



# Étude des effets d'un plasma froid d'air ambiant sur la physiologie des graines d'Arabidopsis

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Sorbonne Université

ED 564 – Physique en Ile-de-France

Laboratoire de Physique des Plasmas (UMR 7648)

Laboratoire de Biologie du Développement (UMR 7622)

## **Investigating the effects of cold ambient air plasma on Arabidopsis seed physiology**

Présentée par

**Jonas AUGUST**

Thèse de doctorat de Physique et Biologie

Spécialité Physique des Plasmas et Physiologie Végétale

Dirigée par Thierry Dufour et Christophe Bailly

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Que l'aventure continue !

(A lire en chantant)

## List of abbreviations

ABA: abscisic acid

APX: ascorbate peroxidase

AUX: auxin

$A_w$ : water activity

BET: Brunauer-Emmett-Teller

BR: brassinosteroids

CAT: catalase

CCC: coaxial cylindrical capacitor

CK: cytokinin

C2AP: cold ambient air plasma

DBD: dielectric barrier device

DCFH-DA: 2',7'-Dichlorodihydrofluorescein diacetate

DEG: differentially expressed gene

DMAB: 3-dimethylaminobenzoic acid

DSC: differential scanning calorimetry

D&W: D'Arcy and Watt

EPR: electron paramagnetic resonance

ESR: electron spin resonance

FAO: Food and Agriculture Organization

FDR: false discovery rate

GA: gibberellins

GAB: Guggenheim-Anderson-de Boer

GDW: Generalized D'Arcy and Watt

GO: gene ontology

HPC: High-Performance Computing

HPLC: high performance liquid chromatography

H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxyde

LCR: inductance-capacitance-resistance

MBTH: 3-methyl-2-benzothiazolinone hydrazine

MD: Morphological dormancy

MDA: Malondialdehyde

MPD: Morphophysiological dormancy

MS: mass spectrometry

PAW: plasma-activation water

PEG: polyethylene glycol

PD: physiological dormancy

POD: peroxidase

PY: physical dormancy

RF: radio-frequency

R<sub>H</sub>: relative humidity

RNS: reactive nitrogen species

ROI: region of interest

ROS: reactive oxygen species

SEM: scanning electron microscopy

SOD: superoxide dismutase

TBA: thiobarbituric acid

TCA: trichloroacetic acid

UMI: unique molecular identifier

WC: water content

XPS: X-ray photoelectron spectroscopy



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## Résumé substantiel

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Ce projet doctoral à l'interface entre la physique des plasmas froids et la biologie des semences a été effectué au sein de deux laboratoires de recherche : le Laboratoire de Physique des Plasmas (UMR 7658) et le Laboratoire de Biologie du Développement (UMR 7622) de l'Institut de Biologie Paris-Seine tous les deux localisés sur le campus de Pierre et Marie Curie de Sorbonne-Université à Paris. L'objectif principal de cette étude a été d'étudier les effets d'un plasma froid d'air ambiant (PFA2) sur la physiologie des graines d'*Arabidopsis* et notamment sur la levée de dormance.

Ce manuscrit est composé de 4 chapitres. Le premier chapitre est une synthèse bibliographique qui introduit les plasmas froids de décharge, son utilisation pour l'agriculture, les mécanismes liés à la régulation de la germination et de la dormance et les résultats obtenus montrant les effets des plasmas froids sur les semences tant par leur diversité de procédés que par les mécanismes biologiques associés. Les 3 chapitres suivants sont écrits sous la forme d'articles montrant le cheminement de la thèse, à savoir (i) l'interaction entre le plasma et les graines, en étudiant comment la teneur en eau et les propriétés électriques des graines d'*Arabidopsis* peuvent modifier les propriétés d'un plasma froid d'air ambiant, (ii) la levée de dormance des graines d'*Arabidopsis* par les plasmas froids d'air ambiant en fonction de la transition vitreuse cytoplasmique et (iii) l'étude des mécanismes biologiques et des changements morphologiques induits par un plasma froid d'air ambiant pour lever la dormance des graines d'*Arabidopsis*.

L'agriculture moderne est confrontée à de multiples défis dans un monde en mutation. Si la population mondiale ne cesse d'augmenter et atteindra 9 milliards d'habitants en 2050, on peut légitimement se demander comment nourrir la planète en faisant face à une demande alimentaire croissante et aux problèmes environnementaux. Selon l'Organisation des Nations Unies pour l'alimentation et l'agriculture (FAO), les épidémies de parasites et de maladies transfrontalières des animaux et des plantes sont en augmentation, menaçant les chaînes alimentaires mondiales. À l'échelle mondiale, environ un tiers de tous les aliments produits sont perdus ou gaspillés, ce qui entraîne des pertes pour les agriculteurs et des pressions inutiles sur les ressources naturelles (Food wastage footprint, 2013). De plus, ces problématiques interviennent dans un contexte où le réchauffement climatique, de plus en plus intense, affecte de nombreux écosystèmes et a des conséquences sur la production et les rendements agricoles (Thornton et al., 2014), notamment par l'arrivée de conditions environnementales extrêmes et de stress abiotiques (Pereira, 2016).

La germination des graines et la croissance des plantes sont deux facteurs clés pour le rendement des cultures. Ces facteurs sont affectés par des événements climatiques majeurs tels que la sécheresse, la salinisation des sols et les inondations. Les plantes et la production agricole sont vulnérables face aux stress physiques et biologiques. La pollution et la baisse de la qualité des sols affectent les populations et les écosystèmes du monde entier, exacerbées par le changement climatique mais aussi par les activités humaines. Si les semences fournissent environ 70 % de l'alimentation mondiale, il est nécessaire de développer des solutions innovantes pour le traitement des semences afin de relever les défis environnementaux et de remplacer les produits agrochimiques utilisés aujourd'hui, qui augmentent la pollution des sols et la perte de terres arables. L'amélioration du potentiel de germination et de la vitesse de croissance des semis conduit généralement à une productivité élevée des cultures. Pour cela, le plasma froid apparaît aujourd'hui comme une technologie prometteuse pouvant être utilisée pour améliorer la germination des graines et répondre aux questions soulevées.

Pour répondre à ces enjeux, les solutions existantes reposent sur l'utilisation de produits phytosanitaires, de pesticides et de molécules chimiques qui ne répondent pas aux problématiques environnementales. Plusieurs actions européennes, notamment l'obligation européenne fixée par la directive 2009/128/CE, et au niveau national avec les plans Ecophyto, tentent de répondre à ces enjeux afin de donner un cadre commun et de parvenir à une réduction de l'utilisation des produits phytopharmaceutiques pour une agriculture compatible avec le développement durable. Ces plans visent à accélérer le retrait des substances les plus dommageables pour l'environnement, à favoriser la diffusion du biocontrôle, à renforcer la prévention de l'exposition du public aux pesticides, à soutenir la recherche et l'innovation et à accompagner les agriculteurs dans la transition écologique. Le développement de procédés plasma serait une solution pour répondre à ces enjeux en s'inscrivant dans la réalisation de ces politiques environnementales (Waskow et al., 2021).

*Arabidopsis thaliana* est une espèce faisant partie de la famille des Brassicaceae. C'est l'espèce majoritairement utilisée en biologie des semences pour des études de génétique moléculaire en raison de la singularité de ses caractéristiques. En effet, l'*Arabidopsis* présente un petit génome concentré sur 5 chromosomes, complètement séquencé (Kaul et al., 2000) et composé d'environ 120 Mb d'ADN qui est 4 et 20 fois respectivement plus petit que le riz et le maïs. Elle présente un cycle de reproduction rapide (3 mois maximum), une taille relativement modeste (15-20cm pour la plante et ~0,1 mm pour la graine) et une production de graines abondante (environ 5000 par plante) (Bewley et al., 2006). Comme

toutes les graines orthodoxes, le développement des graines d'*Arabidopsis* varie selon trois phases dépendant des conditions environnementales (Baud et al., 2002) : (i) l'embryogénèse caractérisée par les divisions et l'élongation cellulaire (augmentation de la teneur en eau), (ii) l'accumulation de réserves (diminution de la concentration en eau par expansion des cellules contenant les réserves et (iii) la maturation tardive (dessiccation) pendant laquelle un blocage physiologique transitoire de la germination peut se mettre en place : la dormance. Elle est définie pour des semences viables par l'inhibition de leur germination sous des conditions environnementales *a priori* favorables (Bewley, 1997). Cette non-germination des graines dépend des caractéristiques intrinsèques des espèces mais aussi des habitats et des conditions de développement. Il existe deux sortes de dormance : la dormance primaire et la dormance secondaire. La dormance primaire se développe au cours du processus de maturation des graines sur la plante-mère (Bewley and Black, 1994) comme pour les graines d'*Arabidopsis* utilisées dans cette étude qui présentent une dormance physiologique. Les graines présentant une dormance primaire peuvent également entrer dans un processus d'inhibition de la germination (dormance secondaire) lors de l'imbibition si les conditions germinatives sont défavorables (Hilhorst et al., 2010). Ces deux types de dormances sont régulés par des réseaux de signalisations hormonales impliquant l'acide abscissique (ABA) inhibiteur de germination et l'acide gibbérellique (GA) régulateur de croissance et stimulateur de germination (Bewley, 1997). Les principaux facteurs externes de la régulation de la germination et de la dormance des semences sont la lumière, l'oxygène, la température et la teneur en eau des graines (Probert, 2000). Pour les graines d'*Arabidopsis*, la dormance s'exprime le plus fortement pour une germination des graines à l'obscurité au-dessus de 20 °C. La dormance des semences s'élimine généralement de façon progressive lors de la conservation au sec, c'est-à-dire quand les semences sont en anhydrobiose et donc présentent très peu d'activité métabolique. Pour des graines d'*Arabidopsis* dormantes, on peut observer une levée de dormance à 25 °C mais sa cinétique dépend de la température et de l'humidité relative de stockage (Basbouss-Serhal et al., 2016). La teneur en eau et la température de stockage peuvent jouer sur la viscosité intracellulaire des graines et donc sur les processus métaboliques impactant leur physiologie. Ce sont des facteurs clés pour la levée de dormance ou la perte de qualité germinative. D'un point de vue de la physique de la matière condensée, les graines sèches peuvent être assimilées à des matériaux solides amorphes en raison de leur capacité à exister soit à l'état vitreux, soit à l'état caoutchouteux en fonction de leur température et de leur teneur en eau. Après la maturation sur la plante mère, les

graines orthodoxes survivront à une dessiccation sévère en entrant dans un état de repos vitreux (Buitink and Leprince, 2004). Dans un état vitreux amorphe, aucune réaction enzymatique ne peut théoriquement se produire autre que les oxydations ou peroxydations liées aux espèces réactives de l'oxygène (ROS) (Buitink and Leprince, 2008) (Ballesteros et al., 2020). Lorsque la teneur en eau ou la température des graines augmente, le cytoplasme entre dans un état caoutchouteux amorphe qui permet une plus grande mobilité cytoplasmique (Buitink and Leprince, 2008). Les mécanismes de levée de dormance des graines à l'état sec, c'est-à-dire lorsque les graines sont à l'état vitreux, sont en grande partie inconnus. Plusieurs études montrent qu'au cours de la post-maturation, la production de ROS non enzymatiques au sein des graines pourrait participer à l'atténuation de leur dormance (El-Maarouf-Bouteau et al., 2013). La production de ROS est supposée favoriser la signalisation oxydative lors de l'imbibition ultérieure des graines, modifiant ainsi la signalisation hormonale, l'expression des gènes, la régulation redox et l'oxydation des protéines, qui à leur tour augmentent le potentiel de germination (Bailly et al., 2008). A l'inverse, une accumulation excessive de ROS entraîne un potentiel germinatif plus faible et une viabilité plus courte des graines. En conséquence, la teneur en ROS doit rester dans une « fenêtre oxydative » permettant à la fois la levée de la dormance et l'achèvement de la germination. La génération de ROS est donc un enjeu clé pour induire la transition d'un état quiescent d'une graine sèche dormante à un état non dormant. Cependant, l'effet de la mobilité moléculaire sur la libération de la dormance, la diffusion de l'oxygène et la production de ROS non enzymatiques connexes par le biais de réactions d'auto-oxydation dans les tissus des graines desséchées sont encore inconnus.

Les PFA2 sont caractérisés par des propriétés électriques (champ électrique, tension, courant mais aussi espèces chargées comme les ions et les électrons), des propriétés chimiques (génération de radicaux et d'espèces réactives à longue durée de vie, notamment les espèces réactives de l'oxygène et/ou de l'azote), des propriétés radiatives (rayonnements dans les domaines visible et UV) ainsi que des propriétés thermiques. Toutes ces propriétés font des PFA2 des outils polyvalents pour relever les défis de l'agriculture, notamment pour lever la dormance des graines (Lo Porto et al., 2019). Par son potentiel à transférer les ROS vers les graines et à induire une signalisation oxydative, le traitement par plasma froid pourrait jouer sur la fenêtre oxydative, et à son tour lever la dormance physiologique des graines. Le traitement au plasma froid peut agir sur l'homéostasie redox cellulaire, la signalisation antioxydante, l'expression des gènes et le contenu biochimique des graines en améliorant les propriétés de germination et le

développement de la résistance aux stress biotiques, abiotiques et anthropogénique (Starič et al., 2020).

Dans cette étude, un réacteur de décharge à barrière diélectrique (DBD) dans l'air ambiant a été développé pour étudier les effets du traitement plasma sur la physiologie des graines d'*Arabidopsis* à l'état sec et pendant l'imbibition lorsque les processus de germination se mettent en place.

La première partie de ce manuscrit est consacrée à l'étude de l'interaction entre les graines et le plasma afin de comprendre comment la teneur en eau et les propriétés diélectriques des graines d'*Arabidopsis* peuvent modifier les propriétés électriques du plasma d'air ambiant. Dans cette étude, les propriétés de sorption de l'eau des graines d'*Arabidopsis* ont été étudiées en comparant des graines non traitées (groupe contrôle) et des graines traitées au plasma (groupe Plasma). Les isothermes de sorption, donnant la teneur en eau des graines en fonction de l'humidité relative de stockage, ont été tracées en adaptant des modèles théoriques aux points de données expérimentales. Ces modèles ont mis en évidence les conditions du plasma dans lesquelles les paramètres d'hydratation des tissus ont été modifiés, ce qui a conduit à la distinction de trois groupes : faible (3 %), moyenne (10 %) et forte (30 %) teneur en eau. Les variations des propriétés électriques des graines ont été étudiées en fonction de leur teneur en eau (par des mesures de résistance équivalente et de capacité). Ces variations ont également été corrélées aux changements des propriétés électriques de la décharge d'air ambiant en fonction de la teneur en eau des graines pendant le traitement. Dans cette étude, il a été montré que le traitement au plasma modifie les propriétés de sorption de l'eau des graines. En effet, le traitement au plasma a augmenté la quantité d'eau fortement liée à une faible humidité relative et a diminué la quantité d'eau libre à une humidité relative élevée. De plus, en considérant trois groupes de graines caractérisant les trois zones de l'isotherme de sorption de l'eau, il a été montré que la teneur en eau avait un fort effet sur les propriétés diélectriques des graines par une augmentation significative des constantes diélectriques à forte teneur en eau. La présence d'eau libre pourrait être liée à ces changements diélectriques et aux effets sur les propriétés électriques de la décharge de plasma suggérant une accumulation de charge plus élevée pendant le traitement des graines. Cela soulève la question de savoir si la réponse physiologique des graines d'*Arabidopsis* au traitement par plasma peut varier avec la teneur en eau, en particulier à une teneur en eau élevée, et quels pourraient être les mécanismes associés.

Le chapitre suivant porte sur le rôle de la transition vitreuse cytoplasmique sur l'efficacité du traitement plasma sur la levée de dormance des graines d'*Arabidopsis*. L'objectif de ce travail est d'étudier l'effet du PFA2 sur la levée de la dormance des graines d'*Arabidopsis*, en relation avec les propriétés de l'eau dans les tissus des graines. Tout d'abord, le réacteur DBD a été caractérisé par des mesures électriques, par spectroscopie d'émission optique et par spectrométrie de masse en faisant varier la température dans l'enceinte (25, 40, 50, 60 et 70 °C) permettant d'évaluer l'effet de la température sur la décharge et sur les espèces émissives et non-émissives générées. La transition vitreuse cytoplasmique des graines d'*Arabidopsis* a été déterminée par des mesures de microcalorimétrie différentiel à balayage (DSC) qui fournissent des informations sur la viscosité cellulaire et la diffusion cytoplasmique. Un diagramme état-phase a été construit donnant la température de transition vitreuse en fonction de la teneur en eau des graines. Ensuite, les propriétés physico-chimiques des verres intracellulaires ont été modifiées soit en équilibrant les graines dans une gamme d'humidités relatives, soit en modifiant la température. Nous avons évalué l'efficacité du PFA2 sur la levée de la dormance des graines dans ces conditions. Nos résultats ont démontré que l'effet du PFA2 sur la levée de la dormance des graines dépend de la viscosité cytoplasmique des graines. Nous avons identifié deux approches pour améliorer les propriétés germinatives et lever efficacement la dormance des graines d'*Arabidopsis* par traitement au plasma par une modification de leur viscosité cytoplasmique : soit en augmentant la température du plasma d'air à 60 °C, soit en augmentant la teneur en eau des graines à 30 %. Cet effet du PFA2 repose sur la diffusion des gaz pendant le chauffage ou sur la diffusion des ROS et RNS pour les graines à forte teneur en eau ; une viscosité cytoplasmique plus faible semble renforcer l'effet du traitement sur la levée de la dormance des graines d'*Arabidopsis*. Assurer une transition de l'état vitreux à l'état caoutchouteux après la récolte des graines pourrait donc être une condition préalable à l'amélioration des processus de levée de dormance du PFA2. Nos résultats devraient ouvrir de nouveaux domaines d'utilisation du PFA2 pour lever rapidement la dormance des graines des espèces cultivées.

