duration obviously depends on the parameters to be measured, such as germination, root length, seedlings, or plants, etc. This is less relevant for seed surface chemistry, but timing can be critical when extracting RNA for investigating the molecular biology of seeds.

7.6.5 Growth parameters

A range of data can be collected from macroscopic to microscopic properties. The most obvious are the macroscopic properties which can be measured by eye, such as germination rate, germination probability, numbers of leaves, flowers, or fruits, shoot length, root length, biomass, leaf area, and seed or leaf colour. Other macroscopic parameters include water uptake and contact angle. Bioassays can be performed to gauge disease or stress resistance.

It is also possible to analyze the seed surface using different types of microscopy, like scanning electron microscopy (SEM) (Sera et al., 2010; Kitazaki et al., 2014; Stolarik et al., 2015; Junior et al., 2016; Khamsen et al., 2016; Zhou et al., 2016; Butscher et al., 2016ab; Koga et al., 2016; Gomez-Ramirez et al.,

2017; Wang et al., 2017; Bafoil et al., 2018; Pawlat et al., 2018ab; Stepanova et al., 2018; Los et al., 2019; Cui et al., 2019; Kang et al., 2020; Billah et al., 2020; Dawood, 2020), and atomic force microscopy (AFM) to observe the topology; energy dispersive X-ray spectroscopy (EDX) (Gomez-Ramirez et al., 2017; Cui et al., 2019), X-ray photoelectron spectroscopy (XPS) (Gomez-Ramirez et al., 2017; Stepanova et al., 2018), micro X-ray fluorescence spectroscopy for chemical element distribution (Ambrico et al., 2020), and attenuated total reflectance Fourier transform infrared absorption (ATR-FTIR) spectroscopy to analyze the seed surface chemistry (Wang et al., 2017; Pawlat et al., 2018; see Chapter 4). On a molecular level, the concentration of antioxidant enzymes, protein expression, and metabolites in general, can be measured using spectrophotometry or mass spectrometry, whereas gene expression can be measured using qPCR, or on a larger scale, microarrays or RNA sequencing. Care is needed in selectively interpreting measurements, because some characteristics can improve, whereas others can be degraded; for example, germination may be inhibited by plasma treatment but plant growth can be enhanced (Sera et al., 2010). Therefore, the question of whether biometric parameters, such as improved germination, are necessary to claim that there is a seed plasma effect should be challenged. It may be that the germination does not change, but the plasma treatment can trigger a response on a metabolic level and still alter plant development in later stages. In this context, seeds may be classified as viable in a germination test, which provides the optimum temperature, moisture and light conditions to the growing seedlings, however, they may not be capable of completing their life cycle under a wide range of field conditions. Generally, seeds start to lose vigor before they lose their ability to germinate; therefore, vigor testing is an important practice in seed production programs (Perez-Piza et al., 2019).

7.6.6 Data collection and processing

There is flexibility in collecting plant biometric data, and there is not necessarily a unique and correct manner of doing so. Here, we will briefly mention which points to consider, which time points are appropriate for sampling, how many are necessary or sufficient to make conclusions, and what types of criteria are used to evaluate if there is a plasma effect on seeds. There are also unspoken criteria for how to take measurements. For example, a 1 mm radicle is considered by some as a parameter for successful germination, whereas others count germination as soon as the roots are visible by eye or camera. This aspect partly depends on laboratory culture and infrastructure, but discrepancies can occur if done by eye since it is rather subjective. Automated image analysis could be used (Iranbakhsh et al., 2017; Cui et al., 2019; Kobayashi et al., 2020), although a camera or microscope for very small seeds may not always be available even though it is more precise and objective. Depending on the system, it might not be possible to give a precise recommendation for time point, however, the time point of the analysis should always be recorded and ideally several consecutive measurements should be taken because there may be variations.

For example, during the EDX measurements, high concentrations of potassium were found only once after plasma treatment and other times, did not appear to be different relative to untreated, ozone-treated, or heat-treated seeds. The exact time point was difficult to determine and control since the experiments were performed by a facility, however, this pointed to the importance of using the same time point. Due to the lack of reproducibility of the EDX maps, XPS was performed twice independently to verify the results, which did indicate a slight increase in potassium. This highlights yet again the importance of using a second diagnostic to verify the results to avoid any false conclusions and reduce the probability of overlooking an effect.

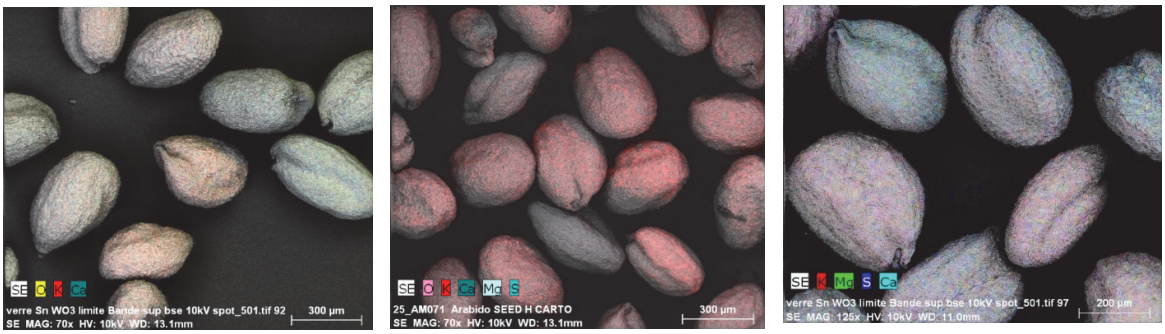

Figure 7.16. SEM-EDX images of plasma-treated seeds. From left to right, untreated, honeycomb SDBD plasma-treated, and ozone-treated seeds are shown, where red represents the concentration of potassium. The highest potassium concentration is after plasma treatment, but this effect was later not reproducible using EDX.

This holds true for any analysis done on the molecular level in a biological system. Different genes may be up- or downregulated depending on the time elapsed following the plasma treatment, and in which developmental stage the treatment and measurement were captured. Furthermore, it is difficult to recommend a particular time point or time range for the frequency of measurements because there may be experimental constraints due to infrastructure, or plant growth conditions. All time points are relevant but it does facilitate cross-comparison if suitable time windows are established i.e. within 48 hours after germination.

After extracting and measuring the results, statistical analysis is the last, but not least, important step before stating conclusions. This has not always been done rigorously in past studies, however, the field has significantly improved over time with recording information and it is now very often a requirement. For the statistical analysis, a variety of statistical programs like SPSS or GraphPad Prism are used. More importantly, the types of tests done should be listed i.e. ANOVA, t-test and the p-value. For experiments that have a weak effect, the type of p-value can dictate whether or not it is a statistically significant effect; examples include the following studies (Sera et al., 2012; Stolarik et al., 2015; Butscher et al., 2016; Kim et al., 2017; Sera et al., 2017; Pawlat et al., 2018; Perez-Piza et al., 2018; Perez-Piza et al., 2019; Lo Porto et al., 2019; Cui et al., 2019; Ambrico et al., 2020).

7.7 Discussion

7.7.1 Protocol and the problem of reproducibility

Plasma treatment of seeds combines two distinct disciplines - plasma physics and seed biology - which have different terminologies and cultural expectations. Biological systems are generally far more complex than physical systems, and variation between nominally identical organisms is inherent (Kobayashi et al., 2020). Nevertheless, even though it is unreasonable to expect highly reproducible results, it is still important to ensure reproducible protocols for the experiments. Otherwise, any protocol discrepancy will exacerbate the natural variation, and could obscure real underlying trends. Clearly, results obtained with even just one different step in two protocols are measurements made on two distinct experiments, and therefore without justifiable comparison. A difference in protocols may be any hidden variable due to unconscious or implicit assumptions and procedures, including preparation and post-treatment of the seeds. If protocols differ in apparently insignificant, unreported details (such as the spectral type of the neon lamps in a phytotron), then scientific validity could be forfeited. Another example is the variety of different methods for seed sterilization. This can be done using ethanol or by other methods, with or without dilution or rinsing. Also, storage can be handled

differently whether in the dark or light, or at different temperatures. No approach is right nor wrong, but the method used must be stated and not left to guesswork on the part of the reader. The most obvious source of protocol discrepancies is missing information in experiment reporting, which renders results useless for a database. The fundamental message of this chapter is therefore to be explicit about every detail in a protocol, in the hope that any missing steps or hidden assumptions will be corrected by experts in these two disciplines.

7.7.2 Obstacles to accurate protocol reporting

Protocols might not be accurately described for several reasons:

1. A complete protocol would be extremely long to publish in an article, although supplementary material could be used. Unfortunately, there is currently no standard protocol for plasma treatment of seeds which could be used to summarize an experiment description.
2. Some laboratory procedures (for example, seed sterilization) are based on laboratory culture, history, and influential personalities, where it might be taken for granted that “everyone does it this way”. Consequently, unconscious assumptions are made, and, by definition, not reported.
3. It is not fully understood which actions (or non-actions) can have important consequences for the final results. What exactly is a necessary and sufficient protocol? Is Fig. 7.2 sufficient?

7.7.3 Recommendations to improve reproducibility

1. Establish protocols for seed preparation, seed plasma treatment, and seed post-treatment. Follow AOSA or ISTA guidelines to find systematic methods already implemented.
2. Multiple diagnostics for the plasma and for the seeds give more points of comparison between experimental results from different laboratories.
3. Objective reporting of results, whether positive, null, or negative. The field can be distorted by reporting only positive results. Definitions of statistics used, and warnings of pitfalls to avoid.

7.8 Conclusions

The aim of this chapter was to present the physical and biological variables necessary for a comprehensive characterization of the plasma treatment of seeds, to aid reproducibility and comparison between experiments. The checklist in Fig. 7.2 has been proposed to record this information systematically. The number of permutations of seed plasma treatments is so large that it is currently impractical to prescribe specific standard protocols. For the foreseeable future, further empirical studies and flexibility in experimental design, while noting the parameters listed here, seems to be the most realistic approach for plasma agriculture.

This highlights the importance of the following points: 1) Any improvements discovered in the laboratory must be sufficiently significant to still provide some industrial advantage in presence of natural variations. This remains a moot point for plasma agriculture today. 2) The best plasma effect that can be obtained is the cumulative effect of small improvements. The literature tends not to mention the necessary fine-tuning of the plasma parameters, which hides the difficulty of entering the plasma agricultural field. Objective reporting is necessary, whether for positive or negative, significant or null, results. The field is distorted by reporting largely positive results. 3) Any protocol discrepancy will add to the natural variability, and could obscure systematic underlying trends. An obvious source of protocol discrepancies is missing information, which renders experimental results unusable in a database. The fundamental aim is to be explicit

about every detail in a protocol. It is recommended to perform a parametric study at the beginning of each experimental campaign to carefully optimize the plasma treatment, and to communicate which variables did, and did not, have an effect on seeds. Furthermore, it would be useful to correlate results across several diagnostics to have an overall understanding of plasma-seed treatments, for example, plasma chemistry coupled with analysis of the seed surface and molecular biology. This would improve the understanding of each seed plasma treatment variable, and help to optimize the design for the desired results. With this, the transfer of this technology to industry will hopefully become a reality in the near future.

Chapter 8

Conclusions and the future of plasma-seed treatments

Abstract

This last chapter provides a detailed overview of what has been achieved in this thesis work and outlines the main contributions to the plasma agriculture field. It is then followed by suggestions for future experiments and advice on how to proceed forward based on what is currently being investigated. It ends with a personal opinion on future applications and includes suggestions of what should be, at the very least, considered before attempting to upscale plasma-seed treatments.

8.1 Achieved results

What has been achieved and concluded in this work was first, the establishment of a bioplasma research facility, followed by the establishment of a plasma-seed treatment and its operating parameters. There are many variables which need to be controlled carefully in the plasma-seed treatment and it is not a straightforward procedure; placing a seed in a plasma does not necessarily mean there will be a visible change.

Using this plasma-seed treatment, a first characterization of the plasma gas chemistry using *in situ* FTIR measurements was performed. It was confirmed that FTIR should be used for long-lived species in conjugation with LIF for short-lived species. Moreover, it seems that NO may be one of the responsible agents for augmenting germination parameters; short-lived species are likely playing an important role in the effects of plasmas on seeds, however, it remains challenging to characterize them. Therefore, multiple diagnostics should be used and multiple variables should be scanned in all studies to avoid misinterpretation of results.

For the first time, a working protocol with guidelines for plasma-seed treatments was developed to improve communication within the field and between disciplines, and simulatenously quicken research progress. It is clear that there is not one universal way to do the treatments so what is most important is careful recording and characterization within each study. Eventually, through collecting this information systematically, it would be possible to do a large meta-analysis and identify the relevant operating parameters.

To execute this properly, an understanding and recommendation of which diagnostics to use is paramount. For this reason, surface analysis techniques for plasma-treated seeds were explored and recommended based on their relative merits. Additionally, it brought awareness to nanoparticle deposition which may be desirable or problematic. This aspect has been neglected or at the very least not yet addressed by the community.

Overall, it was observed that plasma is likely a synergistic effect and unique in its ability to react quickly with surfaces, although more work needs to be done to understand how each parameter in the plasma-seed treatment affects the seed. Therefore, this work contributed towards the first few transcriptomics studies on plasma treated seeds and is the first to include two plasma treatment times in a study. The first impression of the data seems to suggest that, if optimized adequately, plasma treatment time exposure could be a parameter to influence the type of defense response expressed by the plant; potentially attractive for industrial applications. Alternatively, through a second transcriptomic study using a 24-hour delayed extraction time point, it may trigger the same sequence of events but these events might occur on different timescales depending on the plasma intensity. From this data, a tentative hypothesis of how seeds interpret plasma as a stress was proposed for the first time; it appears to be a wounding along with an oxidative stress.

All things considered, it is clear that the complexity in this field is underestimated where there are many positive results but it is questionable whether they are reproducible due to the high number of variables. Plasma technology still has potential in being useful to the agriculture community, although much work is needed to better understand how to tailor the plasma-seed treatment for a predictable output.

In summary, the originality of this thesis is derived from challenging status quo assumptions and breaking down communication barriers by providing the first structured guidelines for designing plasma-seed treatments, which includes advice on productive methods and how to interpret the results. Furthermore, it includes among the first transcriptomic studies which contribute towards our understanding of the mechanisms on a molecular level. By exploring two variables, plasma treatment time as a physical parameter and RNA extraction time as a biological variable, a hypothesis has been proposed addressing how plants interpret plasma treatment, which now remains to be validated by the community.

8.2 Future experiments

To progress in our understanding, future studies should ideally correlate results using methods from different fields. For example, combining material science techniques with biological analysis and recording all plasma parameters diligently to eventually identify trends. The most urgent questions are: which treatment parameters are necessary to see a reproducible beneficial effect on seeds, can these parameters be applied to a plethora of seeds or is it necessary to tailor them to each seed type, how is the plasma treatment affecting the seed on a molecular level and how robust is this effect, is it possible to have these plasma treatments and biological effects consistently reproduced, can the plasma treatment be reliably scaled up for industrial applications, and how does plasma treatment compare to already existing methods, such as acid or mechanical scarification?

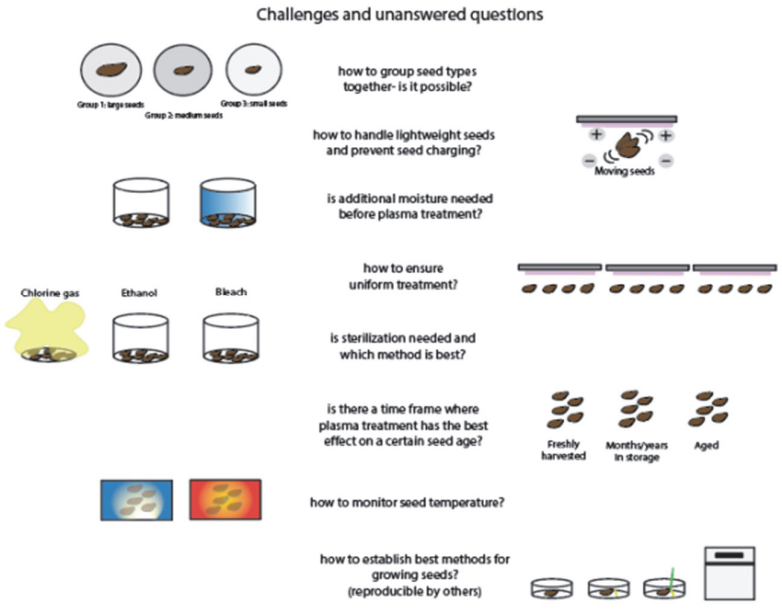

Figure 8.1. A summary of the next questions for plasma-seed treatment design.

In particular, future experiments can include the following:

- Demonstrate that plasma can change the macroscopic properties of plants by using and comparing variously designed plasma sources like a SDBD, VDBD, corona, or plasma jet
- Characterize the plasma compositions and plasma-seed treatment parameters which have positive and negative effects on plant development to identify key differences (presence or absence of components and their concentrations)
    - voltage, current, frequency, electrode gap size, electrode material, dielectric material and thickness, distance, power supply, working gas, power, temperature, RONS spectra
    - measure the results using optical emission spectroscopy, Fourier transform infrared spectroscopy (FTIR), laser induced fluorescence (LIF), two-photon absorption laser induced fluorescence (TALIF)
- Continue to compare protocols for: dry or wet seeds, treating seeds, seedlings, or plants, single intense treatments or multiple mild treatments, gradual or abrupt treatments, freshly harvested seeds or aged seeds, seeds within the same species and family or outside, gaseous plasma or plasma-treated water
- Continue to compare complete plasma treatment in comparison to its separate components: heat, oxidative stress, short lived vs. long lived species, oxygen vs. nitrogen species, UV photons, electric and magnetic fields, with wild-type and mutant plants (see Fig. 8.2)
- Assess results extensively using germination probability, accelerated or delayed development (germination and growth), root length, leaf area, leaf width, chlorophyll content, biomass, gene expression, chemicals indicators for ROS/RNS, protein and metabolic profiles
- Investigate priming and induction of resistance against heat shock, ozone, UV, drought, plant pathogens, or insects with laboratory, greenhouse, and field studies
- Investigate transgenerational memory by comparing the seed size, seed number, time to maturity
    - compare genomes across 2-3 generations to check for mutations (genotoxicity)
    - pool sequencing to look at change in frequency of gene alleles and determine if there is increased somatic recombination (for example, using GUS *Arabidopsis* line)
    - compare transcriptomes, proteomes and metabolomes across 2-3 generations
- characterize residuals after plasma treatment on seed, check for toxic compounds, and analyze vitamin and mineral profiles
- Compare plasma to existing priming methods such as H_2O_2, KNO_3, biostimulants, water soaking, ozone, magnetic and electric fields
- Study how plasma affects the soil microbiome and soil bacteria activity and how plasma-treated plants interact with surrounding biota

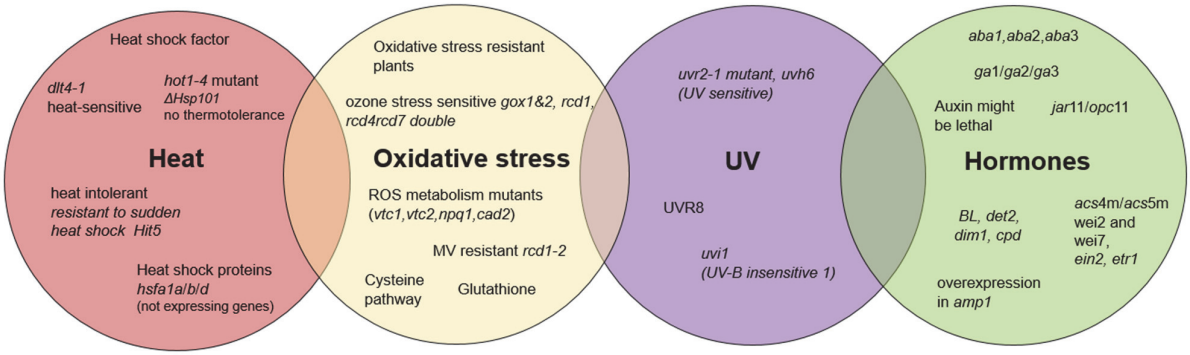

Figure 8.2. A selection of possible mutants to be used in future studies to delineate the causative plasma agent.

8.3 Opinion on the current state of the field

What has been shown to date is a combination of plasma sources, treatment methods and seeds that are able to alter plant parameters. On the one hand, this diversity emerges from individual researchers considering what is relevant for their society and local economy, but on the other hand, this also makes it difficult to standardize current research. We seem to have reached a point where there is potential in this technology as a proof-of-concept, although there may be an inherent bias by publishing solely positive results, giving the impression that finding these setups is simple and it only takes trial-and-error to optimize the treatment conditions. Therefore, it would be helpful for the readers to also publish negative results to know what is not working when designing these treatments to move towards standardization and fundamental understanding.

Although there is flexibility in choosing the type of plasma device, most scientists are using DBD plasmas enclosed in a chamber with air as the process gas. Air plasma is practical and produces a rich chemistry. However, the role of humidity in these plasma-seed treatments is still debated. Is it best to work with dry or wet seeds? Should one control and add or remove humidity, for example, by a gas flow? How do you ensure that humidity will not quench the plasma and damage the plasma device?

Many authors reported evidence, more often than not, that plasma treatment modifies the seed surface in such a way that water uptake increases; this is either done by poration, removal of the topmost layers, or through oxidizing the surface to improve the water interaction. It is not yet clear whether it is through mechanical, chemical means, or both, and whether this is dependent on the plasma treatment, seed type, or both. How much energy needs to be injected into the plasma to achieve these effects? To address this, future studies should continue correlating surface modifications with changes in germination in a systematic manner and clearly record the electrical characteristics. This would clarify if mechanical erosion is necessary to induce a change, under which circumstances mechanical erosion or chemical alterations are needed, and specifically which gas types and electrical properties are needed.

It also seems that most authors agree that RONS are predominantly responsible for the observed plant growth effects. However, it is not yet clear whether it is ROS, RNS, or the synergistic action of both; this will likely depend on the application. It is also not clear yet if it is purely a RONS effect because plasma is a synergy of individual mechanisms. Although most authors agree that the effects are not due to heat, it remains difficult to define temperature in these studies, especially in the presence of a gas flow. Is the seed temperature after

treatment, the plasma temperature, or the temperature of the device measured? Is it certain that an increase of a few degrees is not enough to trigger a heat shock response? Is the relative humidity altered by the plasma heating? For this reason, it would be useful for scientists to include appropriate controls in their studies for heat, ozone, electric fields, and humidity, mimicking what they are using in the plasma treatment where possible. This is more commonly done in plasma treated water experiments but not necessarily with plasma-seed experiments.

Most authors observe changes at the molecular level, although these studies are momentarily very limited. More studies addressing whether the same genes or proteins are activated across different plasma treatments or across different seed types would provide insights into the robustness of plasma-seed treatments. It is also not yet clear whether these effects are long-term and at which point they would be considered genotoxic or adverse for plant growth. The bottleneck or constraint for gene expression studies will be largely limited to already sequenced plant genomes. Nonetheless, these results may be applicable to closely related species.

As a next step, it would be useful to understand how each parameter in the plasma treatment affects the outcome so it is more predictable and therefore possible to control the output. This will require the continued collaborative efforts of biologists, chemists, and physicists. Also, standardized protocols will likely need to be tailored to the seed type, due to the diversity in the seed coat, and build on the existing information in industry or associations, such as AOSA (Association of Official Seed Analysts), with plasma as an additional parameter.

Much of the outcome of the plasma-seed treatment seems to depend on both the plasma setup and seed features. If the seed has many layers that need to be scarified, plasma may help with mechanical erosion through etching, or by melting the wax with the heat produced as a by-product of plasma generation. If the seed is rather permeable, it may functionalize the surface through the addition of chemical groups on the surface to become more hydrophilic and enhance gas exchange to then affect the seed biochemistry. Therefore, it may be that there are several modes of plasma treatment that can be selected. For example, if you need mechanical scarification and erosion, then use high power AC and argon for ion bombardment. If you need a gentle dose with ROS for chemical modification of seed coat, use a nanopulse power supply for better ROS generation and lower temperature. If you want to generate NO or H_2O_2 or if you want to delay or accelerate germination for storage or sowing respectively, choose the appropriate gas type.

Very little has been done in terms of economic analysis other than by Niemira et al. (2012) but it would be useful to mention explicitly the power density, the maximum number of seeds that can be treated to have an effect, and potentially disclose the cost of manufacturing the plasma source so it would be possible to judge which designs would be easiest to implement and scale-up. This does not mean one plasma device is used universally as one of the advantages is the flexibility in design so people can adapt their treatment to their surroundings (seeds may vary in their value or importance depending on the country). By understanding the plasma-seed interactions and mechanisms, this will also help with setting up regulations around this technology since it is still ill-defined.

Lastly, treatment uniformity remains to be addressed. It is far easier to achieve this in low pressure plasmas with a long free mean path, however, bulk treatments are not attractive for industrial implementation. Atmospheric pressure plasma treatments remain challenging as they are not uniform and less controlled. If

the substrate is very close to the surface, the chemistry may not be homogenous but if it is too far, the reactions may die out by the time they reach the surface. Many steps still need to be taken to optimize plasma-seed treatments for applications.

8.4 Opinion on future applications of plasma-seed treatments

As stated in Chapter 2, plasma could be considered as a priming tool (Starič et al., 2020; Holubova et al., 2020; Adhikari et al., 2020; Song et al., 2020ab; Rasooli et al., 2020; Li et al., 2021ab; Simek and Homola, 2021; Attrai et al., 2021; Ranieri et al., 2021; Renáta et al., 2021).

Plasma treatment is a surface treatment, which affects the topmost substrate layer in a nanometer range and therefore, does not penetrate into the bulk properties. Concerning the surface changes in seeds, these effects are mentioned in greater detail in Dufour et al. (2021) but briefly, they include surface modification, decontamination, and surface functionalization. On the surface, it can cause attachment of plasma-produced molecular fragments and surface oxidation whereas within the interior, the diffusion of cations into the seed and removal of chemical inhibitors from the seed envelope has been shown (Volkov et al., 2020; Dauwe et al., 2021).

Although it is mainly a surface treatment, the reactive oxygen and nitrogen species, ultraviolet light, and electric fields can trigger internal changes on a molecular level. Most hypothesize that it is due to the RONS and if dosed adequately, it is considered a low eustress, activating growth and defense (Svubova et al., 2020; Adhikari et al., 2020). One could consider plasma for hormonal, UV, nutri-, chemical, or magnetopriming with the appropriate plasma source, however, biopriming, nanopriming, and genetic engineering will be elaborated on since these are relatively new, underdiscussed, and understudied applications (Fig.8.3).

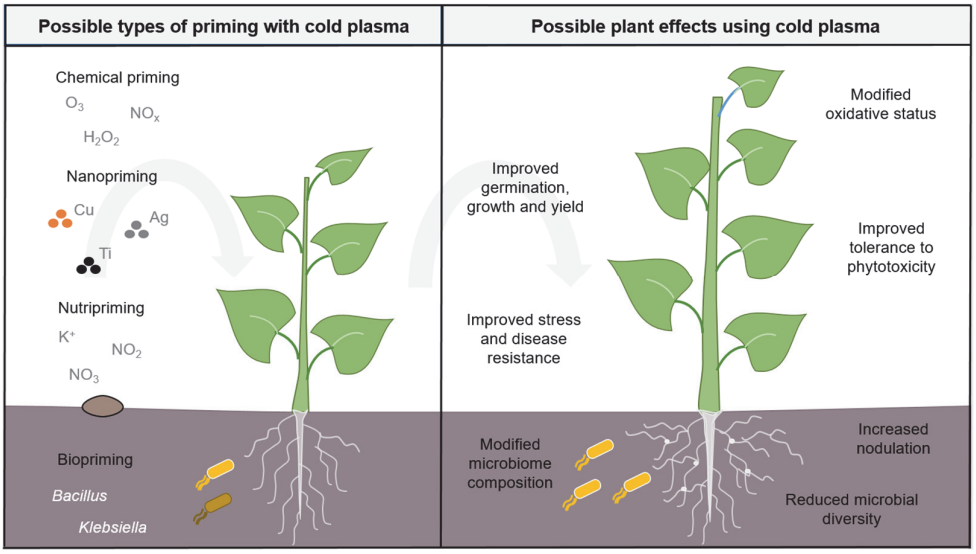

Figure 8.3. A summary of priming possibilities using cold plasma, which includes chemical priming, nanopriming, nutripriming, and biopriming.

For biopriming, plasma interestingly can change the bacterial microbiome composition or the relationship between microbes and plants, regardless if the microorganisms or the plants are treated. On one hand, it has been observed that there is an increase in nodulation, as shown by Pérez-Pizá et al. (2020),

Ivankov et al. (2021), and Mildaziene et al. (2021), by treating the seed or the plant growth promoting bacteria with plasma (Ji et al. 2019). It could be that ROS alter the morphology of the plant, which in turn affects the relationship with the microorganisms. Otherwise, it has been shown that plasma-treated *Bacillus subtilis* had improved replication and motility, and through this improved colonization and modulated phytohormone production, they ultimately increased the biomass and yield of host plants and increased disease resistance (Ji et al., 2019). Similar findings were found in another study using *Klebsiella pneumonia* (Ji et al., 2020).

On the other hand, the plant microbiome composition can be changed after plasma treatment as observed with *Arabidopsis* by Tamošiūnė et al. (2020a). In a second study by the same authors using sunflower seedlings instead, *Firmicutes* were more prevalent in plasma-treated samples with the most variation seen in *Mycobacterium,* which became enriched in the leaf and cotyledon but were reduced in the roots (Tamošiūnė et al., 2020b). Overall, they observed inactivation of non-spore forming bacteria in the cotyledons and leaves of germinated seedlings. They formulated a hypothesis which suggests that the reactive species reduce the microbial diversity, modify the composition which in turn enhances root growth. Because of this, the increase in water uptake can then lead to improved plant growth parameters, such as photosynthesis and lateral organ growth. Los et al. (2020) also observed a reduction in microbial diversity after plasma treatment and therefore, this aspect, as well as the change in microbiome composition, needs to be critically assessed whether it is beneficial or problematic and under which conditions. The implications of this could be that there is a subtle alteration of soil health around the plant and thus, caution should be exercised to avoid selecting for more resistant bacteria which are spore-forming, for example towards the orders *Bacillales* and *Clostridiales* (Lazra et al., 2020). This could complicate matters and make it possible for certain plant pathogens to proliferate with an unfavourable microbiome. Assuming that the composition favours plant growth, there could be an application for biopriming.

For nanopriming, nanoparticles themselves are known to have both negative and positive effects on plants. It could be toxic but it is also possible to stimulate germination by mobilizing storage reserves, enhance growth, increase chlorophyll content, antioxidant levels, nutrient absorption, or improve disease resistance (Acharya et al., 2020; do Espirito Santo Pereira et al., 2021). The mechanisms behind these effects include ROS production, phytohormone crosstalk, and overexpression of aquaporin genes (do Espirito Santo Pereira et al., 2021). Plasma could be a convenient method to apply nanoparticles to the seed surface where the electrodes can sputter nanoparticles but it would be restricted to metals which are compatible with the plasma device, such as copper. However, this aspect has not been explored yet in plasma agriculture and may very well be a less controlled method compared to current synthesis methods. Prior to using this application, the particle morphology, size, and charge of the nanoparticles would need to be carefully assessed but at least plasma would ensure their presence.

Nanoparticles alone or in combination with other plasma components could further enhance the plant effects. Plasma can protect against nanoparticle toxicity and work synergistically to improve growth and plant defense by increasing the expression of antioxidant enzymes (Abedi et al., 2020). Plasma has also been combined with carbon nanotubes to improve growth related performance (Seddighinia et al., 2020). However, nanoparticles require optimization too. Furthermore, from a safety viewpoint, it is questionable whether nanoparticles are desired for health reasons. The long-term consequences and methods for their removal are unknown. Alternatively, it is possible to add thin films of nanocomposites of metals with antimicrobial

properties. It could combine plasma with nanoparticles and work more effectively, control their release on the seed and into the environment, or protect against phytotoxicity as shown by Abedi et al. (2020). If seedborne pathogens are removed with these antimicrobial compounds at the very least, it can indirectly improve plant survival. However, the main questions for this nanoparticle application are whether it is feasible, beneficial, safe, and whether it can be developed into a well-controlled procedure.

In terms of genetic engineering, it is possible to have genotoxic effects on the plant due to the UV photons and high RONS concentrations. Genetic engineering with plasma has only been shown by a few studies and remains understudied (Li et al., 2020). In theory, it could be possible to use it as a mutagenesis tool to screen for plant phenotypes with desired traits since this application has already been proven to work in bacteria (Ottenheim et al., 2018). At the moment, plasma is associated with epigenetic changes since it has been shown in two studies that DNA is methylated after treatment. In one study using rice, the authors found that the promoters of abscisic acid catabolism and alpha-amylase were more active, but did not observe any changes to gibberellic acid production, which promotes germination. Through this, they were able to improve the germination rate of seeds under heat stress; equally well as H_2O_2 solution (Suriyasak et al., 2021). The reason for these changes is likely linked to ROS production since it is known to induce epigenetic changes. The question here is: how would a seed behave in the following generations? At least if it solely affects the epigenetics, it would be less problematic with regulatory issues, quell concerns about transgenerational effects in certain countries, and would likely be accepted by consumers.

8.5 What to consider for industrial applications of cold plasma

The future for plasma applications appears to be full of potential but work still needs to be done to identify which applications are worthwhile to pursue and in other words, which could be competitive with current practices. Assuming that the parameters for the plasma design and plasma-seed treatment are carefully selected and it becomes a well-controlled process, it may be possible to develop it as a Swiss Army knife where several functionalities can exist within the same plasma-seed treatment. If we understand how to control it, it could be possible to produce specifically ozone, NO_x, or H_2O_2 modes by modulating the power and water content. A key treatment parameter is the energy/power since this affects the macroscopic and microscopic properties of the plasma. However, temperature will need to be carefully controlled regardless of the biological application. Since high repetition rates can lead to overheating in both AC and nanopulses, bursting discharge technology will continue to be useful. Admittedly, it could be very difficult to control the RONS composition but it might be possible to operate in an effective range. Depending on the desired output, it could arguably be sufficient to simply have an electric field to stimulate similar responses by operating below the voltage breakdown and therefore, this option could also be embedded in plasma treatments.

These processes will undoubtedly require optimization like all other methods, such as steam or pesticides, so the time and effort to reach this goal should not be underestimated. It will not only require optimizing the plasma-seed conditions but will require treatment uniformity. In terms of uniform results, the response to plasma differs depending on the seed colour, seed age, and even cultivars. For seed colour, Ivankov et al. (2021) used four different coloured seeds and found their response to cold plasma or electromagnetic field treatments could be stronger if preselected for a particular seed colour. Likewise, Koga et al. (2020) and Attri et al. (2021b) demonstrated that grey seeds responded stronger to plasma treatment than brown seeds, as well as, aged seeds compared to freshly harvested seeds.

The gas type and the treatment time need to be selected carefully too. It has been shown that nitrogen was the most damaging to DNA as opposed to air (Tomekova et al., 2020). This is one of the few studies which addresses genotoxicity so overall, the data is very limited. Unless it is intended for a mutagenesis application, the damaging effect could possibly be mitigated by changing the distance and humidity levels. Generally, it is more advantageous to use ambient air at atmospheric pressure as opposed to purchasing nitrogen bottles. However, air and the generation of RONS inevitably leads to oxidation, which can be beneficial when dosed adequately or be problematic in instances where seeds have high oil content. This could lead to lipid oxidation and eventually rancidity, which would reduce the seed lifetime (Afshar et al., 2021). Interestingly, it is possible to increase the oil yield content in plasma-treated seeds (Rezaei et al., 2020), which once again points to our need to understand under which conditions we achieve certain outcomes.

When deciding which macroscopic parameter to use to evaluate the plasma-seed effects, germination rate and probability are often used as a biometric and very often, accelerated effects are favoured over inhibited. However, Mildažienė et al. (2021) investigated the long-term effect of plasma treatment after 3 - 5 years and observed positive growth effects after a few years from seeds with initially inhibited growth. Also, the initial positive effects remained viable for up to one year. Therefore, it is not yet clear which parameter and which timescale should be used to evaluate plasma-seed treatments but this will undoubtedly depend on the context. It is recommended to have a well-balanced overview and therefore, as many parameters as possible should be included in a study to help with cross-comparison.

To translate this into industry, a general roadmap shown in Figure 8.4 includes a selection of parameters necessary to evaluate plasma as a priming tool. More comparisons between current priming methods and plasma would be useful i.e. plasma and H_2O_2 solution (Suriyasak et al., 2021), or plasma and EM fields (Mildaziene et al., 2021). Mujahid et al. (2020) compared plasma and chilling on grape buds and Waskow et al. (2021) compared plasma and electron beam. Despite the attractive short treatment times with electron beam, the doses resulted in root abnormalities.

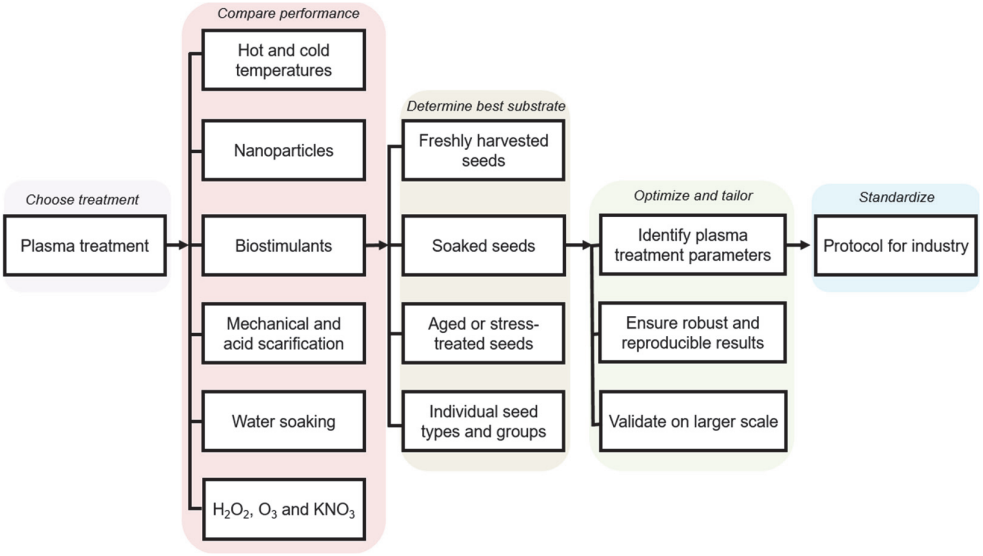

Figure 8.4. A simplified roadmap to evaluate the application of plasma as a priming tool before upscaling.

By comparing two or more methods in a study, it could be possible to pinpoint conditions where plasma either outperforms, performs equally as well, or underperforms relative to other methods. This would simultaneously contribute towards our understanding of plasma-seed mechanisms because of the direct comparison and reduced variation, having been done in the same study. Furthermore, it is very relevant to perform comparisons since it is possible to have the opposite desired effect by changing only one parameter and therefore, it is important to identify the limits of each system. For example, Lu et al. (2021) showed that bioactive compounds increased with a nitrogen plasma but they were otherwise inhibited with an air plasma.

For the substrate, as mentioned before, it appears that the seed responds differently whether freshly harvested or aged (Degutytė-Fomins et al., 2020), and whether it has high or low starting germination capability (Xanthou et al., 2021). It will even respond differently depending on the genotype or cultivar (Sirgedaite-Seziene et al., 2021, Han et al., 2021). Depending on the application, it may be possible to wet and then dry the seeds but in other cases, it may be desirable to avoid this moistening and drying step to save energy and decrease the probability of microbial contamination in humid conditions. However, one advantage of plasma could be improving the germination without necessarily needing additional moisture and subsequent drying and therefore, the treatment times can be very short, as low as 10 s, to see an effect on germination (Terebun et al., 2021) compared to hours or days using other priming methods, like steam. As many are aware, optimization is required to avoid growth inhibition (Sery et al., 2020). There is an optimal value related to power and treatment time, and this has been illustrated as a figure in a review; there is no effect if the exposure is too low or short, and there is a damaging effect if too high or long (Song et al. 2020a).

Additionally, the point at which plasma priming is done, in other words, the window for priming treatment needs to be carefully contemplated. Xu et al. (2020) recommended on high germination percentage seeds either: (1) at the end of the resistant stage without notable viability loss, which is hard to grasp by monitoring; (2) at a slight but identifiable germination percentage decline.

Depending on the desired effect, whether that is promoting growth or resistance, the optimal parameters should be identified, verified independently and then done on a larger scale, noting whether the same results are obtained or whether adjustments need to be made. For example, if humidity should be avoided, this then would require large volumes of controlled dry gas flow and this could make the cost too high on a large scale relative to a simpler and less expensive priming method.

The optimal storage conditions of plasma-treated seeds would also need to be investigated. Storage likely will not deviate far from what is already known, which is to store seeds in a low water content and cool environment (Suma et al., 2013), but nevertheless, this should be verified. Lastly, more toxicological and long-term studies, especially for plasma-treated seeds in storage, need to be performed. Provided that information concerning the points listed above is available, a thorough economic analysis would be necessary to make a decision. Within this evaluation, energy, cost, time, results, and sustainability or life cycle assessment need to be included to determine whether there is enough of a financial incentive to pursue this technology while confirming that it is, in fact, a sustainable technology.

At this point, it would make sense to then standardize a protocol for industry once the application and treatment are clear and robust. It is not to say that plasma is meant to replace all existing methods, but it could be another tool or approach among others to alter plant growth and stress and disease resistance. It could

have the advantage of being a tunable process due to the high number of input variables which other methods might not be able to offer.

For industry applications, plasma applications could fall into several categories: machine sterilization, seed sterilization, or augmenting seed performance. To translate this into reality, for seed sterilization or augmenting seed performance, this can be done either by using a central treatment system with large volumes of falling seeds, or a conveyer system to treat the seeds and later distribute them to users, or by using a portable and easy-to-use equipment for farmers, for example, by attaching the apparatus to existing farming equipment, like a tractor. Undoubtedly, safety precautions must be taken to protect workers from ozone and UV exposure.

In terms of treatment design, it is not yet clear which is the best. The plasma chemistry is dependent on the generation and properties of the streamers (direction, velocity, diameter), but few scientists have included the microscopic properties of their plasma in the papers. Considering the number of variables in plasma-seed treatments, it may not be possible to reduce the complexity into one variable but energy/power seems to be instrumental. One approach to tackle this question could be to study how the chemistry changes with different power inputs and observe which chemistry results in a biological effect. From there on, the parameters which would yield this chemical composition could be tested on different geometries and treatment styles to see if the chemistry and the biological effect would be transferable. Practically speaking, a VDBD or SDBD would likely be the best choices as they are flat and can be easily introduced into a conveyer belt system. Alternatively, they can be done step by step, as shown by Stepanova et al. (2018), or be circulated in a fluidized bed reactor, as shown by Butscher (2016). One hurdle, however, would be to avoid damage to falling seeds so the fluidized bed reactor could be an option but sufficient air flow would be required to ensure that the seeds remain intact. Likewise, it remains a question whether cracks in the seed coat are acceptable, since plasma treatment can erode the seed surface. It can be useful to promote germination but since the seed coat has an important role as a protective barrier, cracks could unintentionally make the seeds more accessible to pathogens. Therefore, this aspect either needs to be optimized or avoided altogether to ensure that the target germination rate of 85% in industry is met.

Provided that scientists now focus on the molecular effects of plasmas, we might understand in detail how plasma-seed treatments work in order to develop plasma-seed treatments into a viable seed processing technology. My hope is that plasma treatments will be another technology useful to the agriculture community specifically, that it can be used as a flexible, well-controlled method for seed priming that could potentially reduce the use of chemical pesticides.

Appendix with supplementary information

Relevant protocols

Seed sterilization protocol

Seeds were transferred to an Eppendorf tube and 800 µl of 70% ethanol was added. After 5 minutes, ethanol was removed and procedure was repeated once more. Afterwards, 800 µl of 96% ethanol was added for 5 minutes. Seeds were then dried in a laminar flow bench on filter paper and were either stored in a new tube or planted directly on agar, which was later wrapped around the edges with scotch tape. When stratification was necessary, the seeds were kept in a cold room for 2 days at 4ºC.

Media preparation

½ MS agar was prepared using 12 g/L of agar for plants, with 4.9 g/L of MS (Murashige and Skoog medium) and deionized water with a pH of 5.8 before autoclaving.

Water agar was prepared using 20 g/L in deionized water with a pH of approximately 6.7 before autoclaving.