Le dernier chapitre de résultats de ce travail a été consacré à l'étude des mécanismes biologiques et des changements morphologiques déclenchés par le traitement plasma sur les graines d'*Arabidopsis* permettant de lever leur dormance. Précédemment, nous avons démontré et expliqué comment de courts traitements au PFA2 pouvaient lever la dormance des graines d'*Arabidopsis* (Chapitre 3). Dans cette partie, l'étude vise à déterminer si les PFA2 peuvent modifier l'architecture interne des graines qui, à son tour, pourrait faciliter la

diffusion des gaz, en particulier la pénétration des ROS. Les PFA2s pourraient ainsi réorganiser le réseau tridimensionnel complexe des espaces intercellulaires de la graine et modifier la circulation des gaz et leurs échanges avec l'atmosphère (air ambiant). À cette fin, des expériences de micro-tomographie aux rayons X et de transcriptomique ont été réalisées. Des expériences notamment liées aux processus oxydatifs et à la physiologie des graines d'*Arabidopsis* ont été réalisées pour déchiffrer certains mécanismes biologiques déclenchés par les PFA2 qui sont liés à la levée de la dormance des graines. Pour cela, des dosages de peroxyde d'hydrogène ( $H_2O_2$ ) et de Malondialdéhyde (MDA) dans les graines sèches, des mesures de fuites d'électrolytes, de respiration cellulaire (par mesure d'oxygène consommé) et de germination en hypoxie. Une étude transcriptomique a également été menée pour montrer la régulation de l'expression des gènes liée au traitement plasma. Cette étude a permis de révéler une séquence d'événements qui pourrait participer à l'effet bénéfique des traitements plasmas sur la germination des graines d'*Arabidopsis*. En utilisant une variété de techniques, nous avons montré que ces traitements, lorsqu'ils sont effectués à l'état sec, modifient les composantes physiques et biologiques des graines qui sont probablement étroitement liées. Nos résultats valident l'hypothèse de la levée de la dormance des graines par des processus oxydatifs en anhydrobiose et ils montrent qu'une augmentation rapide du statut oxydatif des graines est suffisante pour lever la dormance des graines à l'état sec. Ils identifient également un ensemble d'événements biologiques, y compris des modifications des propriétés membranaires, l'activation de la respiration et des changements dans l'expression des gènes qui sont déclenchés par le traitement des graines sèches par le plasma, mais exprimés pendant l'imbibition des graines. L'ensemble de ces résultats devrait permettre de mieux comprendre les bases cellulaires des traitements plasmas sur la germination des graines et aidera à concevoir de nouveaux protocoles de traitement adaptés à d'autres espèces de graines.

Ce travail apporte de nouvelles découvertes dans l'application des plasmas froids pour l'agriculture et en particulier pour le traitement de levée de dormance. L'approche innovante de cette étude multidisciplinaire a permis de mettre en évidence de multiples mécanismes jouant sur l'interaction entre le plasma et la physiologie des graines. Pris ensemble, ces résultats permettent de mieux comprendre l'interaction entre les graines et le plasma en montrant l'influence de l'état physique du cytoplasme de la graine sur l'effet du traitement et ils mettent en évidence les processus cellulaires et les changements morphologiques associés au traitement par plasma sur la germination des graines. Ceci

## Résumé substantiel

aidera à concevoir de nouveaux protocoles de traitement adaptés à des semences d'autres espèces. Dans le dernier chapitre, les résultats de la thèse sont discutés de manière globale et mis en perspectives avec des pistes de travail pour approfondir les questions issues de cette problématique.





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# **Chapter 1:**

## **General introduction: state of the art**

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# 1. Plasma physics: from cosmos to laboratory towards agriculture applications

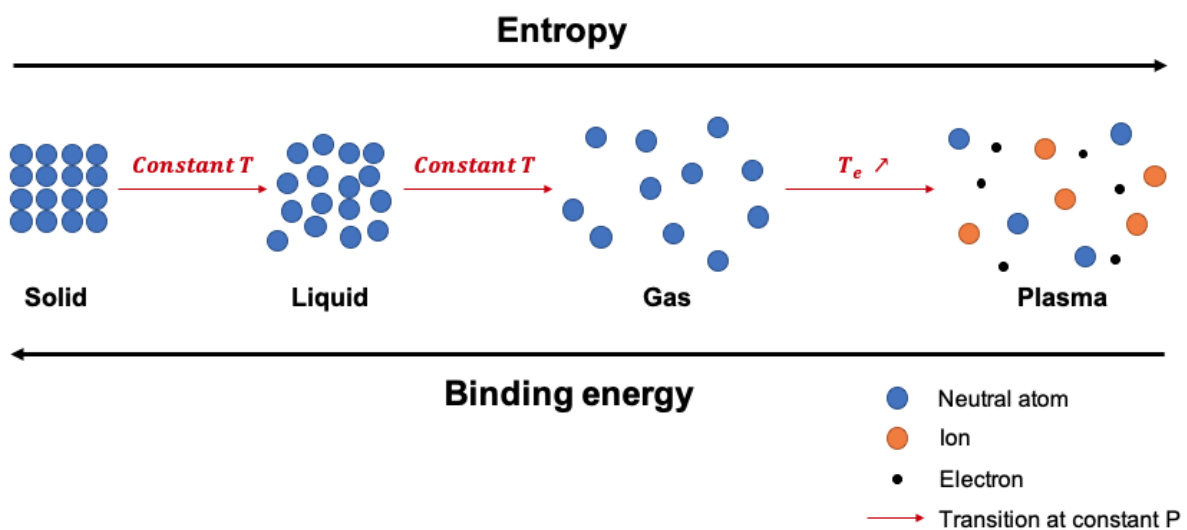
## 1.1. The plasma state: a recent history of matter

First considerations on the conception of matter were developed by the pre-Socratic Greek philosophers Thales, Anaximander and Anaximenes from Miletus (sixth century B.C.). They were the founders of speculative philosophy thinking of the origin and the formation of the Universe passing from myth (mythos) to reason (logos), passing from Cosmogony to Cosmology. One can consider them as the first physicists with their study of nature (philosophy concept of phusis). The distinction between the concept of matter in one hand and the concept of space and time in the other one, started from Ionian physics; matter deploys its structure in space and its dynamics in time. They wanted to define a primary principle (archê) unifying matter properties to explain the formation of Cosmos and the emergence of life. For Thales, water was the source of all things. Anaximander assigned the meaning of the first principle to apeiron: an unlimited substance without qualities. On another hand, Anaximenes (disciple of Anaximander) considered air. Other Ionian philosophers such as Heraclitus opted for fire while Empedocles considered the four elements: earth, water, fire and air (Staszak, 1993). A century later, Leucippus was the first ancient scientist to develop the concept of atomism: matter is composed of fundamental indivisible components called atoms. Nevertheless, these conceptualizations were solely based on speculative physics and presented limitations throughout history. Mathematization and emergence (Castel, 2018) of new concepts and principles in physics, as Archimedes principle or inertia principle of Galileo formalised later by Newton, have permitted a second fundamental distinction between emergent properties of matter (degrees of freedom) and its fundamental properties (irreducible properties, as the inertial mass and the electric charge in plasma physics).

We all know that matter constituting our nearby environment is in the form of three states linked by phase transitions: solid, liquid and gas. It was from the 20<sup>th</sup> century that physicists introduced a fourth state of matter: plasma. A primitive definition of plasma consists to consider it as the state of matter containing a significant fraction of electrical free charges (Rax, 2005). Plasma physics encompasses all the concepts and methods related to this state of matter and was born, strictly speaking, in the 20<sup>th</sup> century. Even though ionised gas in discharge tubes were studied from the 19<sup>th</sup> century, the term plasma was indeed introduced

in 1928 by Levi Tonks (American physicist) and Irving Langmuir (American chemist). This term was used to designate the physical state corresponding to the part of ionised gas containing balanced charges of ions and electrons (Langmuir, 1928). It has been necessary to wait for all the paradigm shifts known in the history of physics, from Newton's law of gravitation to the advent of thermodynamics with its two fundamental principles, to understand matter transformation with concepts of temperature, heat and entropy. And from the development of the formal theory of electromagnetism by Maxwell equations to the postulates of Einstein in the framework of his theory of relativity (gathering the concepts of matter with energy) as well as from the development of Quantum Mechanics to elaborate plasma physics. Plasma theory is indeed closely linked to this history process combining microscopic, mesoscopic and macroscopic manifestations based from the understanding of matter structure and electrical, magnetic and optical phenomena described by electromagnetic and atomic theories.

The plasma state results from ionisation phenomena that can only occur if energy is provided to the gas phase. It is convenient to correlate this energy supply with a rise in entropy. The transformations of a solid in a liquid (fusion), a liquid in a gas (vaporisation) and finally a gas in a plasma (ionisation) to produce free ions and electrons populations are achieved with an increase in entropy (Figure 1).



**Figure 1** - Phase transition of matter in terms of entropy and binding energy.



As the entropy increases, the binding energy which defines the different physical phases becomes increasingly weak (from solid to liquid and from liquid to gas). At a given pressure, a phase transition occurs at constant temperature and is characterised by a specific amount of energy called latent heat according to conventional thermodynamics. However, since the gas-to-plasma transition arises steadily with electron temperature increase, it does not constitute a classical phase transition from a thermodynamics point of view (Burm, 2012). Indeed, the plasma state may - or not - be in the thermodynamic equilibrium and its properties are more described by electromagnetism than thermodynamics.

### 1.2. Features and classification of plasma

A plasma can be considered as an ionised gas composed of electrons, ions, neutral species (atoms, molecules, radicals) and photons. Ionisation of neutrals is necessary and can be achieved either through electromagnetic forces or through thermal transfer. A rise in temperature is not the only procedure to generate a plasma. Ionisation by energetic electron impacts sustained by continuous or alternating electric fields or absorption of energetic photons can also lead to the generation of plasma. Since atomic ionisation energies can change from a few eV to dozens of eV, the typical energy required to generate a plasma is the order of eV/electron, i.e. approximately 11600 K/electron.

In a first approximation, we defined a plasma as a state of matter containing a significant fraction of electrical free charges. However, such unique representation remains as a reductionist point of view of plasma physics (Goldston, 2020). Plasma must be considered as a system including both positive and negative charges, and characterised by two essential aspects: a tendency for disorder due to thermal agitation and a tendency for order due to Coulomb interactions (Ichimaru, 1973). These features bring plasma physics to focus on three principles and parameters which, in turn, permit the description and classification of the wide diversity of plasmas: principle of quasi-neutrality, ideality parameter and ionisation degree.

#### (i) Principle of quasi-neutrality

A plasma is a dynamic and complex system that evolves in such a way as to minimise its coulombic forces which apply between charged particles. It comes down to a charge's rearrangement reflected by an electrical screening. The quasi-neutrality hypothesis is a fundamental concept in plasma physics inherent to collective effects. One can assume that  $n_e = Zn_i$  where  $n_e$  is the electronic density,  $n_i$  is the ionic density and  $Z$  is the charge. A free

charges system behaves as a dielectric. Under an external electric field, such a system spatially organises its charges to create an opposite electric field as a Lenz-Faraday type law: the effects oppose their causes.

The neutrality of plasma can be defined by the following parameter:

*Neutrality parameter:*

$$\epsilon = \frac{n_e - Zn_i}{n_e + Zn_i} \quad (1)$$

The screening phenomenon can result from an electronic response to an intern electronic perturbation. The time scale describing the transition between rapid processes inducing charge separation and slow processes inducing rearrangement is the plasma frequency or Langmuir frequency defined as:

$$\omega_p = \sqrt{\frac{n_0 e^2}{\epsilon_0 m_e}} \quad (2)$$

where  $n_0$  is the homogeneous charge density,  $e$  the elementary charge,  $\epsilon_0$  the dielectric constant and  $m_e$  the electron mass.

The characteristic length of screening resulting from a rearrangement of charges after a charge separation is the Debye length defined as:

$$\lambda_D = \sqrt{\frac{\epsilon_0 k_B T_e}{n_0 e^2}} \quad (3)$$

where  $k_B$  is the Boltzmann constant and  $T_e$  is the electronic temperature. The Debye length is the characteristic length below which a charge separation can exist while the Debye sphere is the corresponding characteristic volume  $\sim \lambda_D^3$ . If  $x \ll \lambda_D$  the hypothesis of quasi-neutrality can no longer be assumed.

## (ii) Ideality parameter

If the quasi-neutrality principle allowed to identify the collective behaviour parameters of plasma (e.g. plasma frequency, Debye length), it cannot describe the competition between the tendency for disorder and the tendency for order occurring in the plasma state. To this end, one can introduce the ideality parameter or plasma parameter  $\mathcal{E}$  defined as the ratio between the average potential energy  $\ll E_p \gg$  of plasma and the average kinetic energy  $\ll E_c \gg$  of plasma.

Let  $n$  and  $T_e$  be the charge density and the electron temperature of plasma respectively. In this system, the number of charges  $N$  in the Debye sphere is such as  $N \sim n\lambda_D^3$ . The average kinetic energy of such a plasma can be estimated thanks to the equipartition theorem as  $\ll E_c \gg \sim k_B T_e$ . As the average distance between charged particles is proportional to  $n^{-\frac{1}{3}}$ , the average potential energy is therefore proportional to  $\frac{e^2}{4\pi\epsilon_0 n^{\frac{1}{3}}}$ .

*Ideality parameter:*

$$\mathcal{E} \equiv \frac{\ll E_p \gg}{\ll E_c \gg} \approx \frac{e^2 n^{\frac{1}{3}}}{\epsilon_0 k_B T_e} \sim N^{-\frac{2}{3}} \quad (4)$$

This parameter allows distinguishing weakly coupled plasmas and strongly coupled plasmas. If  $N \gg 1$ , i.e  $\mathcal{E} \ll 1$ , disorder and kinetic energy are dominant in such weakly coupled plasmas. These classical types of plasma can be considered to be kinetic or ideal in which collective phenomena such as screening effect are significant. Conversely, the condition  $\mathcal{E} \geq 1$  corresponds to strongly coupled and non-ideal plasmas in which the Coulomb potential is dominant and the structure can be fluid or crystalline.

### (iii) Ionisation degree

The principle of quasi-neutrality enables to further define the nature of plasma as a quasi-neutral combination of charged particles with collective behaviours and energetically characterised by the ideality parameter.

As a plasma is composed of charged particles, a key parameter to quantify the ionisation problem under an entropic and statistical aspect is the ionisation degree  $\alpha$ . This parameter is defined as the ratio between the electron density  $n_e$  and the total density of electrons and neutral species  $n_n$ . One can also define it with the ion density  $n_i$  in accordance with the principle of quasi-neutrality.

*Ionisation degree:*

$$\alpha = \frac{n_e}{n_e + n_n} = \frac{Zn_i}{Zn_i + n_n} \quad (5)$$

From this parameter, one can differentiate partially and weakly ionised plasmas for  $\alpha \ll 1$  from strongly and fully ionised plasmas for  $\alpha \approx 1$ .

Depending on the ionisation degree, the distinct species in a plasma (electrons, ions and neutral species constituting the gas phase) will differentiate or homogenise in terms of

collective behaviour and energy. Plasma physics is then divided into two categories: cold and hot plasma.

### **Cold plasma**

The term cold plasma comprises all the weakly ionised plasmas. In most cases, cold plasmas are generated by inducing a discharge in a gas. And the ionisation degree remains weak and may vary from  $10^{-1}$  until  $10^{-7}$  approximately. As a consequence of this inertial difference between light species (electrons) and heavy species (ions and neutrals), cold plasmas are not in thermodynamic equilibrium. The applied electric field will accelerate much more efficiently the electrons which will have a much higher kinetic energy than ions, atoms and molecules (Bittencourt, 2004). For that reason, the electron temperature is totally different from the ion and neutral temperatures.

$$T_i \approx T_n \text{ and } \frac{T_e}{T_i} \gg 1 \quad (6)$$

More precisely, the electron temperature can reach  $10^5 \text{ K}$  while the ions temperature remains equivalent to the neutral's temperature corresponding to the ambient temperature or a tad more in most cases.