Protein extraction from plant tissue

Nanoluciferase modified seedlings were grown on ½ MS agar for 14 days (n= 10 - 15 for a total of 4 replicates) and were transferred to an Eppendorf tube with 800 µl of Evian water. The tubes were placed at the desired temperature (22ºC, 31ºC, 34ºC) in a heating block for 2 hours, then 22ºC for 2 hours. After the water was removed, the leaves were drilled while adding 300 µl of protease inhibitor (diluted 300x). The solution was centrifuged at 17,000 rcf at 4ºC for 10 minutes, and the supernatant was transferred to a new tube. The protein content was then determined using the Bradford assay using 10 µl of the sample and 990 µl of the Bradford reagent. Refer to Baptiste (2021) for additional details.

Bradford assay for protein quantification

The calibration curve was determined by using aliquots of Bradford reagent and 1 µg/mL bovine serum albumin (BSA) in order to obtain the R^2 value (Table A1). The solutions were mixed and the absorbance values were measured after 15 minutes at 595 nm with a spectrophotometer. Using this information, the protein samples obtained in the previous section were then quantified. Refer to Baptiste et al. (2021) for additional details.

Table A1. Pipetting volumes for BSA standard curve.

BSA	0 µl	3 µl	5 µl	10 µl	15 µl	20 µl	30 µl
replicate 1	1 mL	997 µl	995 µl	990 µl	985 µl	980 µl	970 µl
replicate 2	x	997 µl	995 µl	990 µl	985 µl	980 µl	970 µl

Luciferase assay

To prepare the buffer, 25 µl of DTT was added into 5 mL of n-luc buffer. In each well of a 96-well plate, 100 µl of the sample was added, which included 68.5 µl of buffer, 30 µl of plant supernatant, and 1.5 µl of nanoluciferase substrate. HIDEX plate reader SW 0511 was used to measure light emission to determine the nanoluciferase concentration. Refer to Baptiste (2021) for additional details.

Oxidative stress candidate search

All 33,000 genes listed in The Arabidopsis Information Resource (TAIR) were used in this study, including the first transcripts. A matlab script was written to extract the normalized fold change values and standard deviation from multiple Genevestigator studies. An arbitrary cut-off criteria was set, which was ≥ 3-fold change under oxidative stress and ≤ 0.1-fold change under heat stress. Gene candidates were then manually selected based on other information sources, such as Genevisible to confirm that oxidative stress was in the top 10 stresses using other datasets, and the *Arabidopsis* Subcellular Database (SUBA) for the gene expression location ie. preferably in the cytoplasm.

To validate these genes experimentally, qPCR primers were designed for exons with the following criteria: a fragment size restriction between 80 - 150 bp with a maximum of 250 bp, 50 - 55% GC content, primer length between 18 - 22 bp with no more than 3 GC in 5 bases at 3', and a melting temperature between 57 - 60°C with a maximum of 64°C with no more than 2°C apart. The primer pair was selected if it was specific to one amplification region (verified with either primer-BLAST and primer3).

Table A2. qPCR primers of oxidative stress specific genes or references genes.

Gene ID	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3'):
AT2G29420	CGA TTC TTC CTC AAG ATC CGT A	TTT CAT CGC CGT CAC ATA AAT C
AT3G49620	GTT GTG GAA GTA GTC GGA AAA C	GAA CTG ATG CGT CAT CTC TTT C
AT5G51830	AGA CGA GAT AAC ATT CCT GAC C	CAA CTG GCT TCA CTT TTA CTC C
AT4G22530	TTA GAC GCA CGA CCT ACT TAT C	CAT TTC ATC TTC CGT CAT CGA C
AT4G34270	GTG AAA ACT GTT GGA GAG AAG CAA	TCA ACT GGA TAC CCT TTC GCA
AT4G26410	GAG CTG AAG TGG CTT CCA TGA C	GGT CCG ACA TAC CCA TGA TCC
AT5G59820.1	GAC ACA GGA ACG AGA GTG GG	TTC AAC GTA GTC ACC GTG GG
AT5G42380	ATA GTA GCG GAA GCA GCT CG	CAC CGG AGA TTT TCC CGT CT
CUL4	CCAGGCCAGAACAAAACTGC	TGTTGGCCAGTACCCTGTTG
SAND	AACTCTATFCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC

30 seeds were planted on ½ MS + vitamins/MES agar plates and grown for 13 days under continuous light. For the heat treatment, seedlings were treated for 35 min at 35°C using 3 mL of preheated Evian water, and kept in an incubator for (+) samples, whereas control had room temperature water added and was covered with aluminum foil to mimic incubator conditions for (-) samples. For the H_2O_2 treatment, hydrogen peroxide solution was used for 2 hours at 20 mM on the seedlings (concentration was determined from literature by Desikan et al., 1999). Due to the photosensitivity of H_2O_2, the plates were also covered with aluminum and kept at room temperature to respect the H_2O_2 lifetime of 5 - 10 hours. For the ozone treatment, seedlings were treated with 250 ppm of ozone for 2 hours using Sterilux (concentrations were based on plasma-generated ozone measurements). Based on the visible change in phenotype, yellowing and whitening of the leaves indicated senescence in the 15-day-old seedlings. it was clear that there would be an effect on the molecular level and therefore, this was verified with qPCR.

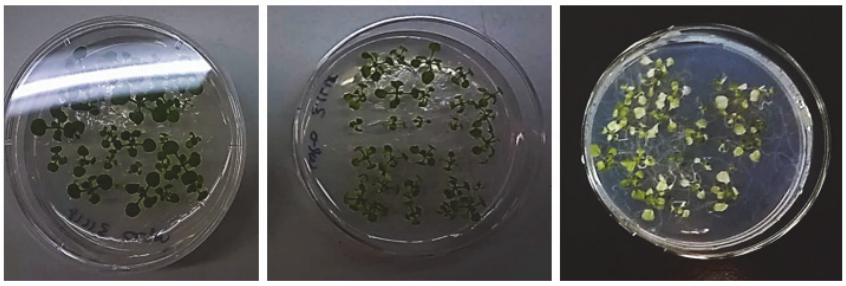

Figure A1. From left to right, untreated seedlings, seedlings treated with 250 ppm of ozone for 2 hours, and seedlings treated with 100 ppm of ozone for 17 hours are shown. Due to the extensive ozone exposure, leaf senescence is visible.

For each treatment, 8 seedlings in triplicates were flash frozen in nitrogen and were later lysed using 3 beads in Qiagen Tissue lyser for 30 s twice at the highest speed. RNA was extracted using Nucleospin RNA Plant MACHEREY-NAGEL following the manufacturer's protocol. The starting RNA concentration was 250 ng for cDNA synthesis, which was done using SuperScript IV reverse transcriptase (Invitrogen) with oligodT20. The cDNA was diluted 8x and then qPCR was done using SYBR (Agilent) and QuantStudio Applied Biosystems (Thermoscientific) with the following cycle: 20 µl with cover temperature at 105°C, hold stage was 95°C for 3 min, then PCR stage was 95°C for 10 s, 60°C for 20 s, followed by melt curve stage at 95°C for 1 s, 60°C for 20 s, and 95°C for 1 s. Two reference genes were used, cul4 and SAND, since they were the least responsive under the given conditions. To compare data across plates, a threshold for qPCR analysis was set to 0.1. The method of 2-ΔΔCT adapted from Bubner and Baldwin (2004) was then applied. Using a template developed by Dr. Elia Stahl, the CT of the target gene was compared to the CT of the reference gene and the value was normalized by setting the efficiency to 2. The mean normalized expression across triplicates (biological triplicates and technical triplicates) was calculated along with the standard error of mean normalized expression.

ATR-FTIR

ATR-FTIR spectra were measured using Nicolet 6700 from ThermoFisher Scientific with the following settings: 32 sample scans, 32 background scans, resolution at 4, sample gain at 8, optical velocity at 0.6329, and aperture at 100. The detector was DTGS KBr, the beam splitter was KBR and it used an IR source.

SEM

Scanning electron microscopy (SEM) images were taken using ThermoFisher Quanta FEG 250. Seeds were fixed onto aluminum stubs on a carbon sticker without additional sample processing. The images were taken at 10 kV in low vacuum with 100 Pa chamber pressure. Large field detector (LFD) and Backscattered detector (BSED) were both used.

EDX

EDX measurements were taken using Bruker 129eV brand detector and Bruker nano Xflash Detector 5030. The software was Bruker's Esprit 1.9 version for EDX spectra. Samples were kept on a carbon sticker on an aluminum stub.

Atomic force microscopy

AFM images were attempted using Cypher VRS, Oxford Instrument with the following specifications: ~30µm x 30µm lateral range of scanning area, 50 mm x 50 mm x 20 mm maximum sample size, µm to Angstrom resolution, photo-thermal (Blue drive) excitation, liquid cell, and heating/cooling stage.

X-ray Photoelectron Spectroscopy

XPS measurements were carried out using a PHI VersaProbe II scanning XPS microprobe (Physical Instruments AG, Germany).

Analysis was performed using a monochromatic Al Kα X-ray source of 24.8 W power with a beam size of 100 µm. The spherical capacitor analyser was set at 45° take-off angle with respect to the sample surface. The pass energy was 46.95 eV yielding a full width at half maximum of 0.91 eV for the Ag 3d 5/2 peak. Curve fitting was performed using the PHI Multipak software.

Bioassays

Spodoptera littoralis experiments were performed with 3-week-old plants in transparent plastic boxes (Fernández-Calvo et al., 2011). Eggs (Syngenta) were hatched to eventually produce mature *S. littoralis*. The caterpillars were then placed on the plants to feed for 7 days. Larvae were then weighed on a precision balance Mettler-Toledo MT5 (Mettler-Toledo) and averaged.

RNA sequencing

The seeds were treated with plasma and planted on agar shortly after (within hours). The germination rate was measured at 48 hours after sowing and were grown for another 4 days until RNA was extracted (6 days after sowing). Total RNA was extracted from three biological replicates obtained from 6-day-old seedlings or 7-day-old seedlings (up to 100 mg) using Precellys (Bertin) and lysing kit with 1.4 mm zirconium beads in 0.5 mL tubes. The settings used were 6,000 rpm for 30 s, followed by a 10 s break, and finished with 6,000 rpm for 30 s, all done at 4°C. InnuPREP Plant RNA kit (Analytic Jena) was used for RNA isolation and quantified with nanodrop (DS-11 Microvolume Spectrophotometer).

RNA quality was assessed on a Fragment Analyzer (Agilent Technologies) and all RNAs had a RNA quality number (RQN) above 7.9. Library preparation and RNA-seq was performed at the Lausanne Genomic Technologies Facility, University of Lausanne, Switzerland (https://www.unil.ch/gtf). RNA-seq libraries were prepared from 400 ng of total RNA with the Illumina TruSeq Stranded mRNA reagents (Illumina) using a unique dual indexing strategy, and following the official protocol automated on a Sciclone liquid handling robot (PerkinElmer). Libraries were quantified by a fluorimetric method (QubIT, Life Technologies) and their quality was assessed on a Fragment Analyzer (Agilent Technologies).

Cluster generation was performed with 2 nM of an equimolar pool from the resulting libraries using the Illumina HiSeq 3000/4000 SR Cluster Kit reagents, then sequenced on the Illumina HiSeq 4000 SR platform (single end) using HiSeq 3000/4000 SBS Kit reagents for 150 cycles (single end). Sequencing data were demultiplexed using the bcl2fastq2 Conversion Software (version 2.20, Illumina). The analysis resulted in approximately 31 - 37 million of 150 bp long single-end reads for each library independently (see Tables S1, S2, and S4, S5 in supplementary information Chapter 6).

After sequencing, raw reads were subjected to quality control (phred score > 20) and adapter trimming using FastQC (0.11.976), and BBDuk. Reads matching ribosomal RNA sequences were removed with fastq_screen (v. 0.9.3). Reads were aligned against the *Arabidopsis* reference genome sequence (Araport11) using STAR v2.7.5 using default parameters (Dobin et al., 2013). FeatureCounts v1.6.2 was used to generate the count matrix and to calculate gene expression values as raw read counts. RPKM were obtained from FeatureCounts (in house script) to make heat maps. Count read values were analyzed using DESeq2 package from R software v1.30.1 (Love et al., 2014) after rlog transformation to identify the differentially expressed genes (DEGs). A t-test was performed to identify differential enrichment between control and treated samples. To identify GO categories of differentially expressed genes, ShinyGO v.0.66 software was used (Ge et al., 2020). The results were based on customized background genes from our RNA-seq, which yield more accurate results for enrichment analysis (Wijesooriya et al., 2021). The transcriptome data are available in NCBI Bioproject Code: PRJNA800224.

Supplementary information

Chapter 2: Mechanisms of plasma-seed treatments

Table S1. Legend describes each category in Table 2 for the type of plasma and seed used in the study as well as the main findings.

Plasma	**Examples**
Geometry	volume or surface dielectric barrier discharge (VDBD/SDBD), radiofrequency (RF), jet
Pressure	atmospheric or low
gas type	oxygen, nitrogen, argon as single or admixtures
Seed type	radish, wheat, rice etc.
Micro(organism)	
bacteria or fungi or insect	native or artificial contamination of a microorganism or organism (pest)
Main findings	
seed coat modification	wettability, water uptake, morphology, surface chemistry
growth parameters	germination rate, root length, shoot length, seed vigour
metabolism	antioxidant enzymes, proline, soluble sugars
disease or stress resistance	plants grown in stressful conditions or include molecular component which contributes towards resistance
Scale	
macroscopic	seen by eye
microscopic	seen using a microscope, mostly light or scanning electron
molecular	enzymes, proteins, DNA, RNA, DNA modification

Table S2. Collection of plasma-seed treatment papers.

Citation	Plasma	Seed type	Microbe	Main findings	Scale
Iranbakhsh et al., 2020	DBD; atm pressure; Ar	Hemp		growth parameters, metabolism	macroscopic and molecular
Kang et al., 2020	1) Arc discharge; low or atm pressure; underwater 2) DBD; low and atm pressure; (0.6-1 atm); not clear	Rice	*Fusarium fujikuroi*	seed coat modification, growth parameters, disinfection/disease resistance	macroscopic
Billah et al., 2020	DBD; low pressure (400 torr); air	Black gram		seed coat modification, growth parameters, metabolism	macroscopic and molecular
Rezaei et al., 2020	not clear; atm pressure; air	Hyssop		tissue modification (dried leaves)	macroscopic
Koga et al., 2020	DBD; atm pressure; humid air	Radish		seed coat modification (colour)	macroscopic
Ghasempour et al., 2020	DBD; atm pressure; Ar	Catharanthus roseus		growth parameters, metabolism	macroscopic and molecular
Mujahid et al., 2020	DBD; atm pressure; He and O_2	grape cultivar Muscat of Alexandria		growth parameters, metabolism	macroscopic and molecular
Filatova et al., 2020	CCP RF; low pressure (200 Pa); air	Maize, wheat, lupine	Native fungi, *Fusarium culmorum*	growth parameters, metabolism, disinfection/disease resistance	macroscopic and molecular
Prakrajang et al., 2020	not given; not given; Ar	Chili pepper		growth parameters	macroscopic
Kobayashi et al., 2020	DBD; atm pressure; air	Arabidopsis (seedlings)		growth parameters	macroscopic
Dawood, 2020	RF; low pressure; Ar	Moringa		seed coat modification, growth parameters	macroscopic and microscopic

Citation	Plasma	Seed type	Microbe	Main findings	Scale
Sidik et al., 2019	plasma jet/plume; atm pressure; He	Corn and eggplant		growth parameters	macroscopic
Ambrico et al., 2019	DBD; atm pressure; air	Basil		seed coat modification, growth parameters,	macroscopic and microscopic
Seddighinia et al., 2019	DBD; atm pressure; Ar	Bitter melon		growth parameters	macroscopic, microscopic
Gao et al., 2019	DBD; atm pressure; air	Pea		seed coat modification, growth parameters	macroscopic. Microscopic
Mildažienė et al., 2019	CCP RF; low pressure (200 Pa); air	Sunflower		growth parameters, metabolism	macroscopic and molecular
Šerá et al., 2019	DBD; atm pressure; air	Pine		growth parameters, disinfection	Macroscopic
Cui et al., 2019	DBD; atm pressure; air	Arabidopsis		growth parameters, metabolism	macroscopic and molecular
Liu et al., 2019	DBD jet; atm pressure; N_2, O_2, air	Radish, mung bean, wheat, tomato, lettuce, mustard, Dianthus and sticky bean		growth parameters	Macroscopic
Los et al., 2019	DBD; atm pressure; air	Wheat		seed coat modification, growth parameters, metabolism	macroscopic and molecular
Moghanloo et al., 2019	DBD; atm pressure; Ar	Astragalus frida		growth parameters, metabolism	macroscopic and molecular
Pérez-Pizá et al., 2019	needle to plane DBD; atm pressure; N_2, O_2	Soybean		seed coat modification, growth parameters, metabolism	macroscopic and molecular

Citation	Plasma	Seed type	Microbe	Main findings	Scale
Babajani et al., 2019	DBD; atm pressure; Ar	Melissa officinalis		growth parameters, metabolism	macroscopic and molecular
Lotfy et al., 2019	plasma jet; atm pressure; N_2	Wheat		seed coat modification (water uptake), growth parameters	macroscopic
Bafoil et al., 2019	DBD; atm pressure; air	Arabidopsis		seed coat modification, growth parameters	macroscopic, microscopic?, and molecular
Singh et al., 2019	RF; low pressure; (0.40 mbar) O_2 and Ar	Basil		growth parameters, metabolism	macroscopic and molecular
Iqbal et al., 2019	not clear; low pressure; Ar	Wheat		seed coat modification, growth parameters, disease resistance	macroscopic and molecular
Islam et al., 2019	DBD; low pressure (10 Torr); air, Ar, O_2	Rapeseed, mustard		growth parameters, metabolism	macroscopic and molecular
Kabir et al., 2019	DBD; low pressure (10 Torr); air, Ar, O_2	Wheat		seed coat modification, growth parameters, metabolism	macroscopic and molecular
Afsheen et al., 2019	RF capacitive; low pressure; Ar	Wheat	Beetle	seed coat modification, pest resistance	macroscopic
Lo Porto et al., 2019	RF; low pressure (800 mTorr); N_2, O_2	Asparagus		seed coat modification, growth parameters	macroscopic
Jiang et al., 2018	ICCP RF; low pressure (150 Pa); He	Tomato		growth parameters	macroscopic
Hosseini et al., 2018	CCP RF; low pressure; N_2	Artichoke		seed coat modification, growth parameters, metabolism	macroscopic, microscopic and molecular

Citation	Plasma	Seed type	Microbe	Main findings	Scale
Tounekti et al., 2018	DBD; atm pressure; He	Coffee and grape seeds		growth parameters	macroscopic
B. Zhang et al., 2018	CCP glow RF; low pressure (30-200 Pa); air, He	maize, peppers, wheat, soybeans, tomatoes, eggplants, pumpkins		growth parameters, metabolism	macroscopic and molecular
Khatami & Ahmadinia, 2018	gliding arc; atm pressure; air	Pea, Zucchini	Native microflora	growth parameters	macroscopic
Pawłat et al., 2018	DBD plasma jet; atm pressure; He and N_2	Thuringian Mallow		seed coat modification, growth parameters	macroscopic and microscopic
Rahman et al., 2018	DBD; low pressure (10 Torr); air, Ar, O_2	Wheat		seed coat modification, growth parameters, metabolism	macroscopic, microscopic and molecular
Pawlat et al., 2018	gliding arc; atm pressure; N_2	Thuringian Mallow		seed coat modification, growth parameters	macroscopic and microscopic
Hayashi et al., 2016	RF; low pressure (20-80 Pa); O_2, Ar	Arabidopsis, radish		growth parameters, metabolism	macroscopic and molecular
Matra, 2018	Plasma flashlight; atm pressure Ar, O_2	Sunflower		growth parameters	macroscopic
Bafoil et al., 2018	1) DBD; atm pressure; air 2) plasma jet; atm pressure; He	Arabidopsis		seed coat modification, growth parameters	macroscopic, microscopic
Măgureanu et al., 2018	DBD (fluidized); atm pressure; air	Tomato		growth parameters	macroscopic
Štěpánová et al., 2018	DBD; atm pressure; air	Cucumber and pepper		seed coat modification, growth parameters	macroscopic and microscopic

Citation	Plasma	Seed type	Microbe	Main findings	Scale
Iranbakhsh, Ardebili, et al., 2018	DBD; atm pressure; Ar	Wheat		growth parameters, metabolism, stress resistance	macroscopic and molecular
Iranbakhsh, Oraghi Ardebili, et al., 2018	DBD; atm pressure; Ar	Chili pepper		growth parameters, metabolism	macroscopic, microscopic and molecular
Pauzaite et al., 2018	CCP RF; low pressure (60 Pa); air	Norway spruce		growth parameters	macroscopic and molecular
Park et al., 2018	DBD; atm pressure; N_2 and air	Barley		seed coat modification, growth parameters, metabolism	macroscopic and molecular
J. Zhang et al., 2018	DBD; atm pressure; Ar	Soybean		growth parameters	macroscopic
Shapira et al., 2018	RF inductive; low pressure; air	Pepper and lentil		seed coat modification	microscopic
Lotfy, 2017b	plasma jet; atm pressure; N_2	Watermelon		seed coat modification, growth parameters	macroscopic
Wang et al., 2017	DBD; atm pressure; air, N_2	Cotton		seed coat modification	microscopic
Kim et al., 2017	corona discharge plasma jet; atm pressure; air	Broccoli	Native Aerobic bacteria, moulds and yeasts, *B. cereus*, *E. coli*, Salmonella spp.	growth parameters, metabolism	macroscopic and molecular
Mildažienė et al., 2017	CCP RF; low pressure (60 Pa); air	Purple coneflower		growth parameters	macroscopic and molecular
Li et al., 2017	DBD; atm pressure; air	Wheat		seed coat modification, growth parameters, metabolism	macroscopic, microscopic, molecular

Citation	Plasma	Seed type	Microbe	Main findings	Scale
Sera et al., 2017	1) gliding arc; atm pressure; humid air 2) microwave plasma discharge; low pressure (140 Pa); Ar, O_2	3 cultivars of hemp		growth parameters	macroscopic
Puligundla et al., 2017a	corona discharge plasma jet; atm pressure; air	Rapeseed	Native	growth parameters, metabolism	macroscopic and molecular
Puligundla et al., 2017b	corona discharge plasma jet; atm pressure air	Radish	Native	growth parameters, metabolism	macroscopic and molecular
Gómez-Ramírez et al., 2017	1) DBD; low pressure (500 mbar); dry air 2) RF; low pressure (0.1 mbar); dry air	Quinoa		seed coat modification, growth parameters	macroscopic, microscopic
Guo et al., 2017	DBD; atm pressure; air	Wheat		seed coat modification, growth parameters, metabolism, stress resistance	macroscopic, microscopic, molecular
J. J. Zhang et al., 2017	needle to plane DBD; atm pressure; Ar	Soybean		growth parameters, metabolism	macroscopic, molecular
da Silva et al., 2017	DBD; atm pressure; air	Mimosa		seed coat modification, growth parameters	macroscopic and microscopic
Meng et al., 2017	DBD; atm pressure; air, Ar, O_2, N_2	Wheat		seed coat modification, growth parameters, metabolism	macroscopic, microscopic, molecular
Nalwa et al., 2017	glow discharge; low pressure (0.2 mbar); O_2	Bell pepper		seed coat modification, growth parameters,	macroscopic, microscopic
Junior et al., 2016	plasma jet DBD; atm pressure; He	Mulungu		seed coat modification, growth parameters	macroscopic, microscopic