### **Hot plasma**

A thermodynamic equilibrium is reached when energy in plasma increases such that the ions kinetic energy becomes equivalent to that of the electrons. The plasma is therefore defined by a unique temperature:

$$T_i \approx T_e \quad (7)$$

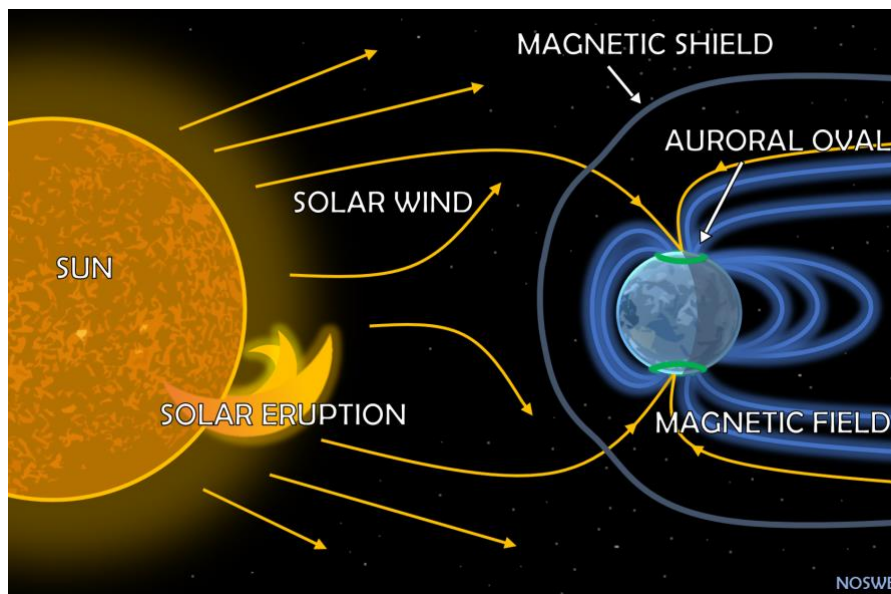
These types of plasma are called hot plasma or thermal plasma in which the temperature can reach dozens of millions of Kelvins.

Both cold and hot plasmas exist in nature but are also produced by humans for industrial applications.

### **Natural plasma**

On Earth, the plasma state is almost non-existent. Earth's matter is dominated by liquid, solid and gas forming our oceans, continents and atmosphere. However, on a cosmic scale, the plasma state represents almost 99.9 % of the physical state of visible matter. Natural plasma represents a wide domain in plasma physics.

Lightning during storms are the typical examples of natural thermal plasma on Earth. The Earth's atmosphere is principally composed of gas. However, from an altitude of the order of hundred kilometres, the UV radiation from the Sun is responsible for the existence of an ionised layer called ionosphere whose charged density is of several tens of thousands of electrons and ions per cubic centimetre. Higher than the ionosphere is the Earth magnetic shield called the magnetosphere which corresponds to a magnetised plasma structured by the Earth's bipolar magnetic field. It is supplied by solar wind (the continuous flow of charged particles from the sun which permeates the solar system) that is responsible for the apparition of aurora borealis on the Earth's poles. As all the stars, the Sun is a big, dense and hot sphere of totally ionised plasma, structured in distinct layers. The rest of the visible Universe is also constituted by a large diversity of plasmas, e.g. as nebulae, neutron stars, pulsars, ...

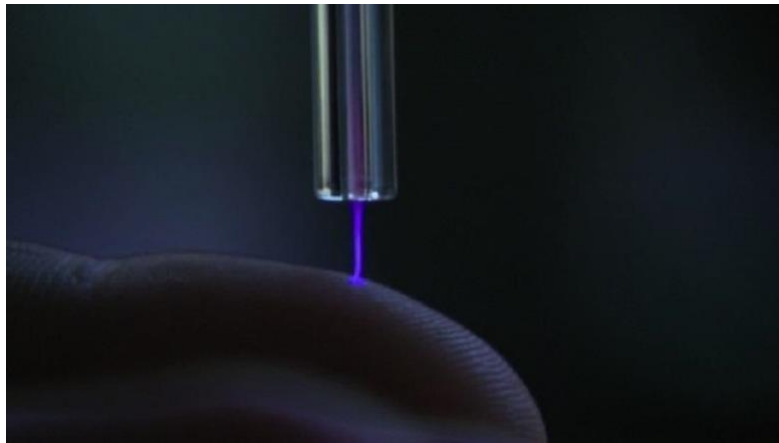


**Figure 2** - Principle of aurora borealis (Northern Lights / Aurora Borealis – Norwegian Centre for Space Weather (NOSWE))

### Industrial plasma

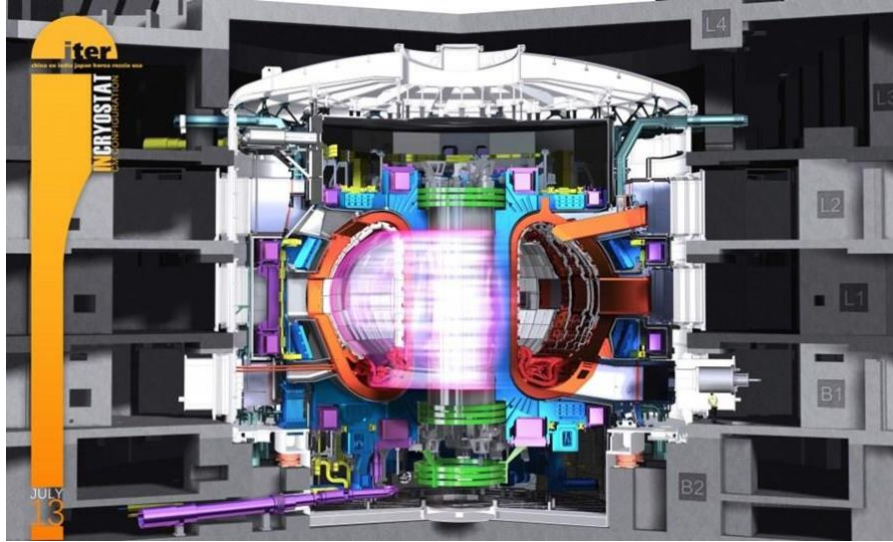
Cold plasmas are studied in laboratories to develop industrial or biological applications thanks to their high chemical reactivity. The plasma physics of electrical discharges depend on various parameters such as the nature of the gas, the pressure (typically from a few mTorr to atmospheric pressure), the features of the experimental setup e.g. geometry and

materials of the electrodes, type of energy source and its parameters such as frequency and voltage (Roth, 2019). In all cases, the electromagnetic energy of low-temperature plasma reactors is converted into other forms of energy: light energy for low-power lighting, kinetic energy for spatial propulsion and chemical energy in which plasma is the source of chemically active species utilised for various industrial applications such as surface treatments (sterilisation, etching, texturing) and gas processing (depollution, syngas production, ...), as well as for applications applied to life sciences and environment (medicine, food processing, liquid activation) (Domonkos et al., 2021) (Bekeschus et al., 2019) (Laroussi, 2005).



**Figure 3** - Cold plasma source for biomedical application ([Le plasma froid, un nouvel espoir contre le cancer | Sorbonne Université \(sorbonne-universite.fr\)](#))

Hot plasma setups are also engineered in laboratories for energy production by two types of processes based on the principle of fusion: controlled thermonuclear fusion and inertial fusion. The thermonuclear fusion of light elements (reaction of Deuterium and Tritium) is developed through devices called *Tokamak* (toroidal geometry). In such a system, the hot plasma is trapped within intense magnetic fields and reaches millions of Kelvin to produce reactions of fusion as it happens in stars. The inertial fusion is based on plasma trapping by applying intense lasers.

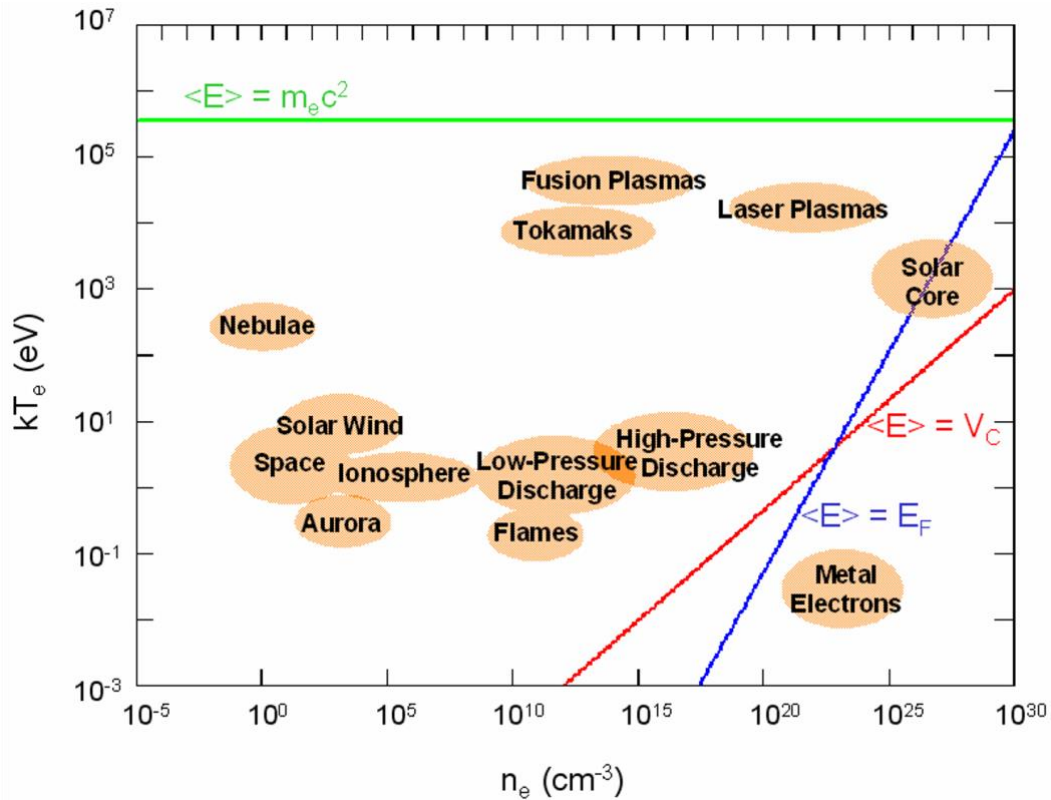


**Figure 4** - Schematic view of the Tokamak core developed for the ITER project ([ITER - une énergie pour notre avenir](#))

### n-T diagram

The main features of the plasma state can be summarised as the electron temperature  $T_e$  and the electron density  $n_e$  or charged density. As represented in Figure 5, the diversity of plasmas can be represented within a unique diagram where the energy  $kT_e$  is plotted as a function of  $n_e$ .

The ideal and non-ideal plasma transition is represented by the red line corresponding to the average Coulomb potential  $V_c = \frac{e^2 n_e^{\frac{1}{3}}}{4\pi\epsilon_0}$ . The non-relativistically and the relativistically plasmas transition is represented by the green line and the transition between non-degenerate and degenerate plasmas is represented by the blue line with the typical Fermi energy  $E_F \sim n_e^{\frac{2}{3}}$ .

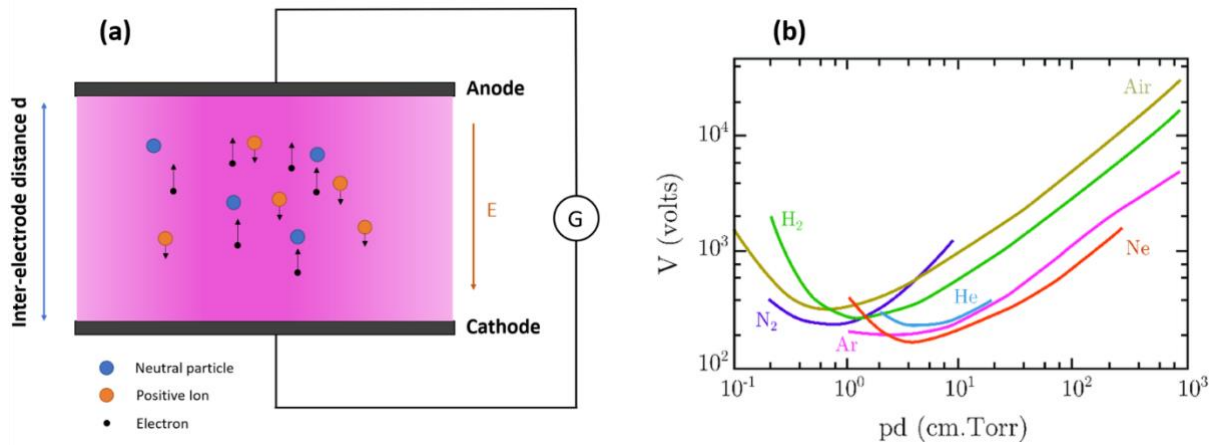


**Figure 5** - Plasma classification in terms of electron temperature and density (Möller, 2006).

### 1.3. Cold plasma physics

Cold plasma developed in research laboratories is the source of many applications in the industry and is used in this thesis for application in seed biology. The generation of cold plasma is based on the physics of gas discharges. The mechanisms for discharge apparition depend on parameters such as the nature of the gas, the nature of the electrodes, the inter-electrode distance and the pressure in which the discharge occurs. A cold plasma electric discharge can be generated by applying a sufficiently high electric voltage to a gas so that the resulting electric field has a value greater than the disruptive electric field of the gas. This was observed by Friedrich Paschen in 1889 who established an empirical law giving the breakdown voltage as a function of the pressure-distance product for different gases (Figure 6) in a plan-plan electrodes configuration.





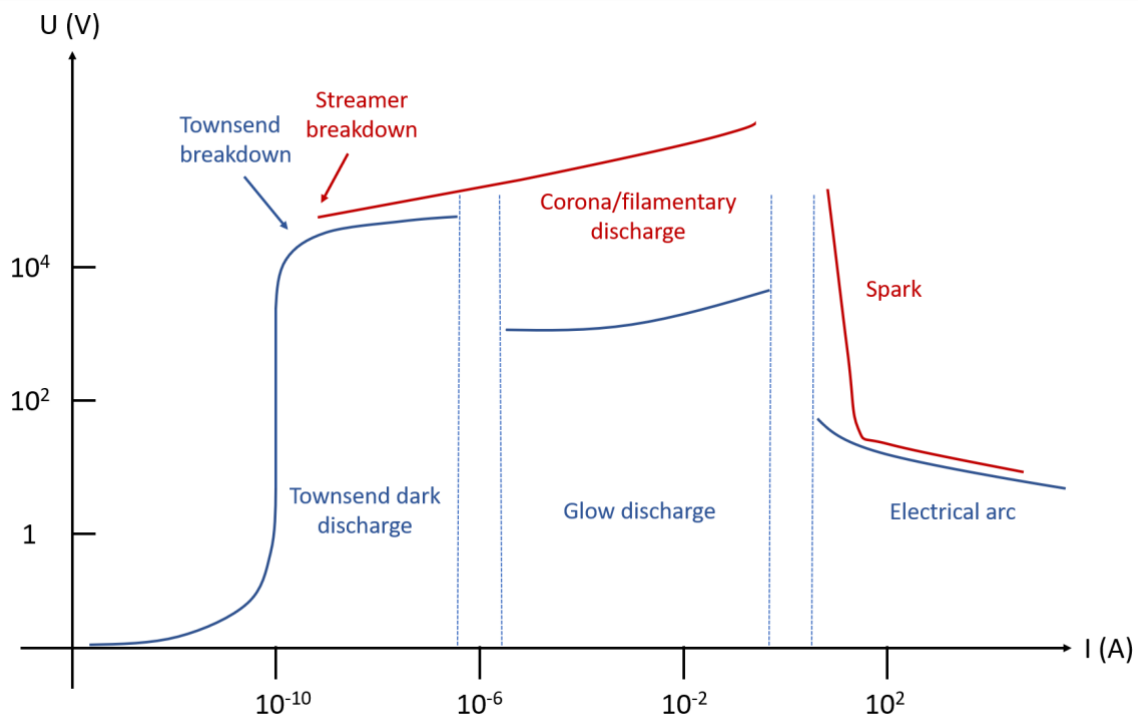
**Figure 6** - (a) Sketch of a plan-plan discharge, (b) Paschen curve: the breakdown voltage ( $V$ , volts) as a function of the product pressure-distance ( $pd$ , cm.Torr) for different gas discharges (Douat, 2014).

From the Paschen curves, one can observe a differentiation in the  $pd$  product depending on the gas nature within the discharge. For each gas, the curve has a minimum delimiting 3 zones in which the discharge behaves differently: (i) pseudo-spark regime for low value of  $pd$  (below  $10^{-1}$  for air discharge), (ii) Townsend regime for  $pd$  product values within the lower limit of the breakdown voltage (between  $10^{-1}$  and 10 for air) and (iii) streamer regime for high  $pd$  product values (above 10 for air). The mechanisms of the two precedent regimes are presented after corresponding to the classical use for industrial and biological applications. The Townsend and the streamer regimes follow a specific current-voltage curve, as shown in Figure 7, both regimes are based on the electronic avalanche phenomenon following the application of a high voltage, whether sinusoidal or pulsed. When an electron acquires an energy higher than the ionisation energy of an atom or molecule, the collision with the electron leads to the production of a second electron which in turn can produce 4 electrons and so on. This avalanche must be sufficient for the discharge to be sustained. The discharge is then capable of being self-sustaining and enters a Townsend or streamer regime depending on the value of the  $pd$  product.