Citation	Plasma	Seed type	Microbe	Main findings	Scale
Zahoranová et al., 2016	DBD; atm pressure; air	Wheat	Native	seed coat modification, growth parameters	macroscopic
Zhou et al., 2016	plasma jet array; atm pressure; He, N_2, air, O_2	Mung bean		seed coat modification, growth parameters, metabolism	macroscopic, microscopic, molecular
Khamsen et al., 2016	hybrid microcorona discharge; atm pressure; air, Ar;	Rice	Native fungi	seed coat modification, growth parameters	macroscopic and microscopic
L. Li et al., 2016	CCP RF; low pressure (150 Pa); He	Peanut		seed coat modification, growth parameters	macroscopic
Gholami et al., 2016	CCP RF; low pressure; air	Ajwain		seed coat modification, growth parameters	macroscopic
Matra, 2016	plasma flashlight; atm pressure; Ar	Radish		growth parameters	macroscopic
Sarinont et al., 2016	DBD; atm pressure; air, O_2, NO, He, Ar, N_2	Radish		growth parameters	macroscopic
Dobrin et al., 2015	DBD; atm pressure; air	Wheat		seed coat modification, growth parameters	macroscopic
Stolárik et al., 2015	DBD; atm pressure; air	Pea		seed coat modification, growth parameters, metabolism	macroscopic, microscopic, molecular
Munkhuu et al., 2015	not clear; not clear; not clear	Clover		growth parameters	macroscopic
Ji et al., 2015	1) DBD; atm pressure; Ar, N_2, air 2) microwave plasma torch for NO; N_2, O_2	Coriander		seed coat modification, growth parameters	macroscopic

Citation	Plasma	Seed type	Microbe	Main findings	Scale
Kadowaki et al., 2014	DBD; atm pressure; air	Arabidopsis		growth parameters	macroscopic

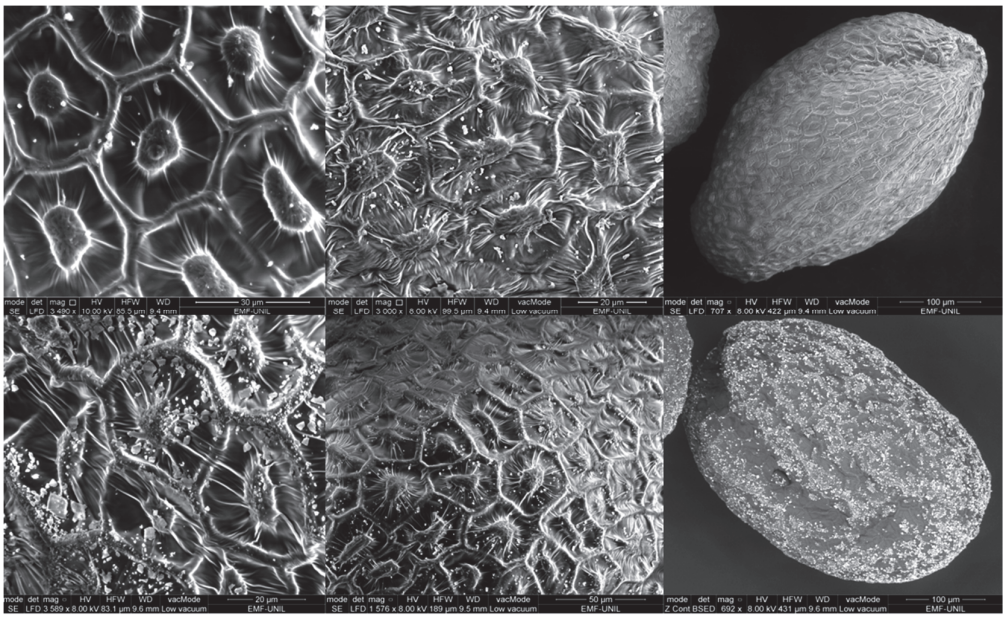

Figure S1. SEM images of *Arabidopsis* seeds (top row) treated for 10 min at 3.4 kV with the nanopulse powered SDBD4 [30°C] and (bottom row) treated for 10 min at 7.8 kV with the AC powered SDBD4 [80°C]. Both conditions lead to high levels of deposition and/or erosion.

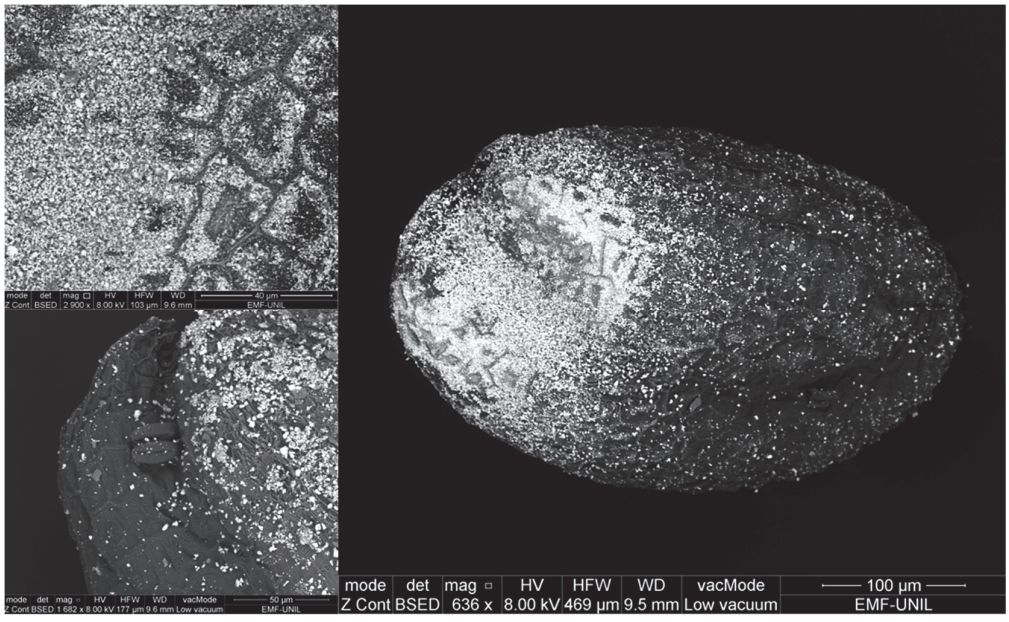

Figure S2. SEM images of *Arabidopsis* seeds treated for 10 min at 7.8 kV with the AC powered SDBD4. Severe erosion and high levels of debris are visible on the seed surface.

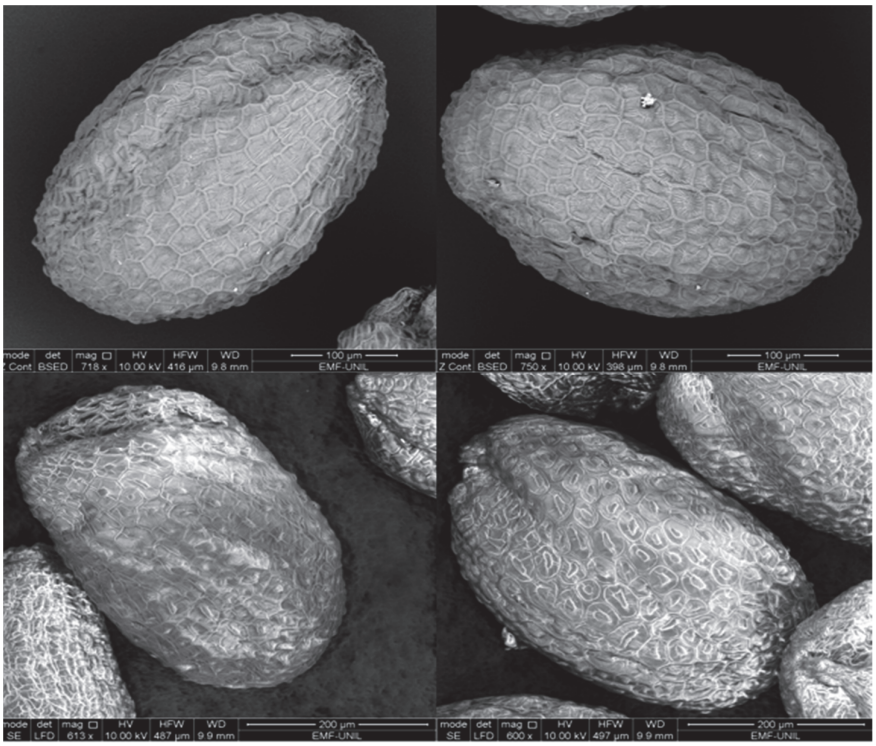

Figure S3. SEM images of *Arabidopsis* seeds (top row) treated for 30 s using the honeycomb SDBD powered by the manufacturer's power supply [<40°C] and (bottom row) treated for 3 min using the honeycomb SDBD powered by the manufacturer's power supply [65°C]. Little deposition is visible with both treatment times, but there may be visible electrostatic seed charging after 3 minutes.

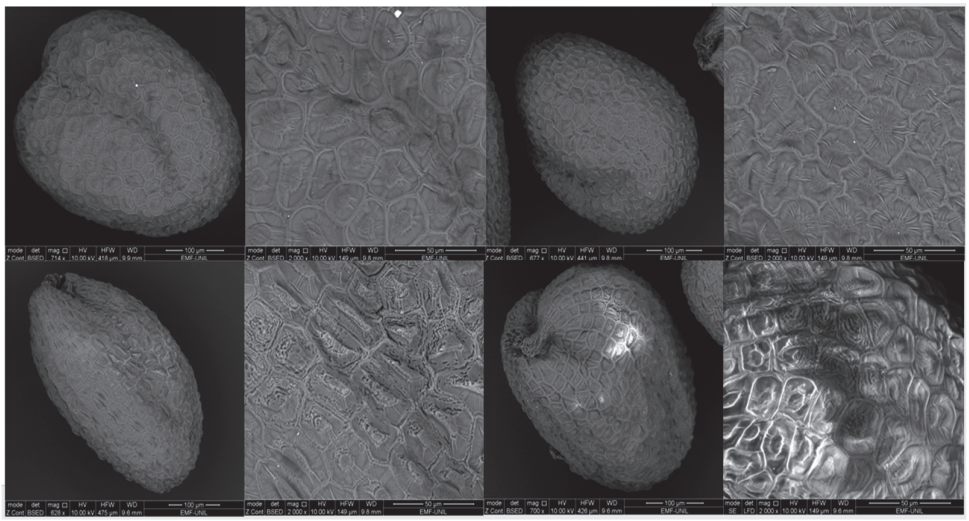

Figure S4. SEM images of *Arabidopsis* seeds (top row) treated for 30 s using the stripes SDBD powered by manufacturer's power supply and (bottom row) treated for 3 min using the stripes SDBD powered by the manufacturer's power supply. Little deposition is visible, however, erosion is visible and only after a 3 min treatment with the stripes SDBD.

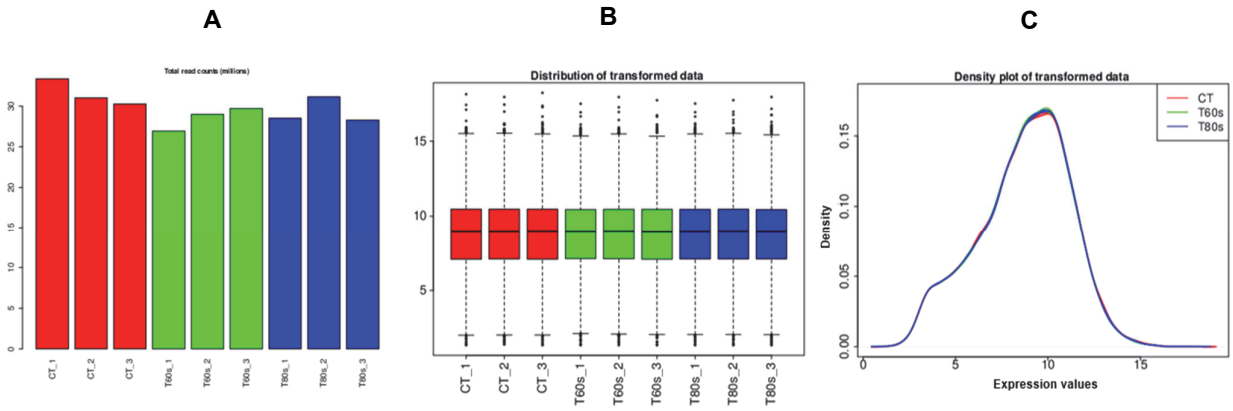

Figure S1. RNA quality control for untreated, control, and 60 s or 80 s plasma-treated samples. (A) Genes retained in each sample (B) normalization of samples (C) density plot to demonstrate that profiles are similar in order to proceed with analysis.

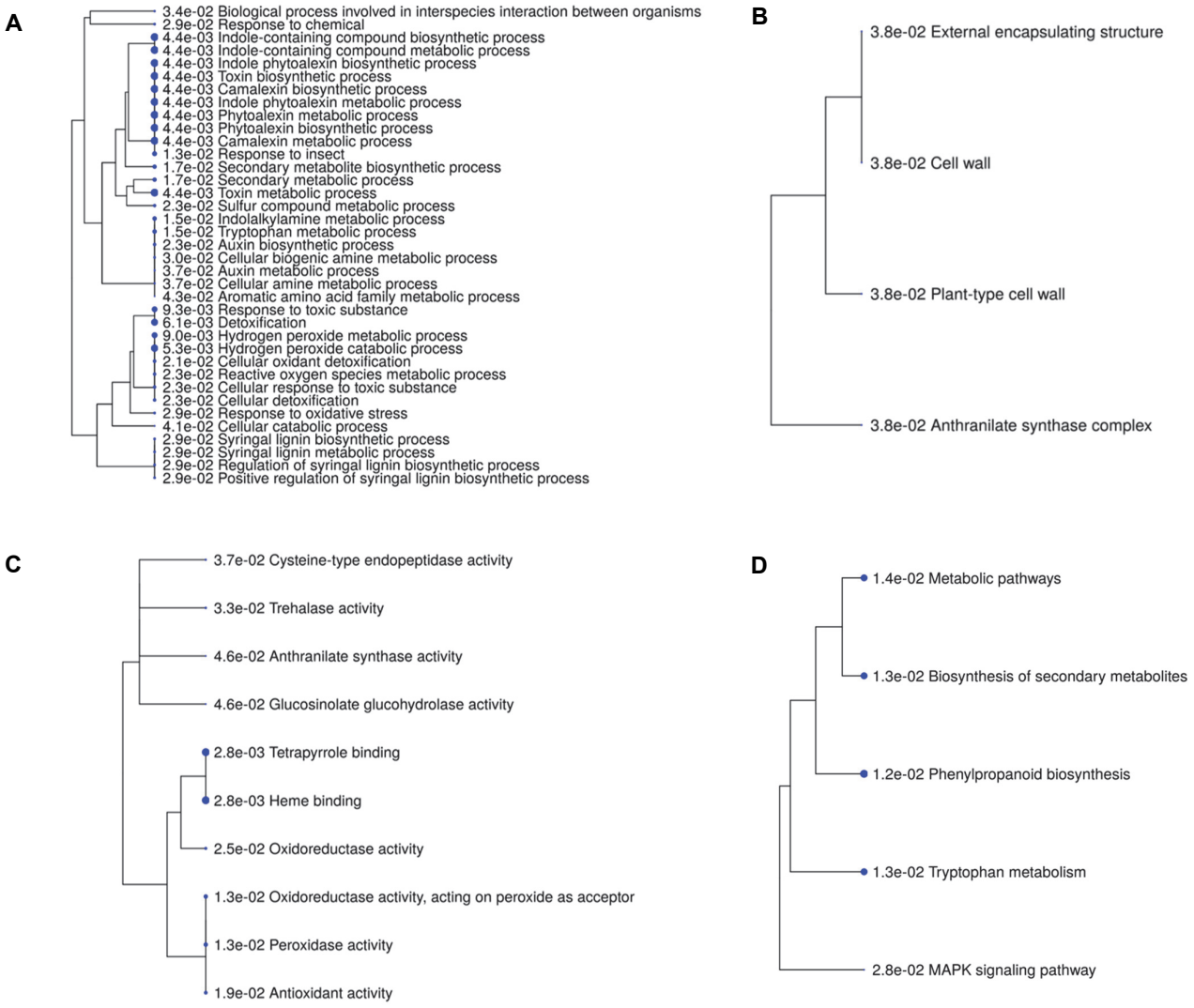

Figure S2. Hierarchical clustering trees for the upregulated genes after 60 s plasma treatment, which summarize the correlation among significant pathways within GO categories (A) biological process (B) cellular component (C) molecular function, and (D) KEGG pathway. Pathways with many shared genes are clustered together. Bigger dots indicate more significant P-values.

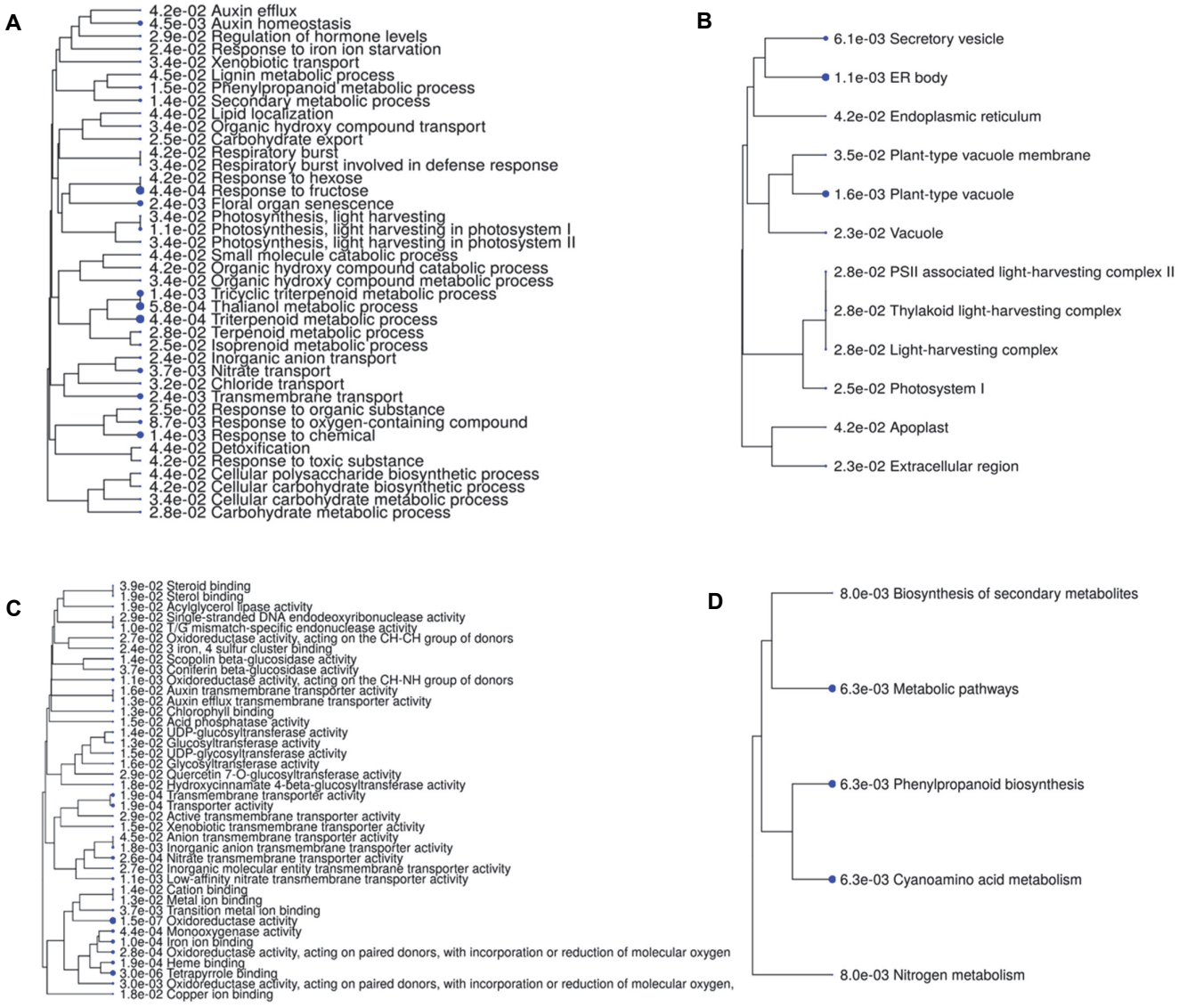

Figure S3. Hierarchical clustering trees for the downregulated genes after 60 s plasma treatment, which summarize the correlation among significant pathways within GO categories (A) biological process (B) cellular component (C) molecular function, and (D) KEGG pathway. Pathways with many shared genes are clustered together. Bigger dots indicate more significant P-values.