At low pressure, the breakdown process leading to the discharge is described by the Townsend regime. This is represented by the blue line in Figure 7 where four main steps can be defined, namely Townsend breakdown, Townsend dark discharge, glow discharge and electrical arc. Before the Townsend breakdown, the voltage is too low and the regime corresponds to a simple collection of charge still present in the environment (from cosmic rays or pre-ionization). The current saturates and is limited by the production of charged

species up to the Townsend breakdown. Then, the electric acceleration is set up with a charge multiplication by electronic impact inducing an exponential increase of the current: the Townsend dark discharge. The transition between the Townsend dark discharge and the glow discharge is electrically unstable. The glow discharge has the characteristic of a high-voltage, low-current discharge with high optical emission in the visible and ultraviolet range with a complex spatial structure and various applications. The glow-arc transition is observed for high current values characterising a high current low voltage discharge. Electric arc has a thermal origin where thermionic emission dominates and a tendency towards thermodynamic equilibrium is established; the discharge is therefore thermally high (thousands of Kelvin).

Above a given pressure-distance product (characteristic of each gas), the electric field formed by the electron avalanche becomes greater than or equal to the external electric field. The discharge becomes filamentary and is described by a streamer-type discharge (in red Figure 7).



**Figure 7** - Current-Voltage diagram in Townsend regime (blue) at low pressure and in streamer regime (red) at high pressure (adapted from Rax, 2005).

When the discharge is initiated, the ion and electron densities become important due to increase of electron avalanches. If the number of electrons generated by the electron avalanches exceeds a critical threshold ( $N = 10^8$ , (Nijdam et al., 2020)), then a space charge field of the same order of magnitude as the external field is created and the discharge can no longer be described by the Townsend model. At atmospheric pressure, the plasma phase is generally unstable and the streamers easily transit to electric arc which is not suitable for biological applications. To avoid electric arc transition, different solutions can be applied (Fridman et al., 2014) such as short pulsed discharges (Shao et al., 2018) controlling the electron avalanche and limiting the electric field or the use of dielectric barriers placed onto an electrode limiting the discharge current (Xu, 2001). In this thesis, an ambient air cold plasma is generated using a dielectric barrier device (DBD) with two planar electrodes and a dielectric barrier (glass plate) placed onto the high voltage electrode forming a gap of 1 mm. The discharge corresponds to a streamer regime characterised by micro-discharges with a duration of approximately ten nanoseconds and an intensity of about one hundred mA throughout the volume formed by the plasma in the DBD reactor.

### 1.4. Cold atmospheric plasmas as a promising technology for the future of agriculture and seed treatment

Modern agriculture is facing multiple challenges in a changing world. If the world population increases constantly and will reach 9 billion people by the year 2050, one can legitimately ask ourselves how to feed the planet dealing with a growing demand for food and environmental issues. According to the Food and Agriculture Organization (FAO), outbreaks of transboundary animal and plant pests and diseases are increasing, threatening global food chains. Globally, around one-third of all food produced is lost or wasted resulting in losses for farmers and unnecessary pressures on natural resources (*Food wastage footprint*, 2013). Furthermore, these issues occur in a context where global warming, which is becoming increasingly intense, affects many ecosystems and has consequences for agricultural production and yields (Thornton et al., 2014), particularly through the arrival of extreme environmental conditions and abiotic stress (Pereira, 2016).

Seed germination and vegetative growth are two key factors of yield crop which are also affected by major climatic events such as drought, soil salinization and floods. Plant and crop production are vulnerable to physical and biological stress. Soil pollution and

declining soil quality are affecting people and ecosystems worldwide, exacerbated by climate change but also by human activities. If seeds provide approximately 70 % of the global food, it is necessary to develop innovative solutions for seed treatment in order to overcome environmental challenges and to replace the agrochemicals used today, which increase soil pollution and the loss of arable land. Improvement in crop germination potential and seedling growth rate generally leads to high crop productivity. Cold plasma appears today as a promising technology used to improve seed germination and respond to the issues raised by the Plasma Applications for Smart and Sustainable Agriculture COST Action (PIAgri-CA19110). The PIAgri action aims to bring together studies from European and global laboratories to develop the potential of cold plasmas as a green alternative for seed treatment, plant treatment, agricultural wastewater treatment, replacement of conventional agricultural processes and to study ongoing plasma treatment processes. The action aims to be innovative and unconventionally constructed to give a new dimension to sustainable agriculture with lower chemical impact, higher yield and sustainability.

Seeds are the most important means of reproduction for higher plants and represent the only mobile form in plant life cycle with pollen. The main functions of seeds are the transfer and distribution of plant genetic material in time and space.

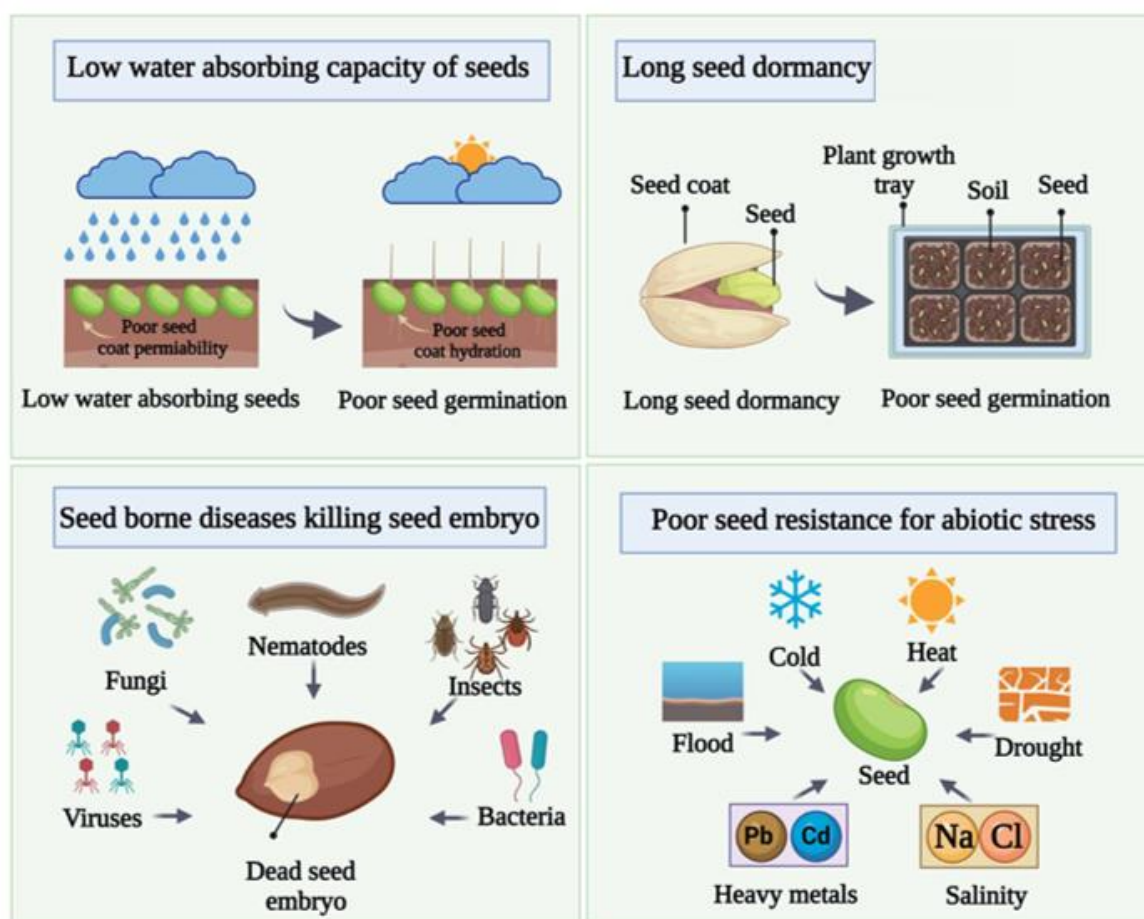
In this context of climate change and environmental degradation due to human activity leading to the depletion of resources, the destruction of ecosystems and the loss of biodiversity, seeds represent a natural capital that is essential to meet environmental and societal challenges for the restoration of ecosystems, the preservation of species and the improvement of the quality of life through a more sustainable and healthy agriculture. It is therefore necessary to find new solutions to replace conventional agricultural practices that have an impact on the environment in order to maintain and improve the quality of seeds. The main challenges associated with seed quality and seed germination are (Figure 8):

- (i) Low water absorbing capacity of seeds: water uptake, the first step in germination, can be impacted by environmental factors in response to heat stresses affecting soil quality and temperature and therefore playing on the permeability of the seed coat (Zinsmeister et al., 2020).
- (ii) Long seed dormancy: this species-intrinsic seed adaptability trait can be influenced by endogenous and external factors. Crops can be affected by

dormancy, reducing crop production, delaying seedling emergence and leading to non-uniform germination.

- (iii) Seed borne diseases killing seed embryos: infection of seeds by pathogenic organisms (viruses, bacteria, fungi) alters seed quality by reducing germination vigour and yields by up to 90 % (Gebeyaw, 2020). Seed health is therefore essential for good farming and good harvests.
- (iv) Poor seed resistance for abiotic stress: drought, salinity, heat waves, cold waves and exposure to heavy metals are all abiotic stresses to seeds that affect average crop yields by more than 50 % (Yadav et al., 2020). Metabolic and physiological responses, molecular signalling pathways, ecophysiology and crop breeding are being studied to understand and respond to the mechanisms of these stresses (Chen et al., 2020).

To meet these challenges, existing solutions are based on the use of plant protection products, pesticides and chemical molecules that do not meet the environmental challenges. Several European actions, in particular the European obligation set by Directive 2009/128/EC, and at national level with the Ecophyto plans, respond to these challenges in order to provide a common framework and achieve a reduction in the use of plant protection products for an agriculture compatible with sustainable development. These plans aim to accelerate the withdrawal of the most environmentally damaging substances, to promote the dissemination of biocontrol, to reinforce the prevention of public exposure to pesticides, to support research and innovation and to accompany farmers in the ecological transition. The development of plasma processes to meet these challenges would be a solution and respond to these environmental policies (Waskow et al., 2021).



**Figure 8** - Seed germination challenges: low water absorption, long seed dormancy, seed-borne diseases and abiotic stress factors (Adapted from Shelar et al., 2022)

## 2. Arabidopsis seed: from anhydrobiosis to germination processes

### 2.1. *Arabidopsis thaliana*: a plant model in biology

In this thesis, the effect of cold plasma on seed physiology is studied on a model species in biology: *Arabidopsis thaliana*. *Arabidopsis thaliana* is a species belonging to the Brassicaceae family. It is the species most commonly used in plant and seed biology for molecular genetic studies because of its unique characteristics. Indeed, *Arabidopsis* has a small genome concentrated on 5 chromosomes, completely sequenced (Kaul et al., 2000) and composed of about 120 Mb of DNA, which is 4 and 20 times smaller than rice and maize respectively. It has a rapid reproductive cycle (3 months maximum), a relatively modest size (15-20 cm for the plant and ~ 0.1 mm for the seed) and abundant seed production (about

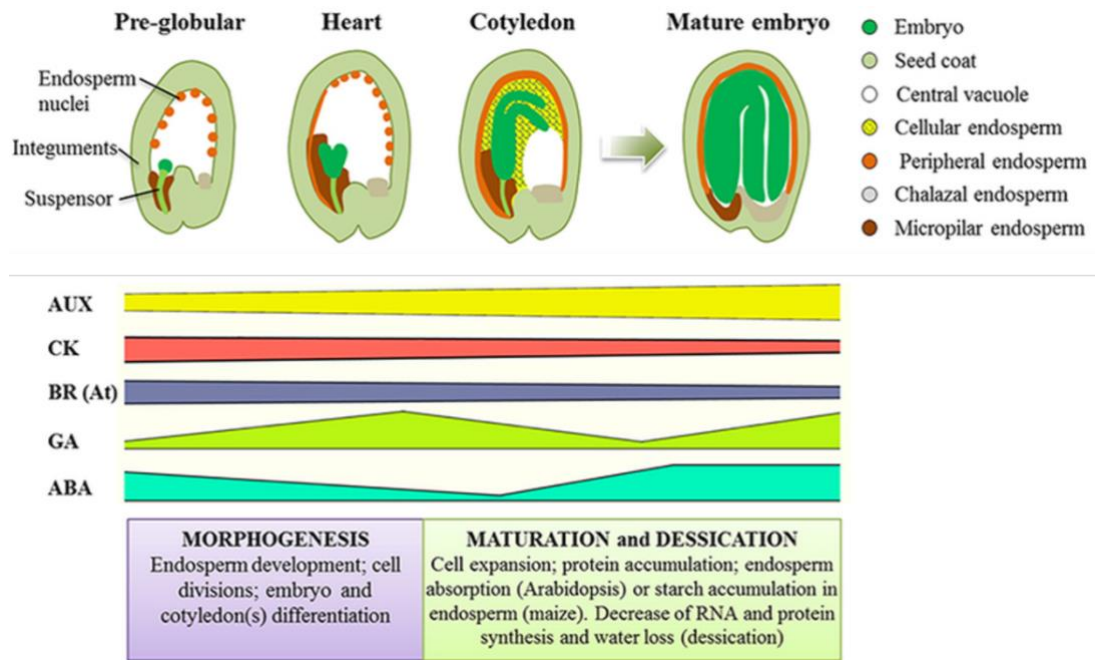
5000 per plant) (Bewley et al., 2006). The scientific community chose *Arabidopsis thaliana* as a model organism in plant biology almost 40 years ago. This has enabled the development of the rapidly growing fields of genetics and molecular biology within plant science (Koornneef and Meinke, 2010). It is important to continue the knowledge of this species, and therefore this model was adopted in this thesis to understand the effects of cold plasma on seeds as well as possible.

### 2.2. Seed development and architecture

Seeds are complex structures to study with a large variability in size, shape, colour, external and internal structure. Seeds are the units of dispersal and propagation of spermatophytes (seed plants) divided into two categories: gymnosperms producing naked seeds, in which the embryo is surrounded by endosperm and protective tissue (conifers and related clades, about 750 species) and angiosperms whose seed is protected within the ovary giving the fruit from a double fertilisation producing additional nourishing tissue (flowering plants, about 250 000 species). Seeds are advantageous for plants with the maintenance of dormancy until appropriate environmental conditions arise and contain adequate food supply until photosynthesis is possible. Variability in seed shape also exists within a given species and is referred to as seed polymorphism. Characteristic of polymorphic seeds is that they differ not only in shape or colour, but also in their germination behaviour and dormancy. Every seed lot is equal to a seed population.

Seed develops after the fertilisation of female gamete by male gamete in the embryo sac of the ovule in the ovary of the flower. Like all orthodox seeds, the development of *Arabidopsis* seeds varies in three phases depending on environmental conditions (Baud et al., 2002): (i) embryogenesis or morphogenesis characterised by cell divisions and elongation (increased water content), (ii) accumulation of reserves (decrease in water concentration by expansion of reserve-containing cells) and (iii) late maturation (desiccation). The development phases are presented in Figure 9 in terms of embryo development stages and hormonal variation. The morphogenesis phase is characterised by a period of embryo growth through cell division processes during which storage products accumulate in embryo and surrounding tissues (seed filing). The histodifferentiation occurs, seed tissues are formed. Then, the embryo ceases to grow during the maturation phase and seed coat is formed: layers issued from maternal integuments differentiate to form the protective tegument. Maturation ends with a phase of desiccation and dormancy induction after the accumulation of the reserves (Gutierrez et al., 2007).





**Figure 9** - Schematic seed development in Arabidopsis with embryo development stages and trend of hormone accumulation during seed development. Seed development takes place in 2 important stages: morphogenesis characterised by cell division and differentiation of the embryonic axis and cotyledons, and maturation & desiccation characterised by cell expansion and reserve filling. The main hormones playing on the embryo development are auxin (AUX), cytokinin (CK), brassinosteroids (BR), gibberellins (GA) and abscisic acid (ABA). (Adapted from Locascio et al., 2014).

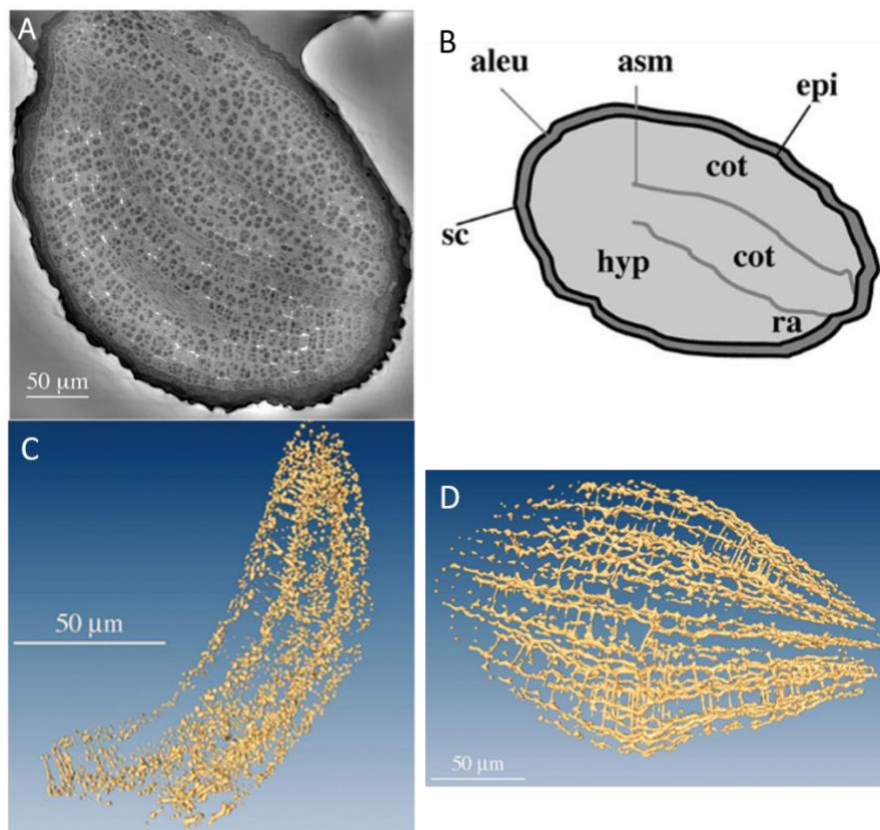
The mature seed of Arabidopsis is composed of three types of endosperm: the single-cell layered endosperm, the micropylar endosperm surrounding the embryo radicle, and the chalazal endosperm. Embryo contains all information for plant development. Food store is composed of nutritive reserves for seed germination: carbohydrates, proteins and lipids. Seed coat separates and protects internal seed content from the environment (coat pigments determine seed colour).

The trend of hormone accumulation during seed development indicates an antagonistic evolution for the level of auxin (AUX) and cytokinin (CK); the first increases during the seed development showing a crucial role whereas the second decreases progressively during the maturation phase after the end of cell division. The brassinosteroids (BR) show the same trend of CK with a strong decrease at the end of maturation. The evolution of gibberellins (GA) over time is characteristic of two stages of seed development: a first peak favouring cell expansion and differentiation of the embryo and a second peak at the end of maturation



to favour germination. Absciscic acid (ABA) functions in opposition to GA and inhibits GA-induced processes. The hormonal balance between the two at the end of maturation will regulate germination processes.

*Arabidopsis* seed architecture and infrastructure have been highlighted by Cloetens et al. (2006) using quantitative phase tomography and revealing seed structures as the hypocotyl and the cotyledons but also intercellular air space within seed tissues forming a pore network in which circulate gases, allowing oxidative signalling and water uptake mechanisms during imbibition (Figure 10). This 3D network reconstructed for the hypocotyl and the cotyledon in Figure 10.C and 10.D, could store oxygen and serve to provide the necessary oxygen triggering germination processes as a transport system for gas exchange in embryos. The network could also serve as a support for the transport of water during imbibition.



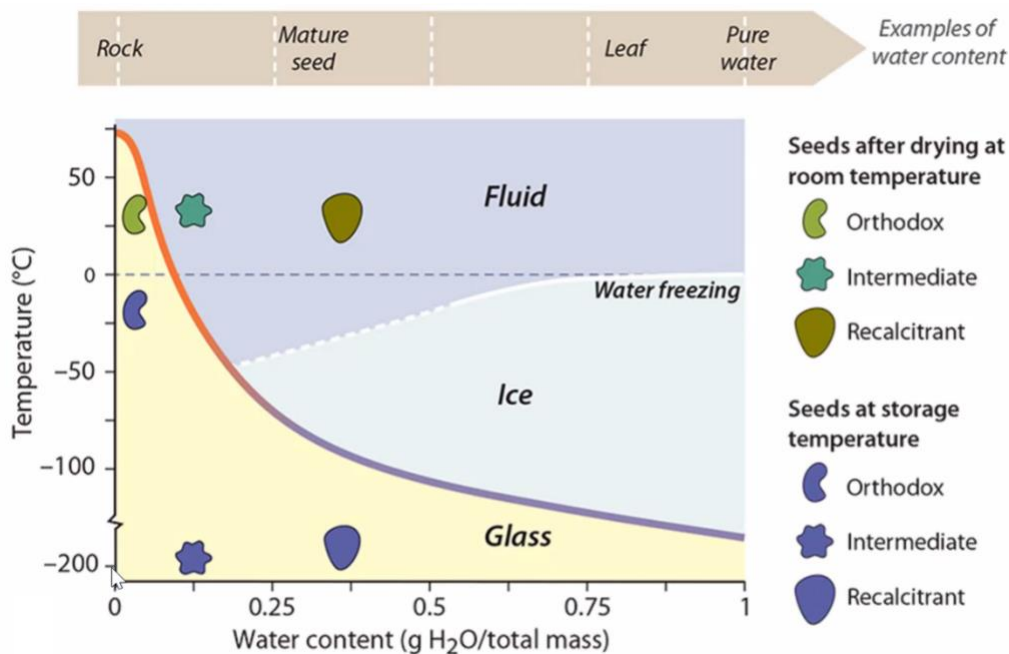
**Figure 10** - *Arabidopsis* seed architecture. (A) longitudinal slice of an *Arabidopsis* seed reconstructed by quantitative phase tomography. (B) Schematic representation of the longitudinal slice. 3D rendering of intercellular air space in the hypocotyl (C) and in a cotyledon (D) highlighted by slices analysis. (Adapted from Cloetens et al., 2006).

### 2.3. Loss of water: metabolism in the dry state (anhydrobiosis)

Seed can be divided into two categories according to their ability to resist desiccation:

- (i) Orthodox seeds which survive drying and acquire high stress resistance with desiccation (water content around 5-10 % at room relative humidity and temperature).
- (ii) Recalcitrant or desiccation sensitive seeds which do not survive drying and cannot be stored for long periods without losing viability (e.g. oak, coconut seed, ...).

From the orthodox group, one can distinguish oily seeds and non-oily seeds from the crop species. The oily one can be dried up to 3-4 % of water content. Examples of oily seeds are rape, sesame, peanut, sunflower, soybean and so on. Non-oily ones can be dried up to 5-7 %. Examples are bean, rice, amaranth, buckwheat, chilly, lentil etc. From the non-orthodox group, two types of species can be considered: intermediate seeds which can tolerate drying up to 12 % as papaya, citrus or coffee and recalcitrant seeds which cannot tolerate drying down to 20 % as mango, cacao, jackfruit or litchi. (Paudel and Poudel, 2020).



**Figure 11** - Orthodox, recalcitrant and intermediate seeds phase diagram showing boundaries between fluid, ice and glass in relation to temperature and water content (<https://saveplants.org/>)

Seeds can be considered as amorphous solid materials owing to their non ordered atomic structure at medium and long range. This particularity means that the tissues of the seeds can vary from a glassy state to a rubbery state (behaving like a fluid) or even in an ice state. This state depends on the temperature and water content of the seeds; it can be characterised by a phase diagram (Figure 11).

For orthodox seeds, tissues are generally in a glassy state and the seed survives in a state of anhydrobiosis. Anhydrobiosis refers to the remarkable ability of some organisms to survive the loss of water and enter into a “quiescent” state in which their metabolism comes reversibly to a standstill. These organisms can survive for a long period of time (even years, decades and centuries). These organisms can be found in animals as well as in plants. Known examples from the animal kingdom are nematodes, tardigrades and rotifers that are able to change shape to enter in lethargy. In plants, there are also some examples such as resurrection plants, lichens or orthodox seeds. Orthodox seeds are indeed capable of surviving during years with a very low metabolism. *Arabidopsis* seeds, the object of study of the Thesis, are part of these organisms. Anhydrobiosis in orthodox seeds can be related to their reduced metabolic activity after their maturation on the mother plant. However, metabolism in such a state remains unknown (Bewley and Black, 2013).

In the dry state, seeds are metabolically inactive (water is not available for biochemical reactions), number of organelles per cell falls, vacuoles deflate, food reserves become dense crystalline bodies, highly tolerant to environmental conditions and can remain viable for long periods, e.g. 2000-year-old date seeds actually produced healthy plants (Sallon et al., 2008). The formation of the glassy state implies very strongly slow molecular mobility (Hoekstra et al., 2001). The remaining water adopts different properties characterised by inhibited or very strongly slowed down degradation reactions, an ametabolic state and new physicochemical properties of the seed.

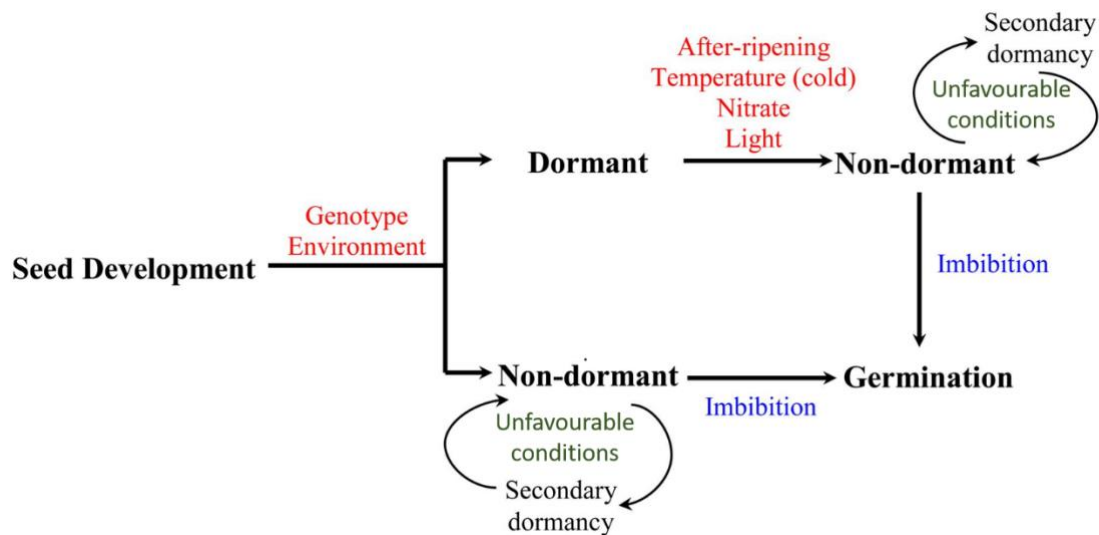
### 2.4. Seed dormancy

In many orthodox seeds, the glassy state allows the species (as *Arabidopsis*) to enter in a physiological dormant state. Seed dormancy can be classified in two major categories (Baskin and Baskin, 1998):

1. Exogenous or coat-imposed dormancy which is Physical Dormancy (PY): physical growth constraint, limited permeability to water and oxygen, limited release of inhibitors from endosperm and seed coat.
2. Endogenous or embryo dormancy of three types: Morphological Dormancy (MD) characterised by an underdeveloped or immature embryo, Physiological dormancy (PD) characterised by a physiological inhibition and Morphophysiological dormancy (MPD).

Seed dormancy can be combined as PY and PD. PD is the most abundant form in seeds of gymnosperms, all major angiosperm clades and in most model species (Finch-Savage and Leubner-Metzger, 2006), including *Arabidopsis thaliana*, *Helianthus annuus*, *Lactuca sativa*, *Lycopersicon esculentum*, *Nicotiana spp.*, *Avena fatua* and several cereals.

Dormancy is defined for viable seeds by the inhibition of their germination under a priori favourable environmental conditions such as temperature, light/dark or water availability (Bewley, 1997). This non-germination of seeds depends on the intrinsic characteristics of the species but also on the habitats and development conditions. There are two kinds of dormancy: primary and secondary dormancy. Primary dormancy develops during the maturation process of the seeds on the mother plant (Bewley and Black, 1994) as in the case of the *Arabidopsis* seeds used in this study, which shows physiological dormancy. Seeds with primary dormancy can also enter a germination inhibition process during imbibition under environmental conditions that are unfavourable: this is the secondary dormancy, as illustrated in Figure 12 (Hilhorst et al., 2010). This second dormancy operates as a safety and survival process during imbibition following conditions such as high substrate salinity, hypoxic conditions (Hoang et al., 2013), prolonged exposure to darkness, far red, blue or white light (Herron et al., 2000).

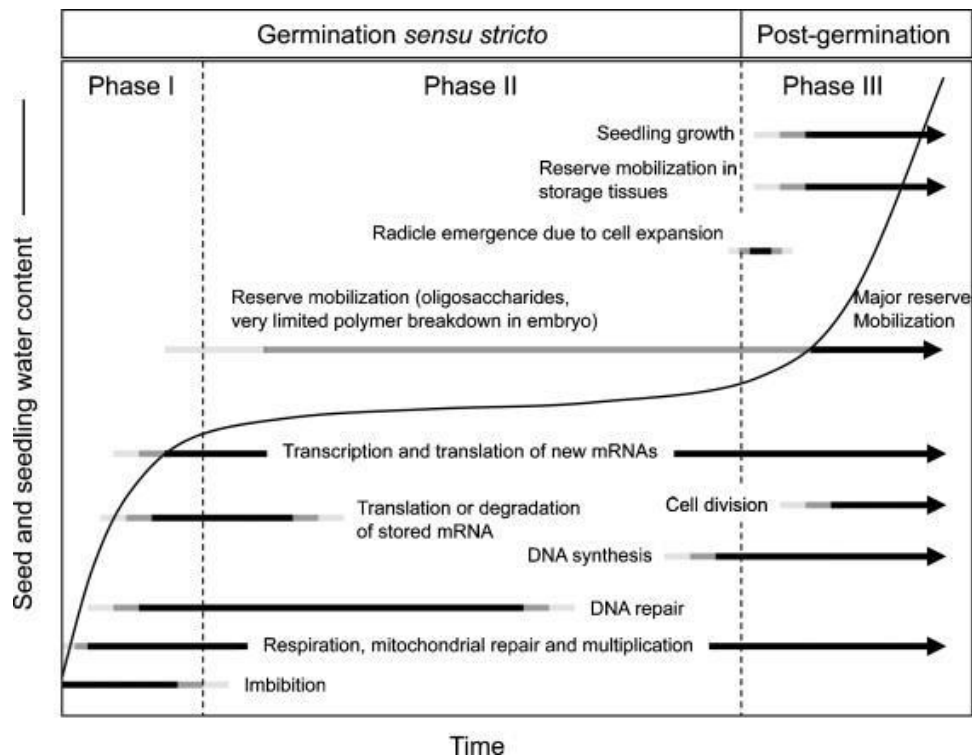


**Figure 12** - Influence of genetic and environmental factors on the regulation and control of seed dormancy and regulation. (Adapted from Gao and Ayele, 2014).

Both types of dormancy are regulated by hormonal signalling networks involving the germination inhibitor ABA (abscisic acid) and the growth regulator and germination stimulator GA (gibberellic acid) (Nambara et al., 2010).

## 2.5. Seed germination

After the release of dormancy and in appropriate environmental conditions (in terms of water abundance, oxygen availability and temperature), seeds acquire the ability to germinate during imbibition. Germination occurs when the seed absorbs enough water to rupture the seed coat. Aerobic respiration is also crucial via oxygen consumption. Temperature regulates germination through enzyme-controlled processes and in some cases, seeds may require exposure to light and even fire (Santana et al., 2010). Germination leads to the elongation of the embryonic axis from a seed, allowing subsequent seedling emergence.



**Figure 13** - Timing of the main events associated with seed germination and subsequent post-germination growth occurring from a few hours to several weeks depending on the species and the germination conditions (Bewley, 1997).

Seed germination begins with reactivation of metabolism through water uptake during imbibition and finishes with the protrusion of the radicle (Weitbrecht et al., 2011). As shown in Figure 13, the water uptake and the resulting change in metabolism leading to germination take place in three phases. Phase I and Phase II define the germination *sensu stricto* and Phase III is the post-germination. Rapid water uptake occurs in the initial phase associated with the hydration of all seed tissues and the beginning of physiological activities. During Phase II, seed water content is fairly constant and metabolic activities increase with substantial transcription of new genes, the synthesis of new proteins, respiration and mitochondrial repair and multiplication. Radicle emergence through the surrounding structures at the end of this phase marks the completion of germination. An increase in water uptake takes place at the post-germination phase in which major seed stored reserves are mobilised to establish young seedling.

## 2.6. External factors regulating germination and dormancy

Germination and dormancy are highly dependent on the environmental factors, especially light, oxygen, temperature and seed water content (Corbineau and Côme, 1995).

### *Temperature*

Temperature is an important parameter that affects reactions triggering germination processes. Its effect on germination depends on the species, the varieties and the geographical distributions. Each species is sensitive to germination over a temperature range characterised by minimum, optimum and maximum values (Probert, 2000). The minimum temperature is determined by following the germination at low temperature but can be complicated to properly estimate by the very slow germination rate. The optimum temperature is identified for the fastest germination. The maximum temperature is related to protein denaturation and generally does not exceed 40 °C for all species. For *Arabidopsis*, the temperature range is between 5 and 25 °C with an optimum at 15 °C. Dormancy is expressed above 20 °C.