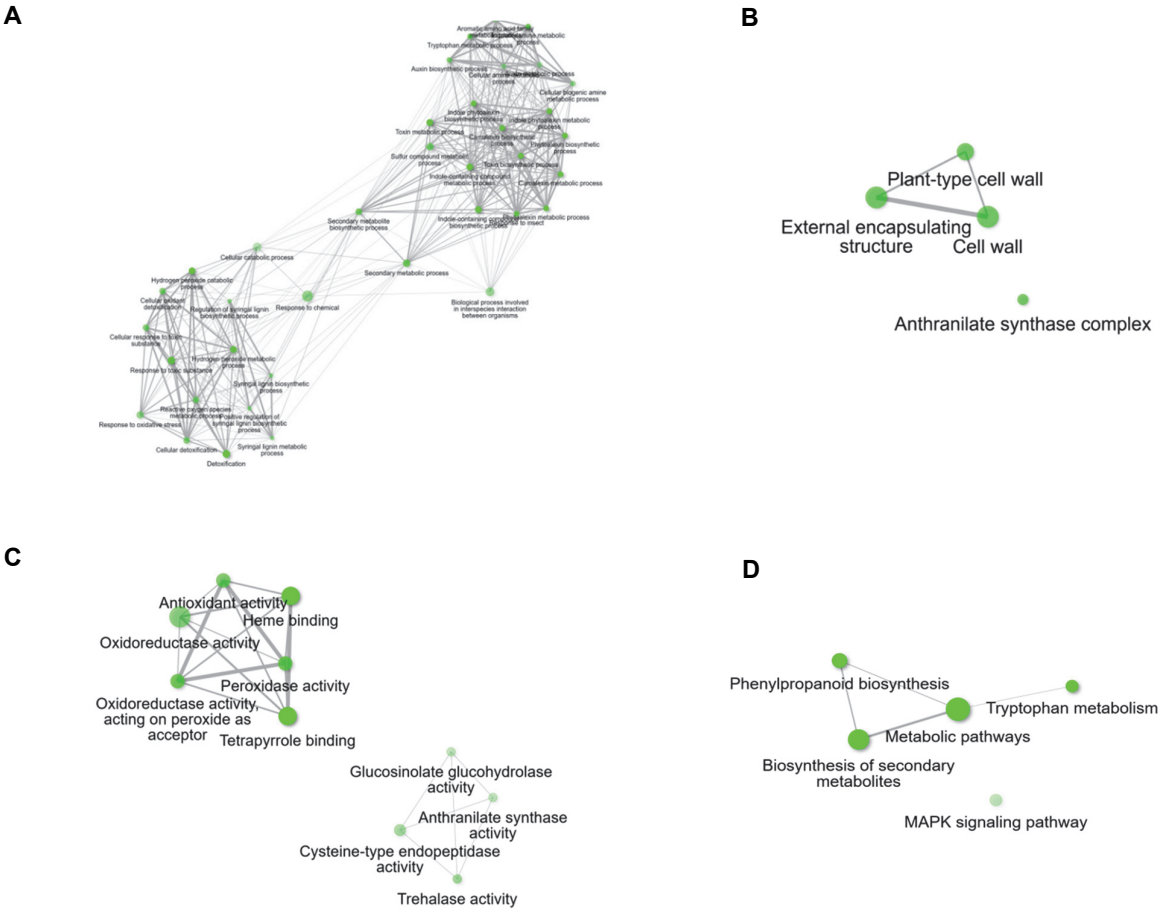

Figure S4. Gene upregulation after 60 s plasma treatment. Network maps show the relationship between enriched pathways in plasma-treated seedlings. From top to bottom, GO categories are in order: (A) biological process, (B) cellular component, (C) molecular function, and (D) KEGG pathway. Two pathways (nodes) are connected if they share 20% or more genes. Darker nodes are more significantly enriched gene sets. Bigger nodes represent larger gene sets. Thicker edges represent more overlapped genes.

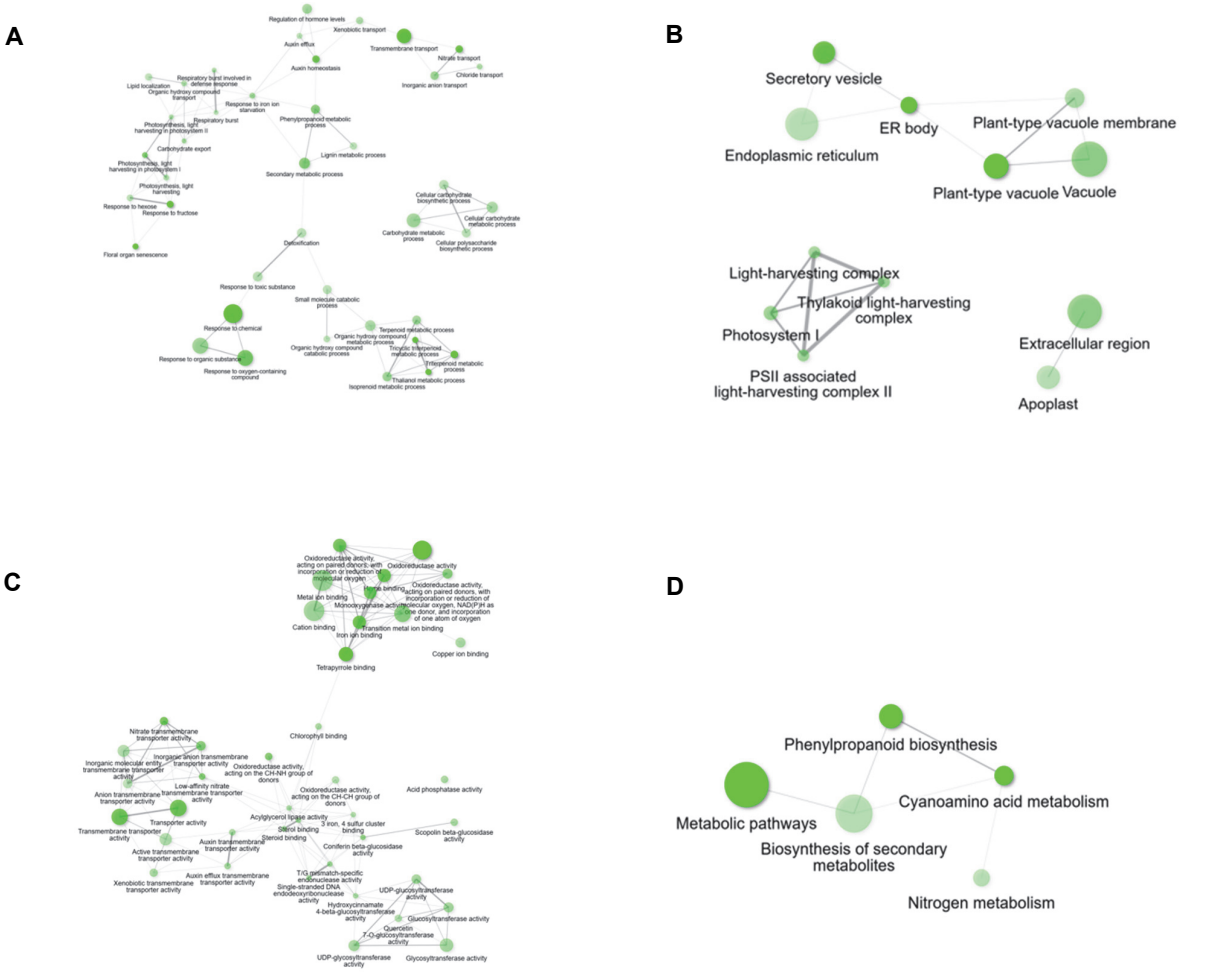

Figure S5. Gene downregulation after 60 s plasma treatment. Network maps show the relationship between enriched pathways in plasma-treated seedlings. From top to bottom, GO categories are in order: (A) biological process, (B) cellular component, (C) molecular function, and (D) KEGG pathway. Two pathways (nodes) are connected if they share 20% or more genes. Darker nodes are more significantly enriched gene sets. Bigger nodes represent larger gene sets. Thicker edges represent more overlapped genes.

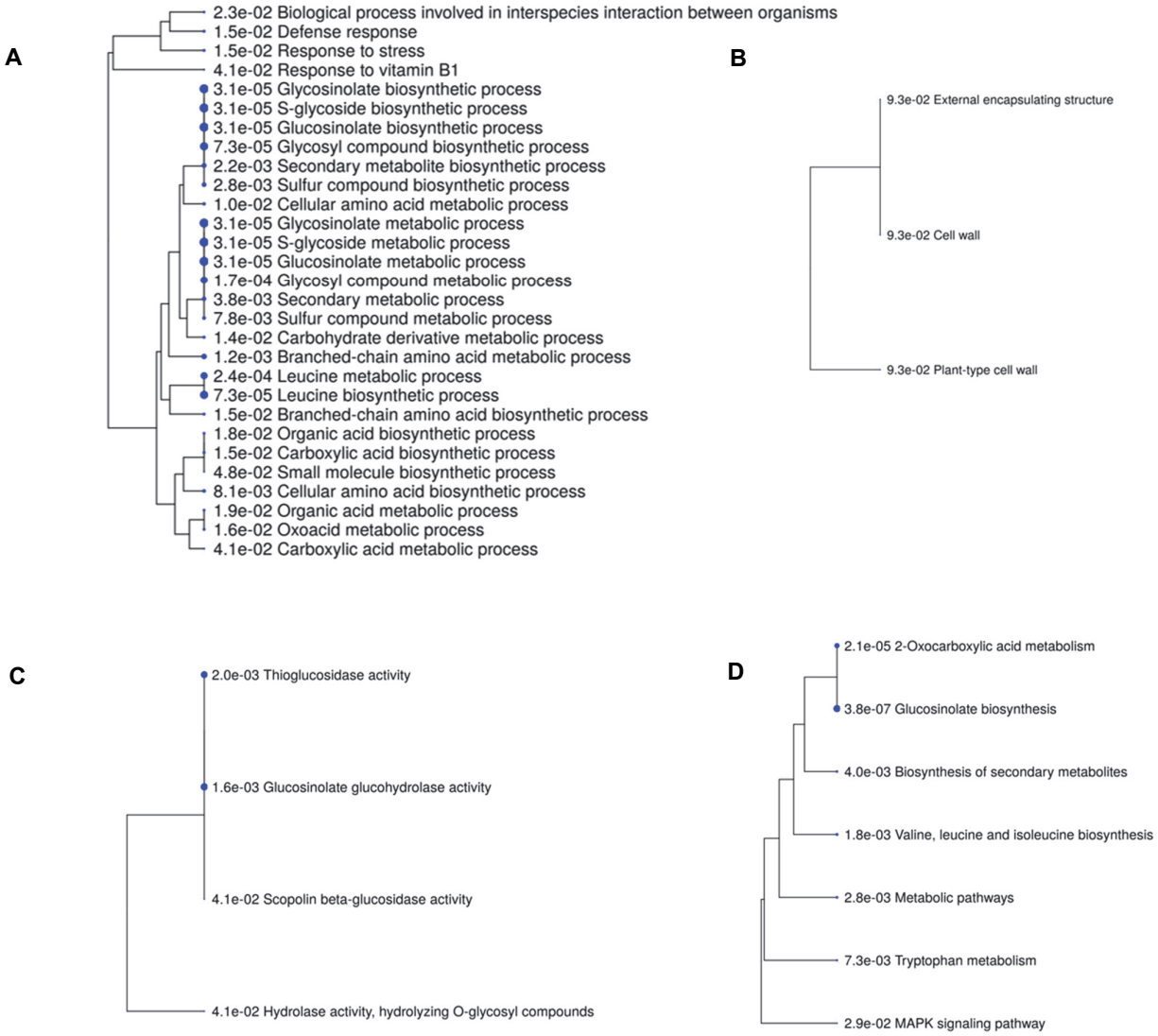

Figure S6. Hierarchical clustering trees for the upregulated genes after 80 s plasma treatment, which summarize the correlation among significant pathways within GO categories (A) biological process (B) cellular component (C) molecular function, and (D) KEGG pathway. Pathways with many shared genes are clustered together. Bigger dots indicate more significant P-values.

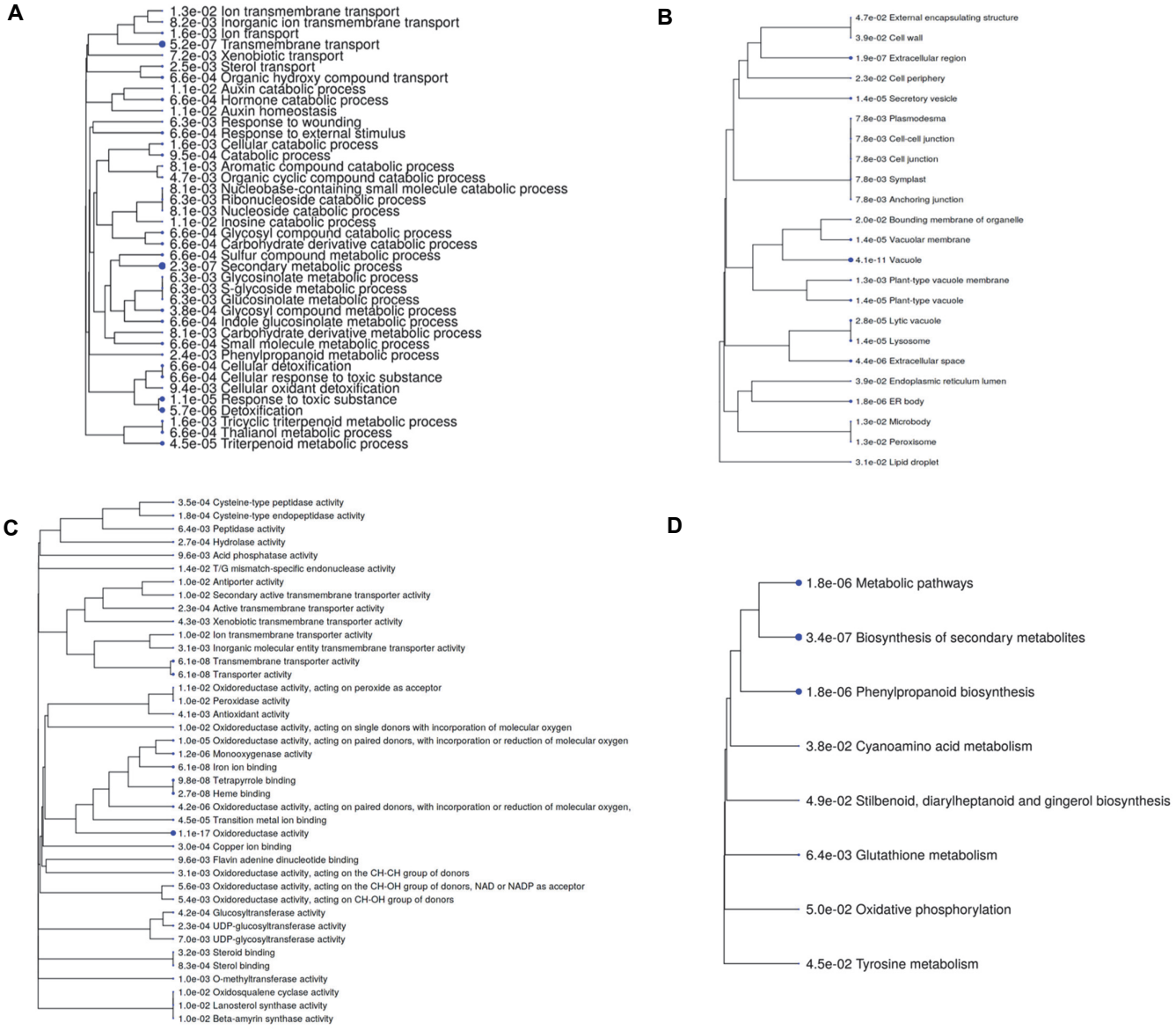

Figure S7. Hierarchical clustering trees for the downregulated genes after 80 s plasma treatment, which summarize the correlation among significant pathways within GO categories (A) biological process (B) cellular component (C) molecular function, and (D) KEGG pathway. Pathways with many shared genes are clustered together. Bigger dots indicate more significant P-values.

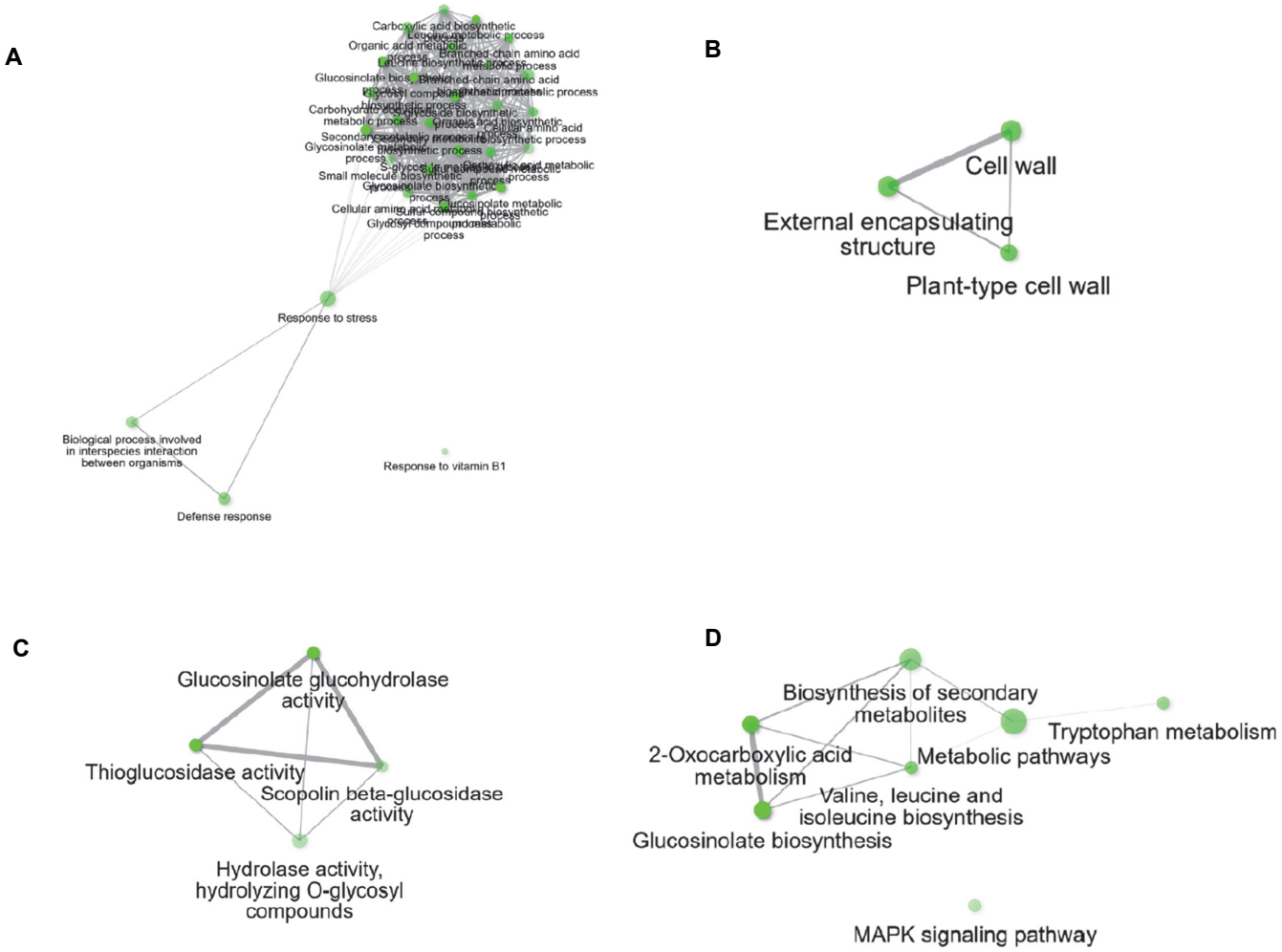

Figure S8. Gene upregulation after 80 s plasma treatment. From top to bottom, GO categories are in the following order: (A) biological process, (B) cellular component, (C) molecular function, and (D) KEGG pathway. Network maps show the relationship between enriched pathways in plasma-treated seedlings. Two pathways (nodes) are connected if they share 20% or more genes. Darker nodes are more significantly enriched gene sets. Bigger nodes represent larger gene sets. Thicker edges represent more overlapped genes.

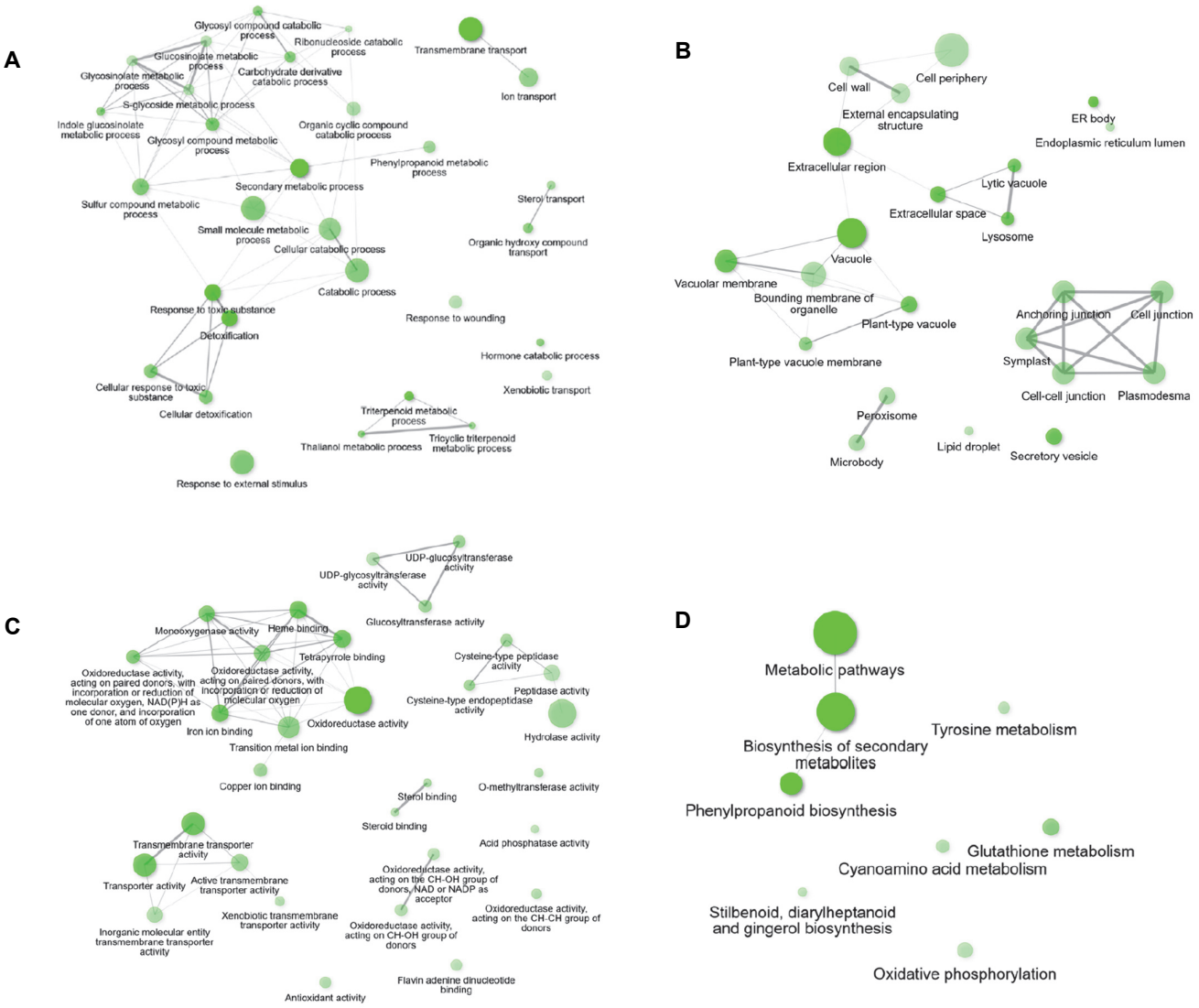

Figure S9. Gene downregulation after 80 s plasma treatment. From top to bottom, GO categories are in the following order: (A) biological process, (B) cellular component, (C) molecular function, and (D) KEGG pathway. Network maps show the relationship between enriched pathways in plasma-treated seedlings. Two pathways (nodes) are connected if they share 20% or more genes. Darker nodes are more significantly enriched gene sets. Bigger nodes represent larger gene sets. Thicker edges represent more overlapped genes.