### *Oxygen*

Oxygen is an important element for the regulation of seed germination. Indeed, the mechanisms of respiration and availability of oxygen during imbibition are essential factors in triggering processes leading to germination. In the dry state, the diffusion of oxygen through the tissues of the seeds can trigger oxidation reactions linked to the aging of the seeds by lipid oxidation, oxidation of proteins or even the decline of antioxidants such as glutathione,  $\alpha$ -tocopherol, and  $\gamma$ -tocopherol (Seal et al., 2010). These mechanisms would play a role in maintaining or breaking seed dormancy during storage under controlled conditions and are dependent on the vitreous state of the cytoplasm. Oxygen diffusion has been shown to be facilitated by the cytoplasmic glassy state in *Pinus densiflora* seeds (Gerna et al., 2022) leading to oxidative reactions and physiological change of seeds. During seed imbibition, oxygen consumption increases and cellular respiration acts on germination. This oxygen requirement is also dependent on environmental factors such as temperature, water potential and light (Corbineau and Côme, 1995). The availability of oxygen in the soil is therefore a factor limiting seed germination by modulating the respiratory activity of mitochondria and metabolism (Borisjuk and Rolletschek, 2009).

### *Water content*

Germination process starts during seed imbibition by activating metabolism from a quiescent state. Water is therefore the first necessity for the acquisition of germinative potential. In a context of global warming, the availability and water intake of seeds are therefore environmental factors affecting germination mechanisms in different



geographical areas of the world (Mondoni et al., 2012) (Gareca et al., 2012). On the other hand, if water is essential for orthodox seeds for germination, its presence in excess can have an opposite effect by limiting the availability of oxygen (Kozłowski, 2012). Too sudden variations in water availability and relative humidity during the germination phases can lead to seed degradation and the activation of pathogens that inhibit germination.

### *Light*

While most seeds do not require light to germinate, light remains an important factor that can affect germination. In addition, light plays a crucial role in the response of plants to their environment. The reception of light signals from seeds and plants varies according to the climate by the alternance of seasons, day and night. These variations can regulate germination and affect the maintenance or breaking of seed dormancy (Yan and Chen, 2020). Light can trigger molecular mechanisms that control the maintenance and release of seed dormancy, as well as seed germination, and regulate hormone metabolism and signalling pathways often linked to ABA and GA (Yang et al., 2020).

## 2.7. Role of ROS in the regulation of dormancy and germination

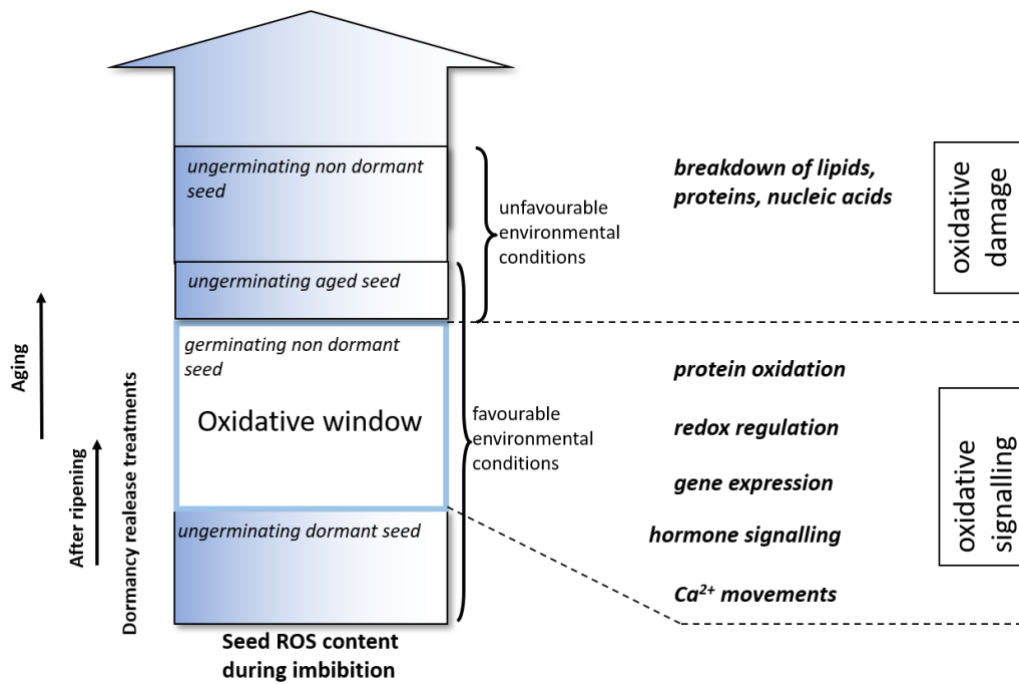
Because of their essential role in molecular signalling, ROS are considered inherent to the response of plants and seeds to their environment. Several studies have shown that during seed after-ripening, the production of non-enzymatic ROS could participate in their dormancy attenuation (El-Maarouf-Bouteau et al., 2013). ROS production is thought to promote oxidative signalling during subsequent seed imbibition, thereby altering hormone signalling, gene expression, redox regulation and protein oxidation, which in turn increase germination potential (Bailly et al., 2008). Conversely, excessive ROS accumulation leads to lower germination potential and shorter seed viability caused by significant oxidative damage, including breakdown of lipids, proteins and nucleic acids. Consequently, ROS content must remain within an "oxidative window" (Figure 14) allowing both the release of dormancy and the completion of germination. ROS generation is therefore a key issue in inducing the transition from a quiescent state of a dry dormant seed to a non-dormant state and associated with germination completion in many seed species as maize, wheat as well as sunflower.

The role of ROS in the hormonal balance between ABA and GA during germination processes have been highlighted in many studies (Ishibashi et al., 2017) (Tuan et al., 2018) (Bahin et al., 2011). If dormant seeds have high ABA/GA ratio, oxidative signalling leads to



a crosstalk between these two hormones with an increase in the level of GA and decreasing the amount of ABA allowing the seed to trigger germination processes. Seed germination is governed by a complex interplay between hormonal pathways determining germination and ROS metabolism. Some specific ROS have been demonstrated to be related with germination processes through molecular and hormonal signalisation. For example, hydroxyl radical ( $\text{OH}\cdot$ ) may be involved in endosperm weakening via polysaccharide degradation through Fenton reaction (Müller et al., 2009). Several studies have also show the role of nitric oxide (NO) as a signalling molecule leading to germination (Arc et al., 2013a) (Bethke et al., 2006) (Bethke et al., 2004), which may act as a common actor in the crosstalk between ABA and ethylene (Arc et al., 2013b). Recently, NO has been shown to be part of a molecular interaction network in Arabidopsis linked to the perception of external environmental signals and internal hormonal signals, in particular with GA through the expression of the EXPANSIN (EXPA) gene, which is linked to cell wall loosening and promotes germination through GA production (Xu et al., 2020).

It is well referenced that  $\text{H}_2\text{O}_2$  is a signalling molecule in plants and especially in the release of seed dormancy interplaying synergistically or antagonistically with plant growth regulators and hormones such as auxins, cytokinin as well as ABA and GA (Nazir et al., 2020). ROS are versatile molecules with toxic effects but they also play a beneficial role in cell signalling of living organisms and seeds. Although their mechanism of action is not yet fully understood, they are at the heart of the physiological regulation of seed germination and dormancy breaking. By direct oxidation of biomolecules, ROS allow the release of seed dormancy during dry storage. They also participate in the perception and transduction of environmental conditions controlling germination during imbibition. They act on hormonal signalling, regulating germination processes through cell expansion events.



**Figure 14** - The dual role of ROS in seed physiology described by the oxidative window model. Seed germination is regulated by the amount of ROS which should remain in the oxidative so as not to be deleterious. (Adapted from Bailly et al., 2008).

## 2.8. Seed dormancy release

After the maturation on the mother plant, seed can enter in a dormant physiological state blocking the processes of germination. There are several methods for breaking seed dormancy, whether embryonic and physiological or integumentary and physical and whether natural or artificial. The mechanisms involved in dormancy release by seed after-ripening are described in this part. Seed after-ripening corresponds to the release of seed dormancy after maturation on the mother plant by a period of seed storage in the dry state under controlled temperature and relative humidity conditions. During this period, physiological changes appear allowing the acquisition of the ability to germinate. This period is related to oxidation processes and non-enzymatic activities leading to change in physiology in which ROS play an important role (Née et al., 2017) (Bazin et al., 2011). After-ripening is the natural way of breaking seed dormancy during seed storage after harvest. Seed after-ripening can lead to decrease in ABA level and sensitivity and an increase of GA sensitivity or loss of GA requirement, the ability of seeds to germinate in darkness (no requirement of light), an increase in seed sensitivity to light, an increase of germination velocity and a promotion of coat and endosperm rupture.

**Table 1** - Some examples of effective conditions of seed after-ripening of various species (adapted from Baskin and Baskin, 2020).

Species	Water content (%DW)	Temperature (°C)	Time
<i>Austrostipa elegantissima</i>	7.1 – 13.2	23	6 months
<i>Avena fatua</i>	10 – 20	40	3 months
<i>Bromus tectorum</i>	7 – 8	20, 30	4 months
<i>Carthamus tinctorius</i>	7.2 – 8.8	24.7	8 months
<i>Conostylis candicans</i>	11.8	23	36 months
<i>Euphorbia esula</i> L.	2.6	30	4-8 months
<i>Helianthus annuus</i>	5	20	1 week
	4	15, 20	3 weeks
<i>Heteropogon contortus</i> (L.) P.Beauv. ex Roem. and Schult.	5.7	30	12 months
<i>Hordeum vulgare</i>	12	38	4 months
	12	27	6 months
<i>Oryza sativa</i> (weedy rice)	6 – 14	25	1.5 months
<i>Oryza sativa</i> (weedy rice)	10.8	30	2 months
<i>Physaria fendleri</i> (A. Gray) O’Kane and Al- Shehbaz	4.75	35	3 months
<i>Stylidium affine</i> Sond.	5 – 6.1	23	3 months
	8.1 – 12.	23	3 months
<i>Zygophyllum fruticosum</i> DC.	5.7	30	12 months
	5.7	45	10 months

The effectiveness of after-ripening depends strongly on the storage conditions (temperature, relative humidity and water content of the seeds) which can affect the oxidation processes linked to the physiological change and which can be deleterious and affect the quality of the seeds or induce a secondary dormancy. Examples of suitable conditions of seed after-ripening of various species are listed in Table 1 which have been established in several studies (Cruz et al., 2013; Oba et al., 2017; Briggs et al., 1994; Allen

et al., 1995; Leopold et al., 1988; Bazin et al., 2011; Gianinetti and Cohn, 2008; Turner et al., 2009; Baldos et al., 2014; Foley, 2008; Commander et al., 2009).

Other dormancy release techniques can be applied to initiate germination processes. One can cite stratification (cold chilling), use of light and temperature, treatment with ethylene or GA as well as scarification process. Seed stratification involves soaking seeds at low temperatures to alleviate dormancy in a wide range of species (Bewley et al., 2013). This method mimics the processes of seeds being dispersed in autumn experiencing low temperatures over winter thus triggering the ability to germinate in warmer seasons. Temperature therefore influences the regulation of seed dormancy as well as the light that can be used to fully release dormancy (Finch-Savage et al., 2007). Seeds can also be treated with germination regulating hormones such as ethylene or GA. Ethylene is a plant hormone and an essential compound that acts as a regulator in the signalling processes of plant and seed development promoting germination and dormancy breaking (Jurdak et al., 2020) (Corbineau et al., 2014).

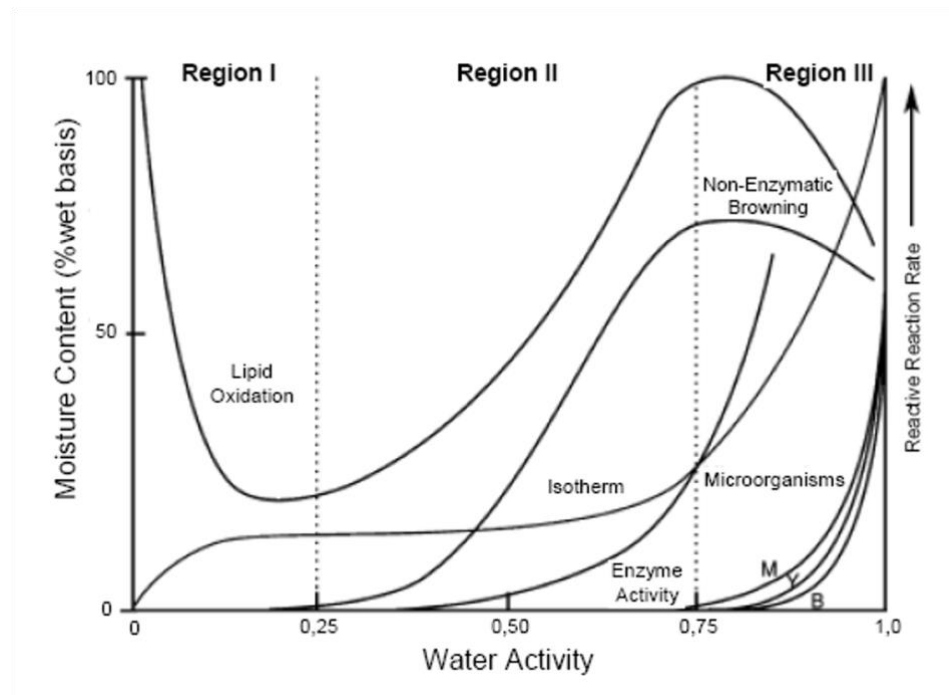
### 2.9. Role of water status and temperature in seed metabolism

As previously seen, water content and temperature play a crucial role in seed after-ripening. One can link this change in physiology to the physical state of the cytoplasm that can exist in the glassy or rubbery state depending on temperature and water content. For orthodox seeds, most often cytoplasm is in a glassy state for normal conditions of temperature and relative humidity (Buitink and Leprince, 2004). This allows the seeds to survive desiccation for long periods of time. Figure 15 describes food and vegetable material deterioration during dry storage as a function of water content and water activity ( $A_w$ ) or relative humidity  $R_H$  ( $A_w = R_H/100$ ) which may be applicable to seed metabolism (Schmidt, 2004). This gives the relative rate of different reactions occurring as a function of water content and water activity.

The relationship between water content and relative humidity is given by the water sorption isotherm. Water sorption isotherms are used to characterise the states of water and the mechanisms of water sorption through tissues (Vertucci and Leopold, 1987a). The curve gives the quantity of water retained by a product (in this case seed) at equilibrium for a given temperature as a function of the relative humidity of the storage atmosphere. It is obtained by placing seeds in a series of hermetically sealed containers over a wide range of relative humidities obtained with saturated salt solutions (Vertucci and Leopold, 1987b). Isotherms are highly specific to biological samples due to their physical and chemical structure and depend on the storage temperature. This relationship makes it possible to distinguish 3

zones associated with regions of water fixation and water binding sites (Figure 15). Region I, at low  $R_H$ , is associated with water strong binding sites. This area reveals a molecular monolayer on which water is strongly bound to the polar groups of certain compounds. This zone generally corresponds to a moisture content of less than 10 % for seeds and allows a high degree of storage flexibility. If lipid oxidations are favoured, there is no enzymatic activity or microbial growth and no non-enzymatic browning. Region II, at medium  $R_H$ , is associated with weakly bound water sites. Water molecules are increasingly free to attach to multilayers becoming available as solvent and reagent for oxidations and electron transport. In this zone, the storage capacity becomes less stable with the initiation of enzymatic activities and non-enzymatic browning. Region III, at high  $R_H$ , is associated with free water in which mitochondrial respiration and other metabolic processes take place. Storability becomes very low and is affected by enzymatic and non-enzymatic reactions and by the development of microorganisms such as bacteria (B), molds (M) and yeasts (Y).

The state of the water and the temperature therefore affect the metabolism during dry storage by oxidative mechanisms, enzymatic and non-enzymatic reactions or by respiratory activities and the development of micro-organisms. Control of temperature and relative humidity conditions can break seed dormancy but also induce aging and loss of viability (Zhang et al., 1995) (Alhamdan et al., 2011) (Bazin et al., 2011) (Basbouss-Serhal et al., 2016) (Hay et al., 2022).

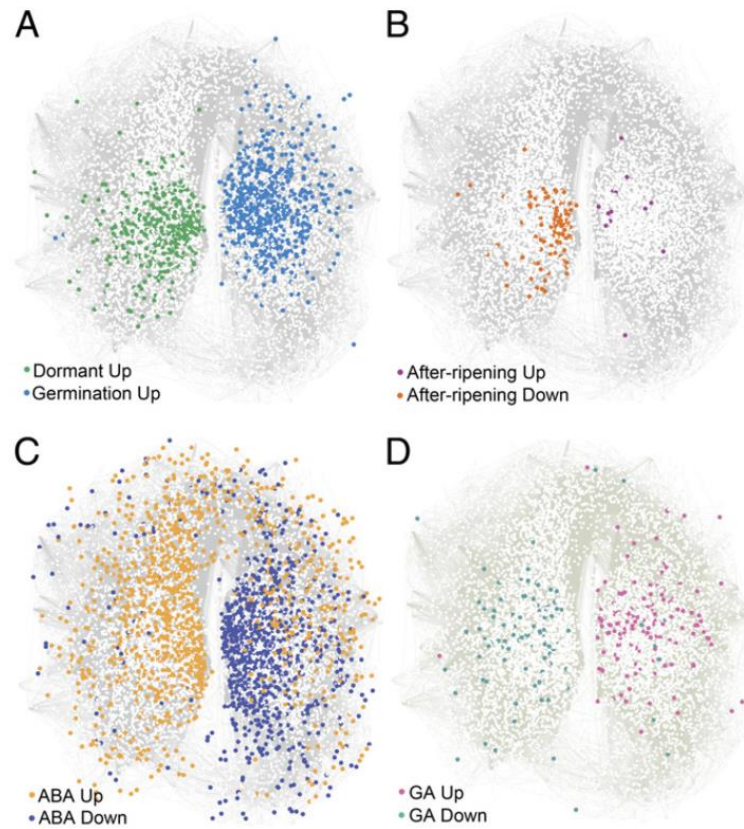


**Figure 15** - Food and vegetable material deterioration during storage in terms of lipid oxidation, water sorption isotherm, non-enzymatic browning, enzyme activity and microorganisms (M: molds, Y: yeasts, B: bacteria) as a function of moisture content and water activity (Schmidt, 2004).

## 2.10. Transcriptional regulation of seed germination

The regulation of gene expression reflects the processes of the development and the reactivation of the cellular metabolism of seeds during imbibition. The development of transcriptomics allows to analyse the transcriptional activity of seeds associated with the germination process from the dry state to imbibition. The expression of genes associated with germination can be analysed through transcriptome analysis and reveals differences between dry and imbibed seeds (Bewley et al., 2013). Such studies reveal thousands of genes that are differentially expressed during the different imbibition phases associated with seed germination. The expression of genes involved in hormone regulation and in the production of signalling proteins and enzymes related to cell wall modification has been demonstrated during seed germination. New omics approaches have enabled the generation of co-expression networks that describe gene regulation and signalling pathways involved in seed germination and dormancy (Bassel et al., 2011) and longevity (Verdier et al., 2013). Figure 16 shows the SeedNet co-expression network of genes associated with *Arabidopsis* seed dormancy and germination developed by Bassel et al. (2011). One can examine topology of the distribution up- and down-regulated genes

associated with dormancy and germination (Figure 16 A), after-ripening (Figure 16 B), ABA application (Figure 16 C) and GA application (Figure 16 D).



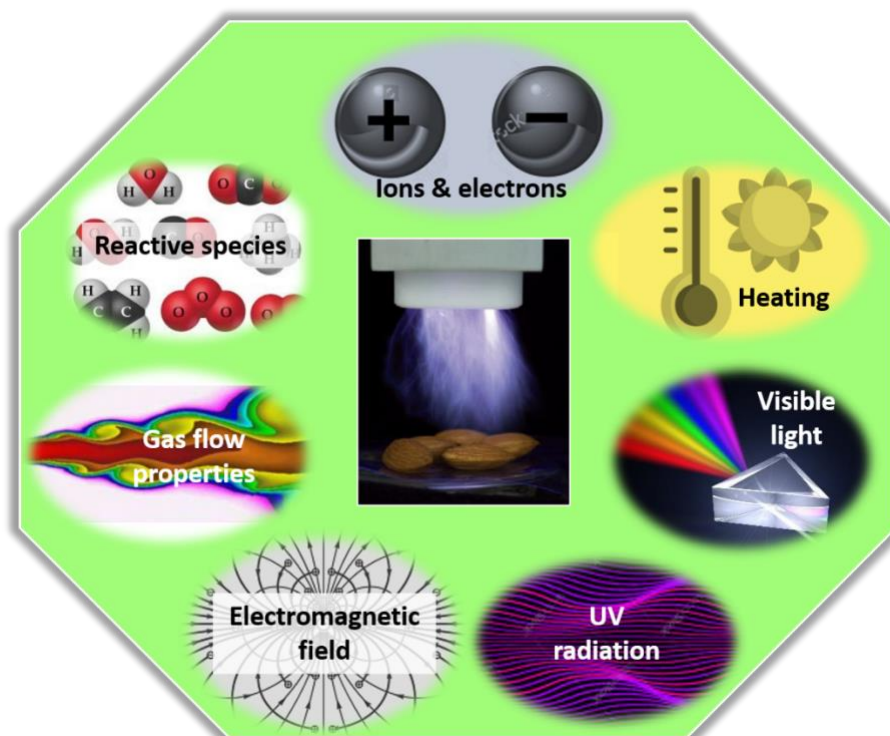
**Figure 16** - The SeedNet network topology of the regulation of germination associated with the distribution of genes related to (A) dormancy (green) and germination (blue), (B) after-ripening up-regulated (purple) and down-regulated (orange), (C) ABA up-regulated (tawny) and down-regulated (blue), (D) GA up-regulated (pink) and down-regulated (green) (Bassel et al., 2011).

This network will be used to illustrate and determine the effect of treatment on the regulation of treatment-specific gene expression that may be associated with dormancy breaking and germination process initiation.

## 2.11. Cold plasmas as a dormancy release tool

As illustrated in Figure 17, cold atmospheric plasmas are characterised by electrical properties (electric field, voltage, current but also charged species like ions and electrons), chemical properties (generation of radicals and long lifespan reactive species, especially reactive oxygen and/or nitrogen species), radiative properties (radiations in the visible and UV ranges) as well as thermal properties. All these properties make cold plasmas versatile tools to meet challenges in agriculture, especially for releasing seed dormancy (Lo Porto et al., 2019). Through its potential to transfer ROS to the seeds and induce oxidative signalling, cold plasma treatment could play on the oxidative window, described in Figure 14, and in turn release the physiological dormancy of seeds. Effects of cold plasma treatment on germination and dormancy release may depend on seed dormancy type. For PY dormancy, caused generally by impermeability of the seed coat (Kelly et al., 1992), the possible effect may lie in the induced change of surface seed properties. For PD dormancy, plasma treatment can lead to a regulation of ROS homeostasis and change in metabolism through phytohormone balance to release dormancy (Adhikari et al., 2020). However, the mechanisms of such possible effects remain unknown. In this thesis, the use of cold plasma in the ambient air as a tool for breaking dormancy allowed to investigate the potential of this technique and the mechanisms linked to breaking of dormancy, in particular by oxidative processes or even structural changes.





**Figure 17** - Cold plasma and its main physico-chemical properties, likely to interplay with seeds upon their treatment.

### 3. Overview of plasma technology for agriculture and seed treatment

#### 3.1. From electroculture to plasma agriculture

The use of electricity in agriculture began more than 200 years ago and can be considered as the beginnings of plasma-agriculture. Indeed, between 1780 and 1787, Abbé Pierre Bertholon wrote a trilogy on the effects of electricity on plant growth: “de l'Électricité dans les végétaux” (Bertholon, 2017). He found that electricity influenced the germination, growth, flowering, fruiting, transpiration and respiration of plants. He developed what he called “electro-vegetometer”, a device for taking “natural electricity from the air” to enrich the soil. This early work led to many experiments on the use of electricity in agriculture, which are non-exhaustively described below, and may have some influence on the development of today's plasma applications for agriculture. The use of atmospheric electricity has also emerged since the 19<sup>th</sup> century by the Finnish scientist K.S. Lemström who introduced, in 1885, the “artificial overhead discharge” to enhance agricultural

productivity (Sidaway, 1975). The idea came to him through the observation of rapid vegetation growth during the short growing season in the Arctic and sub-Arctic regions; attributing this apparent growth enhancement to the particular electrical characteristics of the atmosphere at high latitudes. He developed an electrical discharge from wires suspended above the plants inducing significant growth stimulation drawing the attention of the global scientific community. He published his work in 1904 in "Electricity in Agriculture and Horticulture" (Lemström, 1904) giving details about the benefit effect on harvest and favourable change of plant chemical compounds. This work marked the beginning of 20<sup>th</sup> century "electro-culture" where many studies on the effect of electricity and electrified atmospheres on plants have emerged. For example, experiments on the effect of low current on barley seed growth were achieved in 1923 (Blackman et al., 1923). The use of a needle electrode above the growing barley seedling was investigated and it was found that the exposure has a beneficial effect on the early hours of growth.

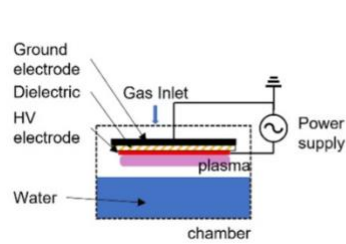
History of the use of atmospheric electricity and air ions on plant growth showed overlap with some modern studies in plasma agriculture. Since the first reported observation of plasma as "radiant matter" in 1879 by Sir William Crookes and the nomination of such state of matter as "Plasma" by Irving Langmuir in 1927, the electro-culture seems to lead research until the first patent on plasma application for agriculture and seed germination in 1994 by Krapivina *et al.* enhancing the germination of soybean seeds with a plasma at atmospheric pressure. From the beginning of the 2000s, the theme of plasma agriculture emerged throughout the globe with different approaches. The diversity of cold plasma treatment processes and methods sometimes lacks clarity to be able to identify common ways of understanding the processes of interaction between the plasma and the treatment sample and its effect on physiology. Process parameters such as exposure time, power, gas and discharge types are important aspects to consider to ensure treatment efficiency and to consider satisfactory commercial applications. Processes using cold plasma are now being investigated in research applied to agriculture for the plasma-activation of water (PAW) that can be used for irrigation of seedlings (Bradú et al., 2020). Besides, direct treatments of seeds and plants are developed for the improvement of germinative properties and the decontamination of pathogens through morphological changes, gene expression and biochemical responses (Attri et al., 2021b). In the following, the various reactors and plasma processes for seed treatment existing in the literature and the effects induced on the physiology will be discussed.

### 3.2. Plasma reactor and processes

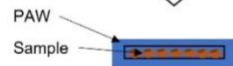
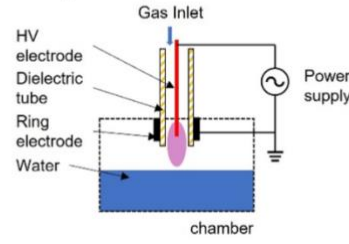
Since the development of plasma-agriculture in recent decades, many plasma processes have been developed by research teams for seed treatment. One can distinguish direct treatment processes (seeds directly exposed to the discharge) and indirect treatment processes (post-discharge treatment or PAW).

#### 3.2.1. Interaction plasma-liquid and activation of chemical species for agronomy applications

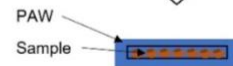
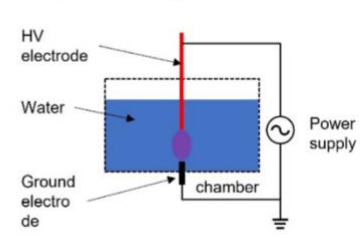
The use of PAW for seed treatment is an indirect approach since the seeds are not directly exposed to the plasma discharge but only to the long lifespan reactive species contained in the aqueous medium. Hence, plasma treatment of water affects the electrical conductivity and pH properties, and induces the generation of long-lived species such as nitrite ( $\text{NO}_2^-$ ), nitrate ( $\text{NO}_3^-$ ), ozone ( $\text{O}_3$ ) or hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The controlled activation of these species and the transformation of water properties can trigger signalling pathways during seed imbibition that promote germination and seedling development as well as the inactivation of microorganisms. Figure 19 shows three types of processes for activating water by plasma, such as the use of surface DBD, plasma jet DBD and pin-to-pin discharge. In the literature, these types of treatments can be found for agricultural applications (Hadinoto et al., 2021) (Liu et al., 2016) (Judée et al., 2018) (Puač et al., 2018b). This indirect approach has also been developed for hydroponic agriculture (Gierczik et al., 2020) and aquaculture (Čech et al., 2020).

**Indirect treatment using plasma-activated water (PAW)****Surface DBD**

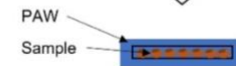
Post process

**DBD plasma jet**

Post process

**Pin to pin discharge**

Post process



**Figure 19** - Sketch of plasma devices developed for indirect seed treatment using plasma-activated water (PAW): surface DBD, DBD plasma jet and pin to pin discharge (adapted from Priatama et al., 2022).

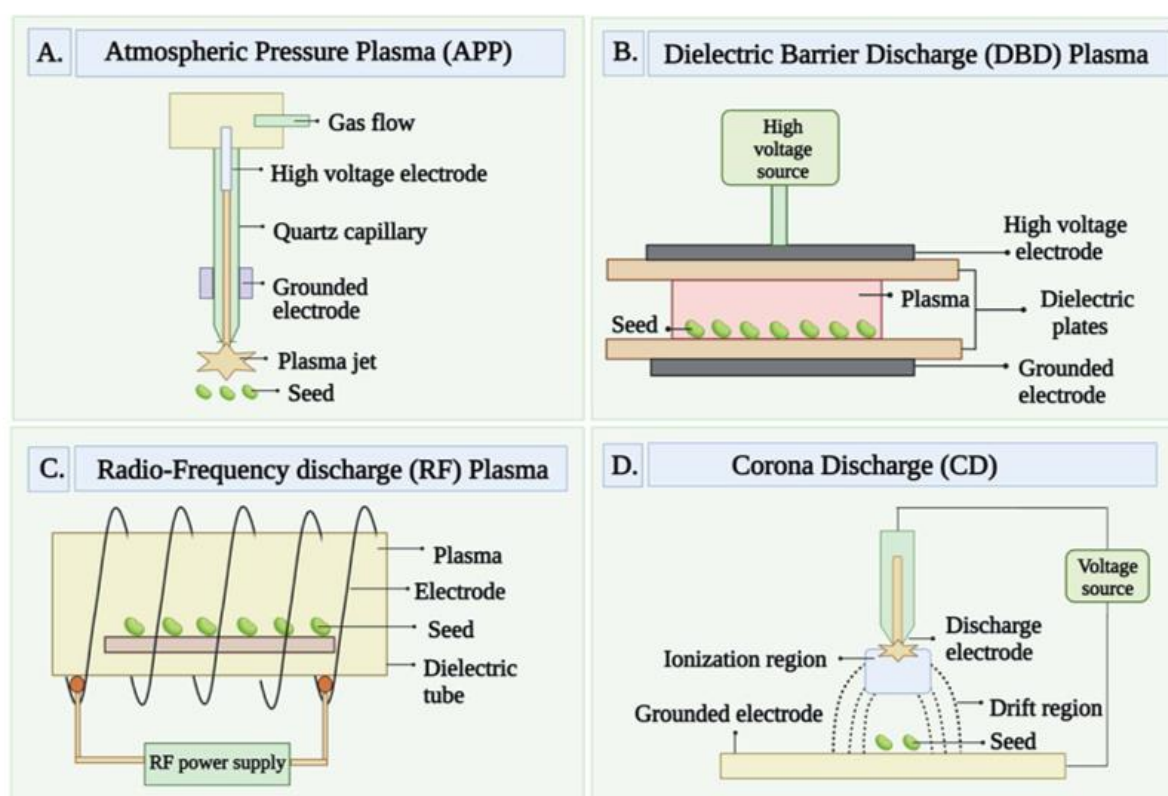
Nitrate and nitrite levels as well as water acidification are considered to be responsible for improved seed and plant development but also for the decontamination of pathogens. PAW is thought to operate as a fertiliser related to the role of nitrogen (Puač et al., 2018a). The chemical characterization of PAWs as well as the study of their effects on seeds and plants is being intensively researched in the scientific community for potential applications in agriculture. However, there are still many questions related to the lack of knowledge and hindsight on the potential benefits, the effectiveness over time and all the mechanisms of operation. Some reviews can be found in the literature on the production and use of PAW in agriculture (Guo et al., 2021) (Gao et al., 2022).

### 3.2.2. Plasma treatment of dry seeds

Generally, plasma seed treatment is carried out in the dry state at atmospheric pressure by directly applying plasma to the seeds for improving germination and inactivating pathogens. Among these processes, a distinction can be achieved between direct and indirect treatments. Direct treatments consist of exposing the seeds to the plasma discharge - whether a glow or a filamentary discharge - and therefore to all its components including radiation, excited atomic and molecular species, radicals, reactive species, electrons, ions, temperature and electric field. Indirect treatments consist of exposing the seeds to an afterglow or a post-discharge, so that the plasma properties are essentially limited to the long-life chemical species so that thermal damages or etching are drastically reduced. In this thesis, the process used is a direct treatment on *Arabidopsis* seeds. Figure 20 shows 4

typical examples of direct seed treatment processes, namely Atmospheric Pressure Plasma jet (Zhou et al., 2016) (Adhikari et al., 2020), Dielectric Barrier Discharge, Radio-Frequency discharge and Corona Discharge (Puligundla et al., 2017) (Kim et al., 2017). The most common processes are DBD and RF discharges and they can be found in a multitude of studies (Meng et al., 2017) (Li et al., 2017) (Stolárik et al., 2015) (Sadhu et al., 2017) (Park et al., 2018) (Li et al., 2016) (Mildažienė et al., 2019) (Guo et al., 2017) (Pauzaite et al., 2018) (Ling et al., 2014), covering a large number of species such as wheat, peanut, pea, mung beans, barley, sunflower as well as Norway spruce and so on.

DBD reactors, as used in this study, consist of two electrodes, at least one of which is covered by a dielectric barrier so as to prevent any arc formation in the inter-electrode gap. The short duration (microsecond) of the micro-discharges generated by DBD are responsible for gaseous temperature that remains typically lower than 50 °C, which makes this process suitable for seed applications and to a larger extent to other life science applications. Cold plasma in DBD is often obtained by applying sinusoidal high voltages ranging from low frequencies (50 Hz) to high frequencies (100 kHz).