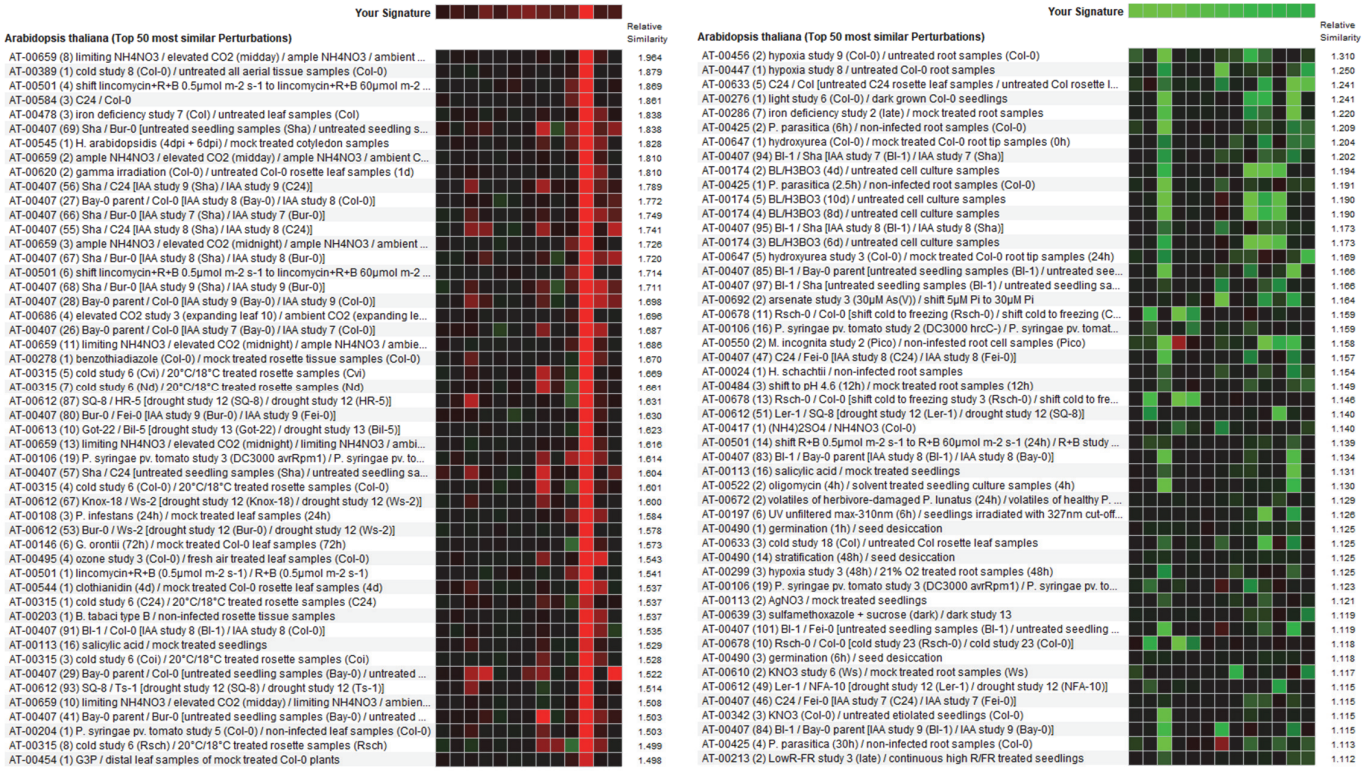

Figure S10. Gene perturbuation study using Genevestigator. Gene signature for 80 s plasma-treated 6-day-old seedlings was investigated for (left) upregulated genes and (right) downregulated genes. Upregulated genes overlap with cold, iron deficiency, gamma irradiation, IAA (auxin), linomycin, drought, *P. syringae*, elevated CO_2, ozone, and salicylic acid. Presence of PR1 is the strongest, however, it is widespread and common to many stresses. Downregulated genes overlap with hypoxia, light, iron deficiency, *P. syringe*, pH, salicylic acid, oligomycin, *P. lunatus*, UV, cold, stratification, KNO_3-$AgNO_3$ with the strongest gene from the signature as Cytochrome P450.

Table S1. Number of NGS-RNA-seq reads before and after quality check on the raw data for 60 s and 80 s.

Sample name	Raw reads	Trimmed reads	Mean GC	Condition
control 1	37031768	34794479	45	control
control 2	33884449	32507959	45	control
control 3	33288964	31830410	45	control
60 s 1	29560591	28386155	45	60 s
60 s 2	31755491	30348719	45	60 s
60 s 3	32244438	31058363	45	60 s
80 s 1	31068712	29937596	45	80 s
80 s 2	33745593	32428165	45	80 s
80 s 3	31108401	29729217	45	80 s

Table S2. Number of clean reads mapped against *A. thaliana* genome for 60 s and 80 s.

Sample	Input reads	Unique	Multi	Unmapped	Mismatch ratio	Assigned GTF
control 1	34794479	33792317 (97.1198%)	555033 (1.59518%)	447129 (1.28506%)	0.06%	33379423 (95.9331%)
control 2	32507959	31460285 (96.7772%)	672683 (2.06929%)	374991 (1.15354%)	0.06%	31023336 (95.433%)
control 3	31830410	30686662 (96.4067%)	681168 (2.13999%)	462580 (1.45326%)	0.06%	30273545 (95.1089%)
60 s 1	28386155	27435554 (96.6512%)	679223 (2.3928%)	271378 (0.956022%)	0.06%	26931867 (94.8768%)
60 s 2	30348719	29391853 (96.8471%)	567223 (1.86902%)	389643 (1.28389%)	0.06%	29009927 (95.5886%)
60 s 3	31058363	30157945 (97.1009%)	587937 (1.89301%)	312481 (1.00611%)	0.06%	29726503 (95.7118%)
80 s 1	29937596	28988426 (96.8295%)	662731 (2.21371%)	286439 (0.956787%)	0.06%	28514100 (95.2451%)
80 s 2	32428165	31571882 (97.3594%)	522820 (1.61224%)	333463 (1.02831%)	0.06%	31166918 (96.1106%)
80 s 3	29729217	28722931 (96.6152%)	627181 (2.10965%)	379105 (1.27519%)	0.06%	28292247 (95.1665%)

Table S3. Comparison with Cui et al. 2021 paper.

Variable	Cui et al. paper	Waskow et al. paper
Plasma device	Hexagonal mesh SDBD	Grid of stripes SDBD
Plasma device materials	Copper and FR4	Metal paste on alumina ceramic
Plasma-seed gap distance	5 mm	3.7 mm
Feed gas	Ambient air	Synthetic air (80:20 N_2:O_2)
	No flow given	2 L/min controlled flow
	RH 65%	RH 2%
Operating parameters	8 kHz, 5 kV	10 kHz, 8 kV,
	2.5 W consumption	2-3 W consumption,
	0.055 W/cm^2	0.03 - 0.08 W/cm^2
Power supply	Continuous sinewave	AC 10% duty cycle
		modulated at 500 Hz
Temperature	28.5°C	31.8°C
Seed storage and handling	vernalized and stored with silica gel,	stored in a locked Eppendorf tube dry conditions at room temperature,
	sterilized seeds,	not sterilized,
	used ½ MS agar plates	used water agar
Plasma treatment procedure	grew seedlings until 4 days,	plasma treated dry seeds,
	transplanted to water,	planted on water agar,
	plasma treated,	
	planted on ½ MS agar	
Seed sample size	35 seeds	30 seeds
Light cycle	23°C, 16 h light	23°C, continuous light
Plasma treatment time	60 s	60 s and 80 s
RNA extraction	48 hours after plasma treatment	144 hours after plasma treatment
	extracted from 6-day old seedlings	extracted from 6-day-old seedlings

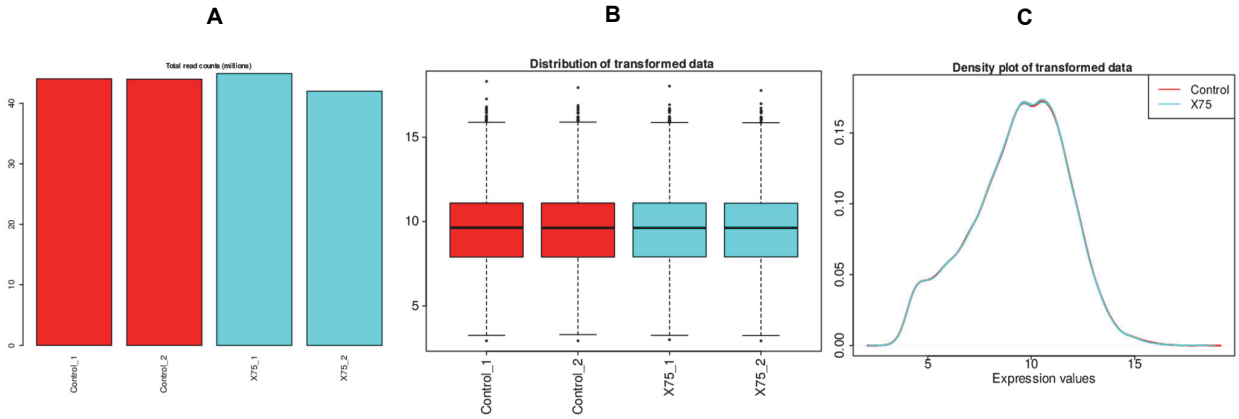

Figure S11. RNA quality control for untreated and 60 s plasma-treated samples (A) Genes retained in each sample (B) normalization of samples (C) density plot to demonstrate that profiles are similar to proceed with analysis.

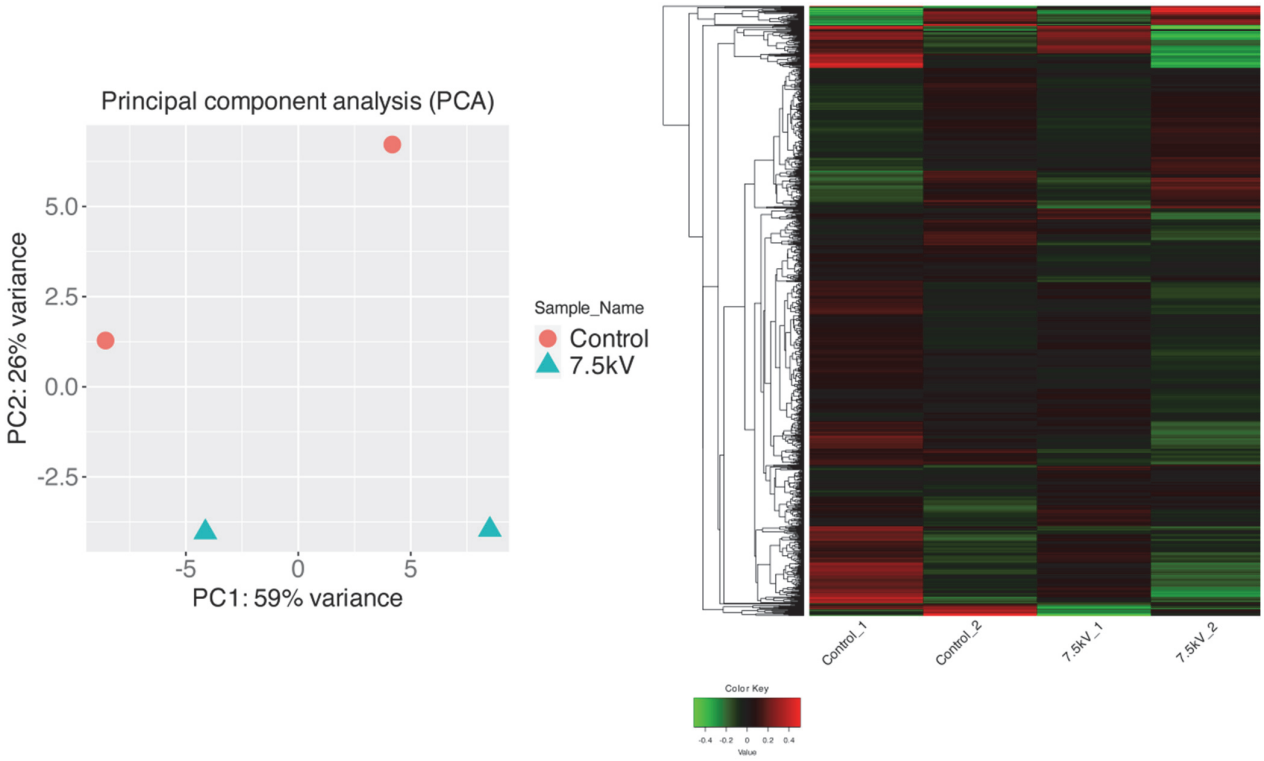

Figure S12. (Left) Principal Component Analysis (PCA) conducted on the normalized gene expression values of the 7-day-old samples at 7.5 kV. X- and Y-axes show PC1 and PC2, respectively, with the amount of variance contained in each component, which 59% and 26%. Each point in the plot represents a biological replicate, representing 30 seedlings, with a total of 4 biological replicates in the plot. Symbols of the same colors are replicates of the same experimental group where orange represents the control which are untreated A.thaliana seeds grown into seedlings and blue represents 7.5 kV plasma-treated *A.thaliana* seeds grown into seedlings. (Right) Heat map of the expression patterns (Z-scaled reads per kilobase of exon per million reads mapped (RPKM)) of the full transcriptome for 7-day-old samples at 7.5 kV. Hierarchical clustering of the relative expression profile of the top 2000 variable genes selected based on the lowest standard deviation using Euclidean distance. Individual samples are shown in columns, and genes in rows. The left vertical axis shows clusters of genes. The color scale represents the relative read count of genes: green indicates low relative read counts; red indicates high relative read counts; black indicates zero (no change).

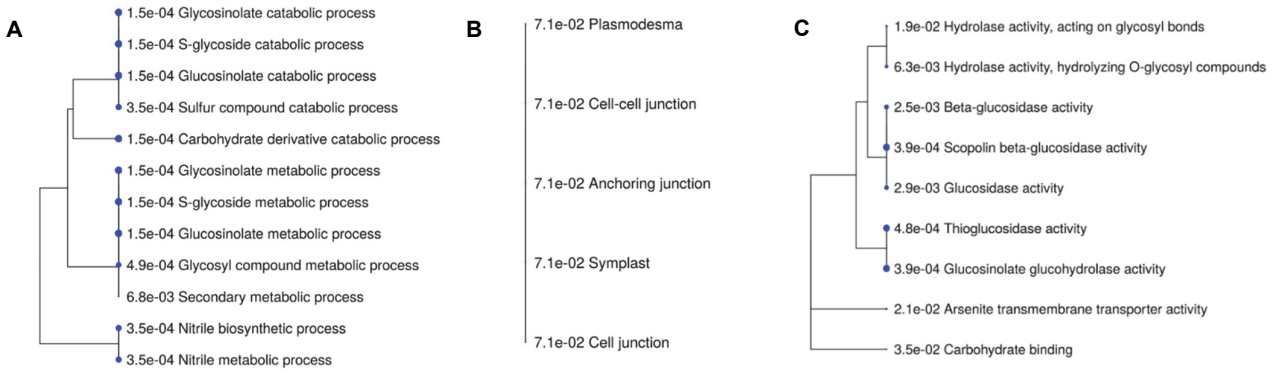

Figure S13. Hierarchical clustering trees for the upregulated genes after 7.5 kV plasma treatment in 7-day-old seedlings, which summarize the correlation among significant pathways within GO categories (A) biological process (B) cellular component, and (C) molecular function. Pathways with many shared genes are clustered together. Bigger dots indicate more significant P-values.

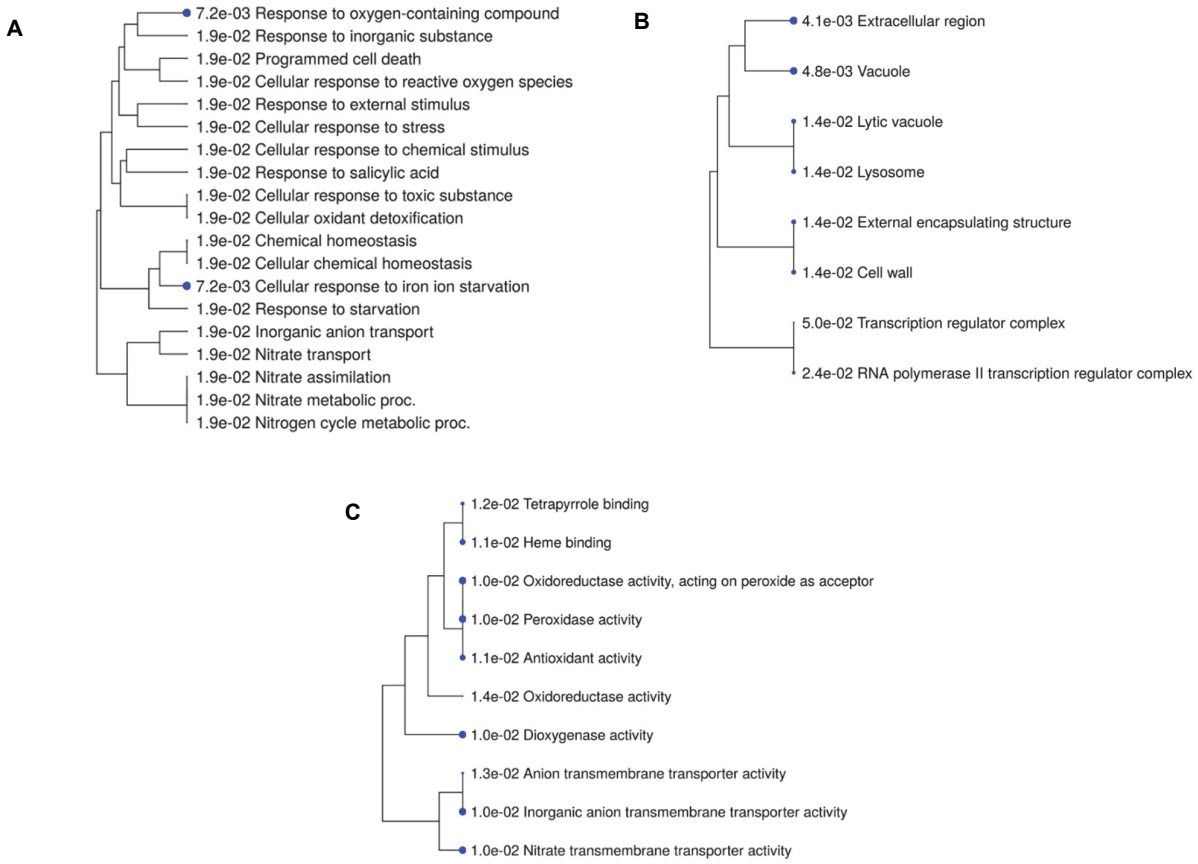

Figure S14. Hierarchical clustering trees for the downregulated genes after 7.5 kV plasma treatment in 7-day-old seedlings, which summarize the correlation among significant pathways within GO categories (A) biological process (B) cellular component, and (C) molecular function. Pathways with many shared genes are clustered together. Bigger dots indicate more significant P-values.

Table S4. Number of NGS-RNA-seq reads before and after quality check on the raw sequencing data for 7-day-old seedlings treated with 7.5 kV plasma.

Sample name	Raw reads	Trimmed reads	Mean GC	Condition
control 1	46625605	45466669	44	control
control 2	46523629	45454673	44	Control
control 3	37351249	36364586	44	Control
7.5kV_1	47453047	46351468	44	7.5kV
7.5kV_2	44547099	43457657	44	7.5kV
7.5kV_3	49914543	48712288	44	7.5kV

Table S5. Number of clean reads mapped against *A. thaliana* genome for 7-day-old seedlings treated with 7.5 kV plasma.

Sample	Input reads	Unique	Multi	Unmapped	Mismatch ratio	Assigned GTF
control 1	45466669	44491934 (97.8562%)	590239 (1.29818%)	384496 (0.845666%)	0.08%	44055690 (96.8967%)
control 2	45454673	44550817 (98.0115%)	631984 (1.39036%)	271872 (0.598117%)	0.08%	43983665 (96.7638%)
control 3	36364586	35579837 (97.842%)	502708 (1.38241%)	282041 (0.775592%)	0.08%	35238242 (96.9026%)
7.5kV_1	46351468	45439262 (98.032%)	601432 (1.29755%)	310774 (0.670473%)	0.08%	44940088 (96.955%)
7.5kV_2	43457657	42405516 (97.5789%)	660139 (1.51904%)	392002 (0.902032%)	0.08%	42010580 (96.6701%)
7.5kV_3	48712288	47418251 (97.3435%)	746002 (1.53145%)	548035 (1.12504%)	0.08%	47118110 (96.7274%)

Table S6. List of DEGs using a 60 s, 7.5 kV plasma treatment with (left) upregulated (right) downregulated genes with the corresponding fold changes.

Upregulated genes			Downregulated genes		
TAIR ID	**Gene description**	**Log2FC**	**TAIR ID**	**Gene description**	**Log2FC**
AT5G62340	plant invertase/pectin methylesterase inhibitor	2.143896252	**AT3G28345**	encodes an ATP-binding cassette (ABC) transporter	-0.402876008
AT1G26240	proline-rich extensin-like family protein	1.615083811	**AT1G66200**	encodes a cytosolic glutamate synthetase	-0.410588965
AT2G43610	chitinase family protein	1.413598141	**AT3G01420**	encodes an alpha-dioxygenase involved in protection against oxidative stress and cell death	-0.447290809
AT1G66270	BGLU21 encodes a beta-glucosidase that has a high level of activity against the naturally occurring secondary metabolite scopolin.	1.3941619	**AT3G13610**	encodes a Fe(II)- and 2-oxoglutarate-dependent dioxygenase family gene F6'H1	-0.481556848
AT1G21890	nodulin MtN21-like transporter family protein	1.342477103	**AT4G01950**	putative sn-glycerol-3-phosphate 2-O-acyltransferase	-0.488511631
AT2G45050	encodes a member of the GATA factor family of zinc finger transcription factors.	1.324256008	**AT5G24030**	encodes a protein with ten predicted transmembrane helices. The SLAH3 protein has similarity to the SLAC1 protein involved in ion homeostasis	-0.513357358
AT2G25980	mannose-binding lectin superfamily protein	1.15095165	**AT4G30280**	encodes a xyloglucan endotransglucosylase/hydrolase	-0.516919325
AT4G15160	protease inhibitor/seed storage/lipid transfer protein (LTP) family	1.095722356	**AT3G12700**	encodes an aspartic protease has an important regulatory function in chloroplasts that not only influences photosynthetic carbon metabolism but also plastid and nuclear gene expression	-0.519596719
AT1G74500	encodes a basic helix/loop/helix transcription factor that acts downstream of MP in root initiation	1.094756193	**AT3G49120**	class III peroxidase Perx34	-0.525077223
AT1G67750	pectate lyase family protein	1.035072465	**AT1G14130**	DAO1 is an IAA oxidase expressed in many different plant parts	-0.534107928
AT2G23050	a member of the NPY gene family - involved in auxin-mediated organogenesis.	1.00036748	**AT1G51420**	sucrose-phosphatase 1	-0.544192088
AT5G19340	hypothetical protein	0.894282644	**AT4G36880**	cysteine proteinase1	-0.553001556
AT3G16390	encodes a nitrile-specifier protein	0.868383943	**AT4G19810**	encodes a Class V chitinase that is a part of glycoside hydrolase family 18 based on CAZy groupings	-0.569442323
AT5G19520	mechanosensitive channel of small conductance-like	0.857801365	**AT5G03355**	cysteine/histidine-rich C1 domain protein	-0.656741451
AT2G34020	calcium-binding EF-hand family protein	0.840951663	**AT1G72140**	tonoplast localized pH dependent, low affinity nitrogen transporter	-0.667274669
AT4G28650	encodes one of the two putative eLRR kinase closely related to PXY	0.807505367	**AT1G21910**	encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	-0.687711813
AT3G50640	hypothetical protein	0.766872726	**AT5G06720**	encodes a peroxidase with diverse roles in the wound response, flower development, and syncytium formation	-0.770210545
AT3G16410	encodes a nitrile-specifier protein	0.765286511	**AT1G64940**	member of CYP89A	-0.799393456

AT5G62210	embryo-specific protein 3	0.764303863	**AT5G22355**	cysteine/Histidine-rich C1 domain family protein	-0.824153639
AT1G12080	vacuolar calcium-binding protein-like protein	0.744265471	**AT3G56980**	encodes a member of the basic helix-loop-helix transcription factor protein	-0.848029051
AT3G24240	RGFR1 is a leucine--rich repeat receptor kinase that, together with RGFR2 and RGFR3, binds ROOT GROWTH FACTORS and is required for establishing the gradient of PLETHORA1 and PLETHORA2 essential for proper root growth and development.	0.712304397	**AT1G77640**	encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	-0.875650026
AT1G47600	encodes a myrosinase	0.698065241	**AT4G21680**	encodes a nitrate transporter (NRT1.8)	-0.957209744
AT1G51470	encodes a myrosinase	0.672534672	**AT4G38420**	SKU5 similar 9	-1.033874204
AT4G19030	an aquaporin	0.640964946	**AT5G56080**	encodes a protein with nicotianamine synthase activity	-1.109636627
AT5G15230	encodes gibberellin-regulated protein GASA4	0.630414814	**AT3G24982**	receptor like protein 40	-1.170528146
AT5G02260	member of Alpha-Expansin Gene Family	0.589658285	**AT3G56970**	encodes a member of the basic helix-loop-helix transcription factor family protein	-1.180905902
AT3G12610	plays a role in DNA-damage repair/toleration	0.589518673	**AT3G21500**	encodes a protein that has very high sequence similarity to 1-deoxy-D-xylulose-5-phosphate synthase proteins but does not possess appreciable activity in vitro	-1.231018665
			AT3G02885	GASA5, is involved in the regulation of seedling thermotolerance.	-2.488621571
			AT5G45890	senescence-associated gene 12 (SAG12) encoding a cysteine protease influenced by cytokinin, auxin, and sugars.	-2.490121601

Chapter 7: Reproducibility

Table S1. Legend with a description for each variable in plasma-seed treatments.

Category	Examples of what is included
Plasma source	
electrode material	copper, aluminum, stainless steel
dielectric material	alumina, teflon, glass, kapton
Geometry	DBD, jet, corona
Pressure	atmospheric or low
Waveform	AC, DC, nanosecond
gas type	Ar, N_2, O_2, air
gas flow or static	L/min or injection
Configuration	enclosed, semi-open, open
Plasma-seed treatment	
distance	mm or cm away from plasma
Orientation	seeds on or under electrodes
voltage, current, frequency	kilovolts, amperes, kilohertz
treatment time	seconds to minutes
power or power density	Watts for treatment and power density when given dimensions
seed movement	shaking, rotating
temperature and humidity	incremental increase or max. temp reached; in open air or controlled
Seed type	
Seed pre-treatment	
source of seeds	market, institute, farm
Storage	none, cool or room temperature, dark or light
Preselection	healthy and uniform looking
sterilization prior to plasma	none, ethanol, sodium hypochlorite
imbibition	soaking in water
Stratification	synchronizing germination in moist, dark environment for few days prior to plasma treatment
Seed growth methodology	
post-retention time	waiting 24 hours after plasma before planting seeds into growth substrate
growth medium	agar, filter paper, sand, soil
growing conditions	incubator, box, climate chamber, greenhouse
light conditions	artificial with photon flux and wavelength spectra, natural, dark

Table S2. A collection of plasma-seed treatment papers.

Citation	Plasma source	Plasma-seed treatment	Seed type	Seed pre-treatment	Seed growth methodology
Information provided	geometry; configuration; materials; pressure; gas type; flow rate; waveform; frequency	distance; power; dimensions; treatment time; temperature		source; storage; preselection; sterilization; preparation	post-retention time; growth substrate; chamber; light conditions
Jiang et al., 2018	ICCP RF; enclosed; material not given; low pressure (150 Pa); He; 13.56 MHz	15 mm away (range within 1.5-10 cm); 60, 80, 100 W; 1200 mm x 180 mm x 20 mm; 15 s; 25ºC	Tomato Solanum lycopersicum L. cv. Shanghai 906	received from enterprise; bank; stored in fridge; not given; no sterilization mentioned; no soaking mentioned	sowed immediately; filter paper, in vermiculite and perlite 1:1, soil with fertilizer; light incubator, greenhouse; artificial?, natural light
Pauzaite et al., 2018	CCP RF; enclosed in stainless steel vacuum chamber; copper electrodes; low pressure (60 Pa); air; 5.28 MHz	20 mm gap but not specified; 120 mm diameter, 0.053m3 reactor; 0.35W/cm3; 2,5,7 min; <37ºC; water cooled electrodes	Norway spruce	received from bank; stored dry, dark for 6 months at 10ºC; preselection of seeds; not given; not given	waited 4 days; filter paper in Petri, peat seed pots; climate chamber, greenhouse, plant nursery, field; 16 h light*; 60% humidity, distilled water
Mildaziene et al., 2017	CCP RF; enclosed in stainless steel vacuum chamber; copper electrodes; low pressure (60 Pa); air; 5.28 MHz	20 mm gap but not specified; 120 mm diameter, 0.053m3 reactor; 0.35W/cm3; 2,5,7 min; <37ºC; water cooled electrodes	Purple coneflower	received from botanical garden; stored before in dry and dark for 6 months at 10ºC; preselected quality seeds; not given; cleaned but no details	not given; plastic cassette pH 5.5-6.5; greenhouse; 16 h light*
Kadowaki et al., 2014	DBD; enclosed in glass and acrylic, copper electrodes and glass dielectric, atm pressure; air; DC pulsed or AC; 5 kV-7 kV	static? depending on voltage 0.37-1.4 W – say 20-50J/cm3 for germination, used energy not treatment time	Arabidopsis	source not given; storage not given; preselection not given; no sterilization mentioned; no soaking before treatment	no post retention mentioned; Petri dish with water; Incubator; 16 h light
Khamsen et al., 2016	Hybrid microcorona discharge; enclosed in metal and glass chamber; metal tips; atm pressure; air, Ar; 2.5 L/min; 14 kVpp DC and HV AC sinusoidal source; 700 Hz	5 mm -1 cm range but using gap distance 7 mm, ~4.8 W 30 cm2; ~27ºC	Rice	source not given; seeds stored before at 5ºC for 9 months; preselection done; not given; not given	no post-retention mentioned; blotter paper = filter paper?; plant chamber; 8 h light 85% humidity
Hosseini et al., 2018	CCP RF; enclosed in stainless steel chamber 270 mm diameter; electrode 110 mm water cooled; low pressure; N2; 20 sccm; 13.56 MHz	on electrode; direct; reactor 270 mm diamater and 110 mm in diameter; 10 W; 3,10,15 min; no temp given	Artichoke	seeds from institute; not given; preselection done; not given; not given	not mentioned; filter paper; plant chamber; 12 h light

Citation	Plasma	Plasma-seed treatment	Seed type	Seed pre-treatment	Seed growth methodology
Sang Hye Ji et al., 2015	1) DBD; enclosed in glass and other layers with cooling system; SiO2 dielectric; atm pressure; Ar, N2, air; 1 L/min; AC; ~0.6 kV; ~9.8-12.5 mA; 15.4 kHz 2) microwave plasma torch for NO; enclosed; N2 10 L/min and O2 50-300 sccm; 2.45 GHz	1) 2 mm; no dimensions; ~7.5 W (from previous study); 30 s, 1 min, 3 min; no temp given 2) no distance given; no dimensions; 400 W; 5,10 min; no temp given	Coriander	not given; not given; not given; not given; water soaked seeds for 1 hr, 100 rpm	not mentioned; filter paper, vermiculite; plant chamber; 16 h light 50% humidity
Dawood, 2020	RF; enclosed in stainless steel chamber 25cm x 36 cm height; stainless steel electrodes and ceramic dielectric; low pressure; Ar; 13.56 MHz	gap 5 cm between electrodes; 100 W; 1,5,10,15 min; no temp given	Moringa	not given for any category	waited 24 h before sowing; filter paper in Petri dish; not given; dark
Iranbakhsh et al., 2020	DBD enclosed; copper electrodes and glass dielectric; atm pressure; Ar; 2 L/min; 8 kV AC; 13 kHz	gap 4 mm between dielectrics; 94.98 cm2; 80 W; 0.84 W cm2; 0, 40, 80 s; no temp given	Hemp	not given; not given; not given; not given; seeds soaked 6 h in water	not mentioned; filter paper or potted vermiculite and perlite 1:1, sup with Hoagland solution; container not given; light conditions not given
Kang et al., 2020	1) Arc discharge; enclosed in acryl container; tungsten electrodes; DC 10 kV 6,9,12 Hz 2) DBD; enclosed; Al2O3 dielectric, metal electrode; low and atm pressure; (0.6-1 atm); AC; 14.4 kHz; 8 kVp-p; 0.724 cm3	SDBD: 10 mm away; Container 60x85x50mm 51.7 W; 71.5 W/cm3; 10, 30 min; 80-121°C	Rice	not given; stored before dry at 4ºC not given; not given; not given	not mentioned; 0.6% water agar; growth chamber; 16 h light

Billah et al., 2020	DBD; enclosed in bell jar; glass as dielectric and stainless steel electrodes; low pressure (400 torr); air; AC sinusodial bipolar power; 5 kV; 4.5 kHz	60 mm between electrodes (seeds inside); 45 W; 20-180 s; 310 K; rotation of seeds	Black gram	seeds collected from institute; not given; random seed selection not given; not given; not given	not mentioned; filter paper; incubator; in the dark deionized
Koga et al., 2020	DBD; Open?; stainless steel electrode and ceramic dielectric; atm pressure?; air? AC; Voltage? 14.4 kHz	3 mm distance seeds sitting on glass; 3.05 cm2; 3 min	Radish	used seeds harvested in 2017 and 2018	not applicable; looked at seed coat colour and stable organic radicals
Ghasempour et al., 2020	DBD enclosed; copper electrodes and glass dielectric; radius 5.5 cm; atm pressure; Ar; 2 L/min; AC; 10 kV; 13 kHz	gap 4 mm between dielectrics; 80 W; 0.84 W cm2; 0, 30, 60, 90 s; temp not given	Catharanthus roseus	not given; not given; not given; soaked in water 24 h; sterilized with sodium hypochlorite, benomyl, ethanol	not mentioned; water in Petri dish then MS media supp. activated charcoal for seedlings; plant chamber; 16 h light and intensity given
Filatova et al., 2020	CCP RF; enclosed in stainless steel vacuum chamber; copper electrodes; 363 cm3; low pressure (200 Pa); air; 5.28 MHz	distance b/w electrodes 20 mm; 9 W; 0.025 W/cm3; 2,4,5,7 min; <37ºC	maize, wheat, lupine	seeds from institute; no other info given	not mentioned; filter paper or Petri dish; climate chamber; not given? follow up on ref 24-26 for details in protocol

Citation	Plasma	Plasma-seed treatment	Seed type	Seed pre-treatment	Seed growth methodology
Prakrajang et al., 2020	not given; not given; Ar; 3 L/min; DC pulse; 25 kHz	not given; 0.41-0.61 W; 15 s; no temp given	Chili pepper	no information given	not mentioned; soil in tray; greenhouse; not given
Kobayashi et al., 2020	DBD (based on image); enclosed in Petri dish; copper electrodes and glass dielectric; atm pressure; air; 6 kV; 20 kHz	5 mm distance; 7.4 W for 16 cm2; 0.4625 W/cm2; 2-20 s; ~25ºC	Arabidopsis (seedlings)	not given; not given; not given; not given; disinfected but not given protocol	not mentioned; ½ MS agar; illuminated incubator; 16 h light
Sidik et al., 2019	plasma jet/plume; open; stainless steel electrode and glass dielectric; atm pressure; helium; 6 L/min; AC; 20 kHz; 6 kV	10 mm away; power not given; 3, 5,10 min; temp not given	Corn and eggplant	no information given	not mentioned; soil; not given, tray then field; No light conditions given; 82% RH
Seddighinia et al., 2019	DBD enclosed; copper electrodes and glass dielectric; atm pressure; Ar; 2 L/min; AC; 10 kV; 13 kHz	4 mm gap between electrodes; 80 W; 94.98 cm2; 0.84 W/cm2; 60,120 s; 27-29ºC	Bitter melon	not given; not given; not given; soaked in water; disinfected with sodium hypochlorite	not mentioned; pots with peat and perlite (1:1 v/v) supp. with Hoagland solution; container not given; 16 h light and light intensity given
Gao et al., 2019	DBD; enclosed in quartz container with small openings; metal electrodes atm pressure air AC	Not clear; 1 cm x 15 cm electrode Quartz container 15x0.8cm; 60-164 W for PAW for 5-20 min; 9-35 W for dry seeds for 1-10 min; temp not given	Pea	seeds from market; not given; preselection random by shaking; not given; not given	not mentioned; filter paper in Petri dish; not mentioned; simulated sunlight after germination
Mildažienė et al., 2019	CCP RF; enclosed in stainless steel vacuum chamber; copper electrodes; low pressure (200 Pa); air; 5.28 MHz	20 mm gap but not specified; 120 mm diameter, 0.053m3 reactor volume; 0.35W/cm3; 2,5,7 min; <37ºC (from other paper); water cooled electrodes	Sunflower	seeds from institute; storage not given; preselection done; not given; not given	waited 4 days before sowing; filter paper or peat; climate chamber; 16 h light
Šerá et al., 2019	DBD; open; atm pressure; air	direct 80W/cm3 0-300 s	Pine	not given; not given; preselection done with floaters; disinfected in bleach, tween20, H2O2 stirred in water for 35 h and dried for 2 h; rotation added	not given; Sabouraud agar; not given; not given; not given

Citation	Plasma	Plasma-seed treatment	Seed type	Seed pre-treatment	Seed growth methodology
Los et al., 2019	DBD enclosed based on ref 27; aluminium electrodes and polypropylene dielectric; atm pressure; air; AC; 80 kV; 50 Hz; 42% RH (from another study)	20-26 mm gap b/w electrodes; 158 mm diamter electrodes; power for 70 kV is 65.2 W; 30, 60,180 s; temp not given	Wheat	organic seeds purchased from local retailer; not mentioned; preselection done; not mentioned; not mentioned	sowing immediately or 24 h after; filter paper; tray; dark
Moghanloo et al., 2019	DBD; enclosed; copper electrodes and glass dielectric; radius 5.5 cm; atm pressure; Ar; 2 L/min; AC; 8-10 kV 13 kHz	gap 4 mm between dielectrics; 80 W; 0.84 W cm2; 30, 60, 90 s; no temp given	Astragalus frida	not given, not given; not given; soaked for 12 h since germination after 24 h; sterilized (no details)	not mentioned; MS medium; germinator; 16 h light and light intensity given
Babajani et al., 2019	DBD enclosed; copper electrodes and glass dielectric; radius 5.5 cm; atm pressure; Ar; 2 L/min; AC; 10 kV; 13 kHz	gap 4 mm between dielectrics; 80 W; 0.84 W cm2; 50, 90, 120 s; no temp given	Melissa officinalis	purchased from company; not given; preselection of homogeneous seedlings before plasma; 48 h soaking; sterilized with sodium hypochlorite	not mentioned; filter paper supp. Hoagland nutrient solution; germinator; 16 h light and light intensity given
Lotfy et al., 2019	plasma jet; enclosed in acrylic chamber; stainless steel electrodes and Teflon dielectric; atm pressure; N2; 14 L/min; RF power; supply 30 mA, 10 kV, 20 kHz; used 2.6 kV	1.7 mm; power not given; 2, 4, 6, 8,10 min; 25.5 - 31ºC	Wheat	seeds from private company; stored at RT and 50% RH; preselection of healthy seeds; not given; not given	not mentioned; filter paper; cup; dark
Bafoil et al., 2019	DBD; open; metallic and any surface for ground electrode, glass dielectric; atm pressure; air; nanosecond monopolar pulsed of 80 s; 10 kV; 10 kHz	direct; 8 cm diameter; power not given; 15 min; temp not given	Arabidopsis	private communication – no contamination so no sterilization	not mentioned; filter paper; not given; 16 h light
Singh et al., 2019	RF; enclosed electrode in glass bottle within a chamber; low pressure; (0.40 mbar) O2 and Ar; 13.56 MHz	not mentioned; 8 L volume 160x160x325 mm chamber; 30-270 W; 10 min; 28ºC; seed tumbling (5 rotations/min)	Basil	source not given; storage not given; preselection done for healthy uniform seeds no soaking or sterilization mentioned	not mentioned; filter paper; not given; 16 h light distilled water 70-80% RH

Iqbal et al., 2019	Not clear; Enclosed? low pressure; Ar; 600-850 V	not mentioned; no dimensions or power given; 1-4 min	Wheat	seeds from research center; not given; preselection of healthy and same size seeds; not given; not given	not mentioned; water in Petri dish; incubator; no light info given distilled water
Islam et al., 2019	DBD; enclosed in vacuum chamber; copper as electrodes and glass as dielectric; low pressure (10 Torr); air, Ar, O2; (for PAW: 0.25 L/min) AC bipolar sinusodial; 5-10 kV; 3-8 kHz	direct from image FigS1; 30 mm apart electrodes; 30 W; 90 s; gas temp 304 K	Rapeseed, mustard	random selection of seeds; no other info given	not mentioned; filter paper for germination; PAW use hydroponic culture pH 6.0; cabinet; 10 h light and intensity given
Pawłat et al., 2018	DBD plasma jet; open with copper electrodes and ceramic dielectric; atm pressure; He and N2; 1.6 dm3/min He and 0.03 dm3/min N2; AC; 3.7 kV; 17 kHz	5 cm away; 1.4/3.4mm x 12 mm; 6 W; 1,2,5,10,15 min; <40ºC	Thuringian Mallow	seeds from plant breeding institute 2009; no other info given	not mentioned; filter paper; not given; 12 h light
Cui et al., 2019	DBD; enclosed in acrylic chamber; copper mesh electrodes and fiberglass dielectric; atm pressure air AC 8.47 kV 7.95 kHz	closely placed under mesh electrode and held by tape; 2.5 W; 0.5,1,3,5,10 min; ~33ºC after 20 min; RH 65%	Arabidopsis	not given; stored at RT for 1 and 10 months; not given; treated with sterilized water, H2O2 for 30 s and dried	not mentioned; ½ MS with sucrose pH 6.2-6.4 and vernalization for 48h at 4ºC; plant chamber; 16 h light
Liu et al., 2019	DBD jet; enclosed in glass dielectric and stainless steel wires; atm pressure; N2, O2, air; 1.5 L/min; AC bipolar square; 20 kV; 500 Hz	5 mm away while in water or soaked in PAW after; 2.5 W; 2,4,6 min; temp <Δ4ºC d	Radish, mung bean, wheat, tomato, lettuce, mustard, Dianthus and sticky bean		not mentioned; filter paper; plant chamber; 16 h light and flux given; 35-45% RH

Citation	Plasma	Plasma-seed treatment	Seed type	Seed pre-treatment	Seed growth methodology
Kabir et al., 2019	DBD; enclosed in vacuum chamber; copper as electrodes and glass as dielectric; low pressure (10 Torr); air, Ar, O2; 0.4 - 0.6 L/min AC bipolar sinusodial; 5-10 kV; 3-8 kHz	direct from image FigS1; 30 mm apart electrodes; ~45 W; 90 s; gas temp 304 K	Wheat	not given information	not mentioned here; moist Petri dishes with salts or hydroponic culture; growth cabinet; 10 h light
Lo Porto et al., 2019	RF; enclosed in stainless steel vacuum chamber; low pressure (800 mTorr); N2, O2; 4,16,20 sccm 13.56 MHz	30 mm gap size?; 150 mm diameter of chamber; 50 W; 1,15,30 min; no temp given	Asparagus	seeds from field (Bari); stratification in sand for ~2 months in the dark; no pre-treatment	not mentioned; filter paper; not given; dark; sterilized tap water
Pérez-Pizá et al., 2019	needle to plane DBD; enclosed? atmospheric; N2, O2; 6 NL/min; AC; 0-25 kV; 50 Hz	10 mm between dielectric and tip; 65-85 W; 60-180 s; no temp given; mechanical movement during treatment	Soybean	seeds from agricultural service; stored in the dark 5ºC; no other info given	after treatment stored in vessels temporarily; vermiculite supp. with Hoagland; greenhouse; 12 h or 16 h light with light intensity
Tounekti et al., 2018	DBD; enclosed in quartz chamber with metal electrodes and quartz as dielectric; atm pressure; He; 2 slm; AC; No voltage mentioned; 10 kHz	within 1 cm gap; 10 cm x 1 cm; 50 W; 30,60,120,240 s; +Δ10ºC	Coffee and grape seeds	no info given	not mentioned; filter paper; growth chamber; 14 h light and intensity given; distilled water
B. Zhang et al., 2018	CCP glow RF; enclosed; materials not given; low pressure (30-200 Pa); air, He; 13.56 MHz	on drive belt; 260mm x 1200 mm; 50-1000 W; 5-90 s; temp not given	Maize, peppers, wheat, soybeans, tomatoes, eggplants, pumpkins	not given source of seeds; not given storage; preselection done with screening; drying seeds; no sterilization mentioned	not mentioned; plates and field; not given other info
Khatami & Ahmadinia, 2018	gliding arc; open; materials not given; atm pressure; air; 5 L/min; AC; 15 kV	1 cm distance; power not given; 30, 60 s; temp not given	Pea, Zucchini	purchased from company; not given; used ripe intact seeds without defects; seeds soaked in water for 1 h; no sterilization mentioned	not mentioned; pots of soil; growth chamber; 16 h light or dark when using dark and drought stress

Citation	Plasma	Plasma-seed treatment	Seed type	Seed pre-treatment	Seed growth methodology
Rahman et al., 2018	DBD; enclosed in vacuum chamber; copper as electrodes and glass as dielectric; low pressure (10 Torr); air, Ar, O2; 0.4 - 0.6 L/min AC bipolar sinusodial; 5-10 kV; 3-8 kHz	direct from image FigS1; 30 mm apart electrodes; ~45 W; 90 s; gas temp 304 K	Wheat	not given; not given; random selection of seeds; not given; not given	not mentioned here; filter paper or hydroponic culture with salts; incubator; 10 h light
Pawlat et al., 2018	Gliding arc; semi-open in reactor with copper rod electrodes with min. space 3 mm and angled 12°; atm pressure; nitrogen; 8 L/min; AC; 680 V – 3.8 kV; 33 mA; 50 Hz	4.5 cm away; Area of plasma not given; 40 W; 1,2,5,10,15 min; 35-45ºC; seed mixing with flow rate; RH 44%	Thuringian Mallow	seeds from plant breeding institute 2009; no storage given; no preselection given; no soaking or sterilization mentioned; 7% seed moisture content	not mentioned here; filter paper in Petri dishes; not given; 12 h light; distilled water
Nakano et al., 2016	RF; enclosed; low pressure (20-80 Pa); O2, Ar; 13.56 MHz	not given; volume 20 L; 60 W; time not given; temp not given	Arabidopsis, radish	no info given	no info given
Matra, 2018	Plasma flashlight?; open cylinderical plastic chamber; atm pressure Ar, O2 2-4 L/min; DC; 8-14 kV	1 or 1.5 cm away; 10 cm x 10 cm; power not given; 1,3,5 min; <50°C	Sunflower	seeds from farm; no other info given	seeds soaked 8 h in water after plasma; moist tissue paper; not given; no light info given; distilled water
Li et al., 2017	DBD; enclosed in plexiglas cylinder with stainless steel electrodes and quartz dielectric; atm pressure; air; 1.5 L/min; AC; 13 kV; 50 Hz	8 mm away; volume 62.8 mL or 100 mm by 8 mm; 1.5 W; 1, 4, 7, 10, 13 min; temp not given	Wheat	seeds from institute; stored at 0–4°C fridge; 10% water content; air dried, cleaned but no preselection mentioned; soaked in water for 5h, disinfected with 70% alcohol	not mentioned here; filter paper; germination chamber; 12h light and intensity given; distilled water

Bafoil et al., 2018	1) DBD; open; any surface for ground electrode, glass dielectric; atm pressure; air; nanosecond monopolar pulsed of 80 s; 10 kV; 10 kHz 2) plasma jet; open; glass as dielectric; atm pressure; He; 3 L/min; nanosecond monopolar pulsed of 80 s; 10 kV; 10 kHz	2 cm away; power not given; 15 min; temp not given; seeds moving with magnetic stirrer	Arabidopsis	No info given	not mentioned; filter paper or soil; culture chamber or growth room; 16 h light; 40% or 75% RH
Măgureanu et al., 2018	DBD (fluidized); enclosed aluminum tape as electrodes and glass dielectric; atm pressure; air; 15 L/min; AC; 13-17 kV; 50 Hz	4 mm gap; 12 mm x 48 mm; 0.55-1.43W or 11-28.6 mJ; 5,15,30,45 min; no heating; seed circulation with gas flow	Tomato	seeds from company; no other info given	not mentioned here; top-of-paper, sand or universal soil; incubator; 12 h light
Štěpánová et al., 2018	DBD; open with metallic electrodes and alumina ceramic; atm pressure; air; AC; 20 kV; 15 kHz	direct; 100 W/cm3 or 8 cm x 20 cm; input 400 W; 4-50 s; <50°C; vibrating movement of seeds;	Cucumber and pepper	seeds from company; no other info given	not mentioned; filtration paper; germinators; 16 h artificial light
Iranbakhsh, Ardebili, et al., 2018	DBD; enclosed; copper electrodes and glass dielectric; atm pressure; Ar; 2 L/min; 8 kV AC; 13 kHz	3 mm gap between dielectrics; 80 W, 0.84 W cm2; 15, 30, 60, 90,120 s; <29ºC;	Wheat seedlings	not given; not given; selected only intact with no visible defects; not given; not given	waited 3 and 6 h after plasma treatment to extract; pots with peat and perlite (1:1); not given; not given
Iranbakhsh, Oraghi Ardebili, et al., 2018	DBD; enclosed; copper electrodes and glass dielectric; radius 5.5 cm; atm pressure; Ar; 2 L/min; 8 kV AC; 13 kHz	3 mm gap between dielectric; 80 W, 0.84 W cm2; 60, 120 s; check previous paper for temp	Chili pepper	purchased from company; not given; not given; sterilized with sodium hypochlorite, detergent; 24 h water soaked or dry seeds	not mentioned here; MS with or without hormones BA and IAA, or soil and perlite and peat (1:1); germinator; 16 h light and intensity given

Citation	Plasma	Plasma-seed treatment	Seed type	Seed pre-treatment	Seed growth methodology
Pérez-Pizá et al., 2018	needle to plane DBD; Enclosed?; Atmospheric; N2, O2; 6 NL/min; AC; 0-25 kV; 50 Hz	10 mm between dielectric and tip; 65-85 W; 60-180 s; no temp given; mechanical movement during treatment	Soybean	selected most infected seeds	not mentioned; top-of-sand; not given; 12 h light
Park et al., 2018	DBD; enclosed with metallic electrodes and alumina ceramic dielectric; atm pressure; N2 and air; N2 3 L/min + air 0.1 L/min; AC sine pulsed 1.5 us, 30 kHz; Not clear	direct; 91.875 cm2 plasma/110.25 cm2 total; net 222.6 W, nominal 400 W; 10,20,40,80 s; temp not given	Barley	not given; not given; preselection done for healthy seeds through visual scanning; dry and 24 h water soaked seeds; no physical or chemical treatment done	not mentioned; filter paper; not given; not given; distilled and deionized water; 50% RH
J. Zhang et al., 2018	DBD; atm pressure; Ar; 1 L/min	12 s-3 min	Soybean	not given info?	not given info?
(Lotfy, 2017b)	plasma jet; enclosed in acrylic box with stainless steel electrodes and Teflon dielectric; atm pressure; N2; 14 L/min; RF power supply with 10 kV, 30 mA, 20 kHz; 3 kV used	7 mm away?; Power not given; 2,4,6,8,10 min; <28ºC	Watermelon	seeds from market; stored at RT in the dark until dry; seeds chosen randomly; not given; not given	not mentioned; filter paper; cup; dark
Wang et al., 2017	DBD; enclosed in a glass container, materials not given; atm pressure air, N2; 1000 sccm; AC; 19 kV; 1 kHz	15 mm gap distance; Power not given; 3, 9, 27 min; temp not given; shaking seeds	Cotton	info not given	waited 24 h before sowing; no germination test
Kim et al., 2017	Corona discharge plasma jet; open; no materials given; atm pressure air? DC; 20 kV; 58 kHz	25 mm away; electrode emission slit: 6mm x 35mm; Power not given; 0-3min; temp not given; seed mixed with gas flow	Broccoli	purchased from a company; no other info given	not mentioned; sprouting chamber; distilled water

Citation	Plasma	Plasma-seed treatment	Seed type	Seed pre-treatment	Seed growth methodology
Meng et al., 2017	DBD; enclosed in plexiglas cylinder with stainless steel electrodes and quartz dielectric; atm pressure; air, Ar, O2, N2; 1.5 L/min; AC; 13 kV; 50 Hz	8 mm away; 100 mm x 8 mm; 1.5 W based on Li paper; 1-19 min; temp not given	Wheat	seeds from research institute; air dried, cleaned, stored at 0–4°C fridge; 10% water content; not given; soaked in water for 5h, disinfected with 70% alcohol	not mentioned here; filter paper; germination chamber; 12h light and intensity given
Nalwa et al., 2017	glow discharge; materials not given; low pressure (0.2 mbar); O2; DC; 500 V 0.2 A	distance not given; no dimensions given; 100 W; 3, 6, 9,12,15 min; temp not given	Bell pepper	no info given	seeds stored after plasma treatment for 0,4,8,12 months; Petri plates; no other info
Junior et al., 2016	plasma jet DBD; open; materials not given; atm pressure; He; 0.03 L/s; No power supply given; 10 kV; 750 Hz	13 mm away; 150 W; 60 s; temp not given; shaking added	Mulungu	no info given no pretreatment mentioned	not mentioned here; sterilized sand; tray; no other info given
Zahoranová et al., 2016	DBD; open with silver electrodes embedded in alumina ceramic dielectric; atm pressure; air; AC sinusosial; 14 kHz; 20 kV	direct; plasma area: 200 mm x 80 mm; 70-100 W/cm; 10-80 s; ceramic <50-55ºC; shaking added	Wheat	3-year-old seeds; stored at 10ºC in the dark; no other info given	waited 24 h after treatment; soil substrate (sand/peat/pearlite 1:1:1 v/v/v); not given; 12 h light and intensity given
L. Li et al., 2016	CCP RF; no materials mentioned; low pressure (150 Pa); He; 13.56 MHz	not clear; 1200 mm x 180 mm x 20 mm; 60-140 W; 15 s; 25°C	Peanut	no info given; says pretreatment in flowchart but no idea what this means	not mentioned; filter paper in petri dish; incubator or field; dark
Gholami et al., 2016	CCP RF; enclosed in pyrex cylindrical tube with mesh electrodes; low pressure; air; 13.56 MHz	no distance given?; 100 mm x 350 mm; 50, 80,100 W; 2 min; temp not given	Ajwain	no info given	not mentioned; Petri dish; germinator; 8 h light; 75% RH; distilled water
Matra, 2016	Plasma flashlight; enclosed in acrylic chamber with copper electrodes and copper tube; atm pressure; Ar; 4 L/min; DC; 0-30 kV	distance not given; chamber 10x15x20 mm3; 90,140 W; 2,4,6 min; temp not given	Radish	seeds from market; no other info given	soaked in water 2 hrs after treatment; no substrate given; no container given; dark

Sarinont et al., 2016	DBD; enclosed in a chamber with stainless steel electrodes and ceramic dielectric; atm pressure; air, O2, NO, He, Ar, N2; AC; 9.2 kV; 0.2 A; 10 kHz; 10-90% RH	3 mm below and -5 to 30 mm away; 60 mm x 20 mm?; 1.49 W/cm2; 3 min; temp not given	Radish	seeds from a company; no other info given	stored for 0, 8,10,12,17 months; tray in incubator; dark; 80% RH; deionized water
Dobrin et al., 2015	DBD; enclosed in a rectangular case with copper HV and aluminum tape ground electrodes and glass dielectric; atm pressure; air; 1 L/min; AC; 15 kV; 50 Hz	direct; 13x6 by 44 mm for plasma area; 2.7 W; 5,15,30 min; temp not given	Wheat	seeds from private collection; not given; preselected healthy seeds; not given; not given	not mentioned; filter paper; incubator; dark; distilled water
Stolárik et al., 2015	DBD; open with silver electrodes embedded in alumina ceramic dielectric; atm pressure; air; AC; 10 kV; 14 kHz;	direct; plasma area: 200 mm x 80 mm; 370 W; 2.3 W/cm2; 60-600 s; temp not given	Pea	seeds from institute; stored in the dark at 10ºC; seeds mixed; not given; not given	waited 24 h after plasma treatment; pots containing perlite substrate, Hoagland nutrient solution added; not given; 12 h light with intensity given; 70% RH
Munkhuu et al., 2015	not well described	not given; not given; 20-280 W; not given	Clover	not sure if soaked before treatment or did treatment with seeds on wet filter paper?	filter paper and field?; not clear

Citation	Plasma	Plasma-seed treatment	Seed type	Seed pre-treatment	Seed growth methodology
Sera et al., 2017	1) gliding arc; atm pressure; humid air; open? 10 L/min; No voltage given; 50 Hz 2) microwave plasma discharge; Enclosed low pressure (140 Pa) Ar, oxygen; 50 mL/min 2.45 MHz	1) Gliding arc: 250 mm away No dimensions given; 180, 300, 600 <50ºC 2) DMP: 10 cm 10 L volume for reactor 500 W 180, 300, 600 s ~25ºC	3 cultivars of hemp Finola, Bialobrzeski, Carmagnola	seeds from seed association konopa; no other info given	not mentioned; filter paper; growing box; dark
Puligundla et al., 2017a	Corona discharge plasma jet; open; no materials given; atm pressure air? DC; 20 kV; 58 kHz	25 mm away; electrode emission slit: 6mm x 35mm; Power not given; 0-3min; temp not given; seed mixed with gas flow	Rapeseed	seeds purchased from company; no other details given	not mentioned; sprouting chamber; no other details given
Puligundla et al., 2017b	Corona discharge plasma jet; open; no materials given; atm pressure air? DC; 20 kV; 58 kHz	25 mm away; electrode emission slit: 6mm x 35mm; power not given; 0-3min; temp not given; seed mixed with gas flow	Radish	seeds purchased from company; no other details given	not mentioned; sprouting chamber; no other details given
Gómez-Ramírez et al., 2017	1) DBD; enclosed with metal electrodes and quartz dielectric; low pressure (500 mbar); dry air; AC; continuous wave mode 8.2 kV; 1 kHz; 2) RF; materials not given but cooled bottom electrode; low pressure (0.1 mbar); dry air; continous waveform; 13.56 MHz	1) 4.2 mm gap; 8 cm diameter electrode; 6.4 W; 10, 30, 60,180, 900 s, + Δ1ºC 2) 2.5 cm gap; 10 cm diameter electrode; 15 W; 10,30,60,180 s; no temp increase	Quinoa	seeds developed by WU and used commercial seeds; not given; no additional pre-treatment	not mentioned; filter paper or soil; growth chamber, 16 h light and intensity given

Guo et al., 2017	DBD; enclosed in plexiglas cylinder with stainless steel electrodes and quartz dielectric; atm pressure; air; 1.5 L/min; AC; 9-17 kV done experimentally but range 0-50 kV; 50 Hz	8 mm away; volume 62.8 mL or 100 mm by 8 mm; 1.5 W; 4 min; temp not given	Wheat	seeds from research institute; air dried, cleaned, stored at 0–4°C fridge; 10% water content; not given; soaked in water for 5h, disinfected with 70% alcohol	not mentioned here; filter paper; germination chamber; 12h light and intensity given
J. J. Zhang et al., 2017	Needle to plane DBD; enclosed in acrylic chamber with metallic electrodes and ceramic dielectric; atm pressure; Ar; 2 L/min; AC; 10.8-22.1 kV; 60 Hz	30 mm gap; 10 cm diameter electrode; 3.4 W – 15.6 W; 12 s; 25ºC	Soybean	seeds from Korea; no other info given	soaked in water for 6 h after plasma treatment; water; bean sprout machine; no light info given
da Silva et al., 2017	DBD; enclosed with metal mesh as electrodes and glass as dielectric; atm pressure; air; DC pulsed; 17.5 kV; 990 Hz	sandwiched?; 0.006 m2; 0.18 W/m2; 3, 9,15 min; temp not given	Mimosa	no info given	seeds stored in desiccator after treatment; sterilized sand; germination box; no light info given

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Academic CV

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Strengths

- Project coordination and management
- Communication between interdisciplinary backgrounds
- Leadership and team building
- Highly motivated, organized, persuasive, results-oriented, problem solver, and quick learner

Education

Doctorate of Science in Biotechnology and Bioengineering
Swiss Federal Institute of Technology Lausanne (EPFL), Switzerland | 2018 - 2022

Master of Science in Microbiology and Immunology with Distinction
Swiss Federal Institute of Technology Zurich (ETH Zurich), Switzerland **|** 2014 - 2017

Bachelor of Science in Biology with Honours
Ryerson University, Toronto, Canada | 2009 - 2013

Languages

- intermediate French (A2/B1)
- basic German (A1)
- native English

Publications

1. **Waskow, A**.*, Guihur, A.*, Howling, A., Furno, I. (2022). Catabolism of glucosinolates into nitriles revealed by RNA sequencing of *Arabidopsis thaliana* seedlings after non-thermal plasma-seed treatment. Manuscript submitted.
2. **Waskow, A**.*, Guihur, A.*, Howling, A., Furno, I. (2022). RNA Sequencing of *Arabidopsis thaliana* Seedlings after Non-Thermal Plasma-Seed Treatment Reveals Upregulation in Plant Stress and Defense Pathways. *Int. J. Mol. Sci.*, 23(6):3070.
3. Avino, F., Von Allmen, M., Howling, A., **Waskow, A**., Ibba, L, Furno, I. (2022). SDBD operation in a humid environment: reversible and permanent degradation. Manuscript in preparation
4. **Waskow, A**., Ibba, L., Leftley, M., Howling, A., Ambrico, P., Furno, I. (2021). An in situ FTIR study of DBD plasma parameters for accelerated germination of *Arabidopsis thaliana* seeds. *Int. J. Mol. Sci. 22*, 11540.
5. **Waskow, A**., Avino, F., Howling, A., Furno, I. Entering the plasma agriculture field: An attempt to standardize protocols for plasma treatment of seeds. (2021) *Plasma Processes and Polymers*. e2100152.
6. **Waskow, A.**, Howling, A., and Furno, I. (2021) Advantages and Limitations of Surface Analysis Techniques on Plasma-Treated *Arabidopsis thaliana* Seeds. Front. Mater. 8:642099.
7. **Waskow, A**., Howling, A., and Furno, I. (2021) Mechanisms of Plasma-Seed Treatments as a Potential Seed Processing Technology. Front. Phys. 9:617345.
8. Novossiolova T. et al. (2021) Addressing Emerging Synthetic Biology Threats: The Role of Education and Outreach in Fostering Effective Bottom-Up Grassroots Governance. In: Trump B.D., Florin MV., Perkins E., Linkov I. (eds) Emerging Threats of Synthetic Biology and Biotechnology. NATO Science for Peace and Security Series C: Environmental Security. Springer, Dordrecht.
9. Waskow, A., Butscher, D., Oberbossel, G. et al. Low-energy electron beam has severe impact on seedling development compared to cold atmospheric pressure plasma. Sci Rep 11, 16373 (2021).

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11. Waskow, A., Betschart, J., Butscher, D., Oberbossel, G., Klöti, D., Büttner-Mainik, A., ... & Schuppler, M. (2018). Characterization of efficiency and mechanisms of cold atmospheric pressure plasma decontamination of seeds for sprout production. *Frontiers in microbiology*, *9*, 3164.
12. Butscher, D., Van Loon, H., Waskow, A., Rudolf von Rohr, P., Schuppler, M. (2016). Plasma Inactivation of Microorganisms on Sprout Seeds in a Dielectric Barrier Discharge. International Journal of Food Microbiology.
13. Johnson-Henry, K.C, Pinnell, L.J., Waskow, A.M., Irrazabal, T., Martin, A., Hausner, M., Sherman, P.M. (2014). Short-Chain Fructo-oligosaccharide and Inulin Modulate Inflammatory Responses and Microbial Communities in Caco2- bbe Cells and in a Mouse Model of Intestinal Injury. Journal of Nutrition.

Accepted invited talks and awards

15th Asia Pacific Physics Conference (APPC-15) | August 2022
Best Student Paper Award, 9th International Conference on Plasma Medicine | July 2022
OLTP online seminar series | February 2022

Teaching experiences

General physics II | 2018-2021
Electromagnetism | 2020

Student supervision

Max Leftley for Master thesis | 2021
Lorenzo Ibba for Master thesis | 2018-2019

Technical expertise

Wet lab

- Fourier-Transform InfraRed spectroscopy (FTIR), Attenuated Total Reflection- FTIR (ATR-FTIR), X-ray Photoelectron Spectroscopy (XPS), Energy-Dispersive X-ray spectroscopy (EDX), Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) all familiar at a basic level
- protein extraction and quantification, qPCR, Western blot, Coomassie Blue, nanoluciferase assay, backcrossing with *Arabidopsis*
- knowledge transfer of CRISPR-Cas, Biolector, metabolite extraction, mass spectrometry with qTOF 6550
- CRISPR-Cas, plasmid construction and isolation, bacterial transformation, yeast transformation
- spread plating, dilution series, plasma decontamination of contaminated wheat grains, native seed microbiota and artificially contaminated seeds, seed germination (ISTA guidelines)
- phenotypic assays, conjugation, Polymerase Chain Reaction (PCR), cloning, flow chambers, Leica Confocal Laser Scanning Microscopy (CLSM)
- DNA extraction, cultivation, PCR, Denaturing Gradient Gel Electrophoresis (DGGE), Fluorescence In Situ Hybridization (FISH), Carl Zeiss CLSM

Project management

- designed and built a bioplasma lab (BSL1), maintained equipment, and acted the biosafety officer
- guided team to develop a successful water sustainability solution (award) by designing team building activities, establishing and maintaining successful team dynamics
- worked in an interdisciplinary team to develop a successful solution for food sustainability (award)
- have been coordinating or managing interdisciplinary projects with collaborations since 2015
- was responsible for the master plan of a 6-day event which involved around 300 people
- trained incoming volunteers or students and formulated solutions for team members' troubleshooting