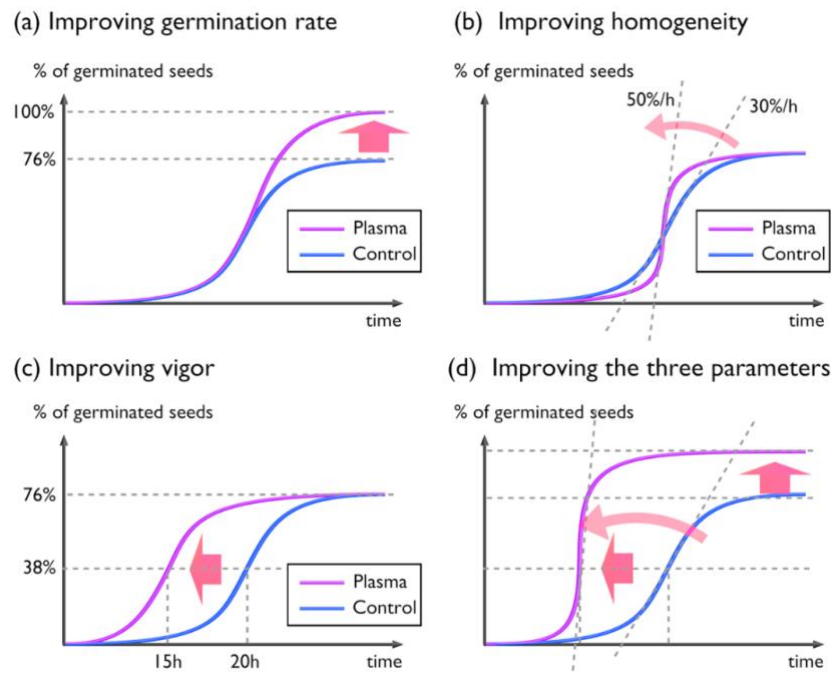
**Figure 20** - Sketch of plasma devices developed for direct seed treatment: Atmospheric Pressure Plasma (APP), Dielectric Barrier Discharge (DBD) plasma, Radio-Frequency discharge (RF) plasma and Corona Discharge (CD) (Shelar et al., 2022).

### 3.3. Cold plasma effects on seeds

In this thesis, the effects of plasma treatment with a DBD reactor are studied on the physiology and morphology of *Arabidopsis* seeds. The different mechanisms of such changes found in the literature are presented in this part. Seeds are in a very dynamic organic state undergoing many biochemical changes over time. Knowledge on seed biochemistry and physiology is crucial for understanding the effects of plasma on germination parameters, seed structure, biochemical and molecular responses through oxidative and hormonal signalling, stress response development and genetics, as discussed in this section.

#### 3.3.1. Effect on seed germination parameters

Improving the germination properties and furthermore the quality of seeds as well as ensuring seed diversity are fundamental issues for tomorrow's agriculture. To evaluate the effects of cold plasma on germination, it is mandatory to monitor different germination parameters so as to compare control seeds and plasma-treated seeds. The parameters of the germination curves on which the plasma treatment (as priming treatment) is applied are described in Figure 21 (Dufour et al., 2021). The plasma treatment can induce the increase of the total germination rate (by primary or secondary dormancy release mechanisms or simply increase the germination properties of a seed lot) but also improve the homogeneity of germination or accelerate the germination speed and play on the germinative vigour. Finally, it is expected that a same plasma treatment could have beneficial effects on these three parameters.



**Figure 21** - Sketch diagrams of comparison between control seeds and plasma-treated seeds germination curves with enhancing of (a) germination rate, (b) homogeneity, (c) vigour and (d) the three parameters (Dufour et al., 2021).

Improvement of the germination rate and the germination speed are the main effects that one can find in all studies (Ling et al., 2015) (Mitra et al., 2014) (Los et al., 2019). No effect on the germination homogeneity was reported in the literature. Plasma treatment therefore appears to be a good tool for improving germination properties. These beneficial effects can be attributed to several mechanisms found at different biological levels impacting seed physiology.

### 3.3.2. Cold plasma effects on seed structure

In many studies, part of the effects induced by cold plasma are attributed to changes in the surface properties of the seed coat. Effects such as etching or morphological changes can be observed by scanning electron microscopy (SEM) while the grafting of oxidised functions on the seed coat can be evidenced by X-ray photoelectron spectroscopy (XPS) (Li et al., 2017). Similar effects, characterised by the deformation of the apparent and typical structures of the envelope and the appearance of cracks, were observed in a similar way (Meng et al., 2017) (Rahman et al., 2018) (Adhikari et al., 2020). It turns out that too much exposure to plasma could completely alter the morphology of the seeds and that these effects are species-dependent, i.e. depending on the plasma composition and



integumentary forms, a same plasma exposure will drive to different biological effects. Studies have also shown a different response of the treatment depending on the colour of the seeds (Attri et al., 2021a) (Ivankov et al., 2021) assuming the fact that the composition of the envelope can impact the effect of cold plasma.

Several studies have reported the effects of cold plasma treatment on seed surface properties of *Arabidopsis* (Bafoil et al., 2019) and it was found that, depending on the dose value, the surface etching can range from slight narrowing of the epidermis to total detachment (Cui et al., 2019). Some seeds need to be notched due to a hard shell to allow the penetration of water; the treatment could have a scarifying effect improving water absorption processes as already shown for quinoa and tomato seeds (Adhikari et al., 2020; Gómez-Ramírez et al., 2017).

Cold plasma treatment can induce changes in seed surface wettability, which is revealed by a significant decrease in water contact angle of seed coat in many seed species (Zahoranová et al., 2018) (Los et al., 2018). This increase on seed wettability can also lead to negative effects on germination as for wheat seeds with different treatment times (Los et al., 2019). It was found that the decrease in contact angle measurement could be associated with chemical changes (Bormashenko et al., 2012) and not only with morphological changes (no structural effect observed by SEM) that transform the seed coat from hydrophobic to hydrophilic state. These effects could improve the water uptake of the seeds during imbibition and accelerate the germination processes.

### 3.3.3. Effect on ROS homeostasis, hormonal balance and enzyme activity

If it is known that the crosstalk between hormones and ROS is at the heart of the regulation of seed dormancy and germination (Bahin et al., 2011), one can legitimately think that cold plasma treatment, source of ROS and RNS, can induce biochemical and physiological responses responsible for improving germination. While ROS-induced ROS release is cellular process in animals and plants (Zandalinas and Mittler, 2018), the ROS and RNS produced during discharge could induce the generation of reactive species within seed tissues by transfer from the outer layers to the seed tissues, triggering oxidative signalling. Assessment of ROS production by plasma treatment is crucial to understand the effects of the treatment but it remains difficult to correlate ROS from the plasma phase (exogenous) and ROS from the seed tissues (endogenous). Few studies have shown whether plasma treatment could trigger ROS and RNS production during imbibition which could be related to improved germination. In the case of imbibed Norway spruce seeds, no evidence of



significant increase in  $\text{H}_2\text{O}_2$  production was found although germination was promoted (Pauzaite et al., 2018). For *Arabidopsis* seeds, one study showed that a short treatment duration (3 min) could lead to increased production of superoxide ion  $\text{O}_2^{\cdot-}$  and NO, resulting in improved germination whereas longer treatments inhibited germination with overproduction of  $\text{H}_2\text{O}_2$  (Cui et al., 2019). So far, no study clearly demonstrates whether a cold plasma treatment could directly induce the production of ROS within the seeds.

Several studies have shown that the synthesis of hormones associated with cell elongation, cell division, seed germination and plant development such as auxin, cytokinin, GA and ABA could be modulated by cold plasma in seeds and seedlings (Zukiene et al., 2019) (Stolárik et al., 2015) (Li et al., 2017). The beneficial effects on germination are related to the increase of some hormones such as auxins and GA but also to the variation of the ratio between concentrations of germination inhibiting hormone, e.g. an increase in GA/ABA ratio (Mildažienė et al., 2019) (Attri et al., 2021a). This hormonal balance illustrates that plasma treatment is a tool to activate hormonal signalling pathways and release dormancy. To measure hormone concentrations, high performance liquid chromatography (HPLC) methods are carried out in most studies (Ivankov et al., 2021) (Degutyte-Fomins et al., 2020) (Zukiene et al., 2019) (Pérez Pizá et al., 2018) but require more detailed analysis by combining them with other diagnostics like mass spectrometry (MS).

Oxidation processes and hormonal regulation can also be linked to antioxidants and enzymatic activity showing the response of seeds to plasma treatment. Antioxidants, both enzymatic and non-enzymatic, allow seeds and plants to respond physiologically to oxidative stress. In the literature, there are various studies on the increase in activity of several groups of enzymes during imbibition induced by plasma treatment, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) associated with better seed germination (Zhang et al., 2017a) (Zhang et al., 2018) (Rahman et al., 2018). For *Arabidopsis* seeds, it was found that plasma treatment did not change the amount of SOD but induced an increase in POD and a decrease in CAT, showing a change in enzymatic activity that could explain the improved germination (Cui et al., 2019). Some studies have also reported an effect on hydrolytic enzymes such as amylase, protease or phytase which can facilitate the use of nutrients for germination as for starch-rich seeds (MacLeod et al., 1964). A plasma treatment-induced increase in these enzymes has been demonstrated for mungo bean seeds (Sadhu et al., 2017) as well as for brown rice seeds (Chen et al., 2016).

### 3.3.4. Effect on plant development and stress resistance

Numerous studies report an effect of plasma seed treatment on the development of resistance to abiotic, biotic and even anthropogenic stresses. Seeds treated with plasma in the dry state would trigger beneficial responses during germination in penalising conditions but also on seeds from plants that have been subjected to these stresses. The treatment of the seeds also allows a better development of the seedlings, inducing adaptive qualities to the stresses translated by a reduction of the oxidative damage, an increase of enzymatic and antioxidant activity regulating a hormonal balance.

In the literature, several studies show the beneficial effect of cold plasma treatment on seeds in response to adverse environmental conditions. Hence, it was shown that the plasma treatment improved wheat seed germination by simulating drought conditions with polyethylene glycol (PEG) solutions limiting water potential and inducing an oxidative response during germination (Guo et al., 2017). The seedlings also developed an adaptability to this stress shown by a reduction in oxidative damage (decrease in  $H_2O_2$  and  $O_2$  level), lipid peroxidation (decrease in MDA) and an increase in antioxidant activity (SOD, CAT, POD) and ABA synthesis. The same results were obtained in other studies, for drought resistant and sensitive rapeseed batches (Ling et al., 2014), for alfalfa seeds and seedlings (Feng et al., 2018) but also on the development of tomato seedlings under punishing conditions (Adhikari et al., 2020). Cotton seed germination has also been stimulated by cold plasma under hot and cold germination conditions (de Groot et al., 2018). An effect on seeds initially exposed to high temperatures during storage has also been reported to restore germination potential by affecting ABA biosynthesis and  $\alpha$ -amylase gene expression suggesting epigenetic regulation (Suriyasak et al., 2021). Cold plasma may also have an effect during salt stress, facilitating germination and development of rice seedlings (Sheteiwy et al., 2019) applying saline conditions during germination. The same effect on germination has been shown in wild *Arabidopsis* seeds and some mutants (Bafail et al., 2019).

Seed and plant development can also be affected by anthropogenic stresses related to human activity, inducing contamination by toxic chemical compounds, heavy metals or nanoparticles. Some studies show that cold plasma treatment of seeds led to an adaptive response to these stresses that can participate in the bioremediation of plants. An effect of cold plasma on the toxic reduction of zinc oxide nanoparticles was demonstrated by an improved development of pepper seeds and plants (Iranbakhsh et al., 2018). This effect was

linked to a promotion of the activity of enzymes such as phenylalanine ammonia-lyase and peroxidases. A similar study showed this effect on lemon balm seeds, reducing the toxicity of selenium nanoparticles (Babajani et al., 2019). Plasma-treated pea seeds developed resistance during seedling development and decreased DNA damage upon contamination with toxic zeocin molecules (Kyzek et al., 2019).

Studies have also shown adaptability of plants from plasma-treated seeds to biotic stresses and pathogen contamination. For example, the plasma treatment of soybean seeds initially infected with fungal pathogens like *Diaporthe/Phomopsis*, drove to higher resistance and a reduction of the negative effects on seedlings and plant development (Pérez-Pizá et al., 2019). Similar studies have been conducted in maize, narrow-leaf lupin and winter wheat species (Filatova et al., 2020) in which infection with fungal pathogens, such as *Fusarium culmorum*, was reduced and antioxidant activity was enhanced. An initial reduction of the pathogen load on the seeds induced better development and resistance during the development of the plant at different stages. Plant defence mechanisms were also established, promoting seedling growth and increasing yields.

### 3.3.5. Effect on the regulation of gene expression

Several studies have shown that cold plasma treatment of seeds induces gene regulation during plant development that characterises improved growth and stress resistance. For example, wheat seed treatment induced regulation of the LEA 1, SnRK2 and P5CS genes in relation to oxidative resistance and drought adaptability (Guo et al., 2017). Similar results were found with wood sage (*Salvia nemorosa*) seeds (Ghaemi et al., 2020). Expression and activation of AREB1 and WRKY1 genes were measured in the leaves from treated seedlings, hence assuming their role in stress tolerance.

Few studies have been carried out on the regulation of seed genes induced by plasma treatment during seed imbibition involving germination processes. The gene regulation induced by plasma treatment was demonstrated for heat-stressed rice seeds (Suriyasak et al., 2021). OsNCED2 and OsNCED5 genes involved in ABA biosynthesis have been down-regulated although OsABA8'OH1 and OsABA8'OH3 genes involved in ABA catabolism and OsAmy1A, OsAmy1C, OsAmy3B, and OsAmy3E genes associated with  $\alpha$ -amylase have been up-regulated. Gene regulation was also studied with microarray analysis (Hayashi et al., 2016). It was found that oxygen plasma can modulate the gene expression of

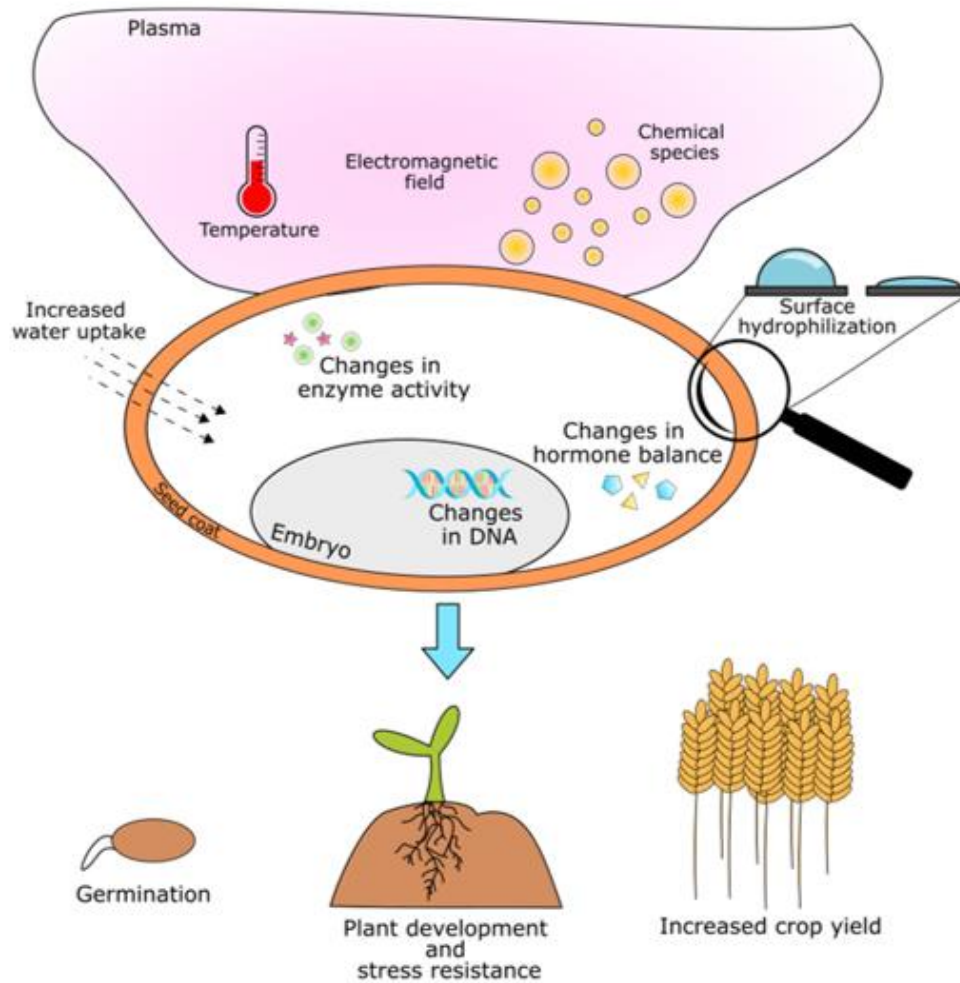
Arabidopsis seeds implicated in cell growth, stress response, photosynthesis and hormone response. In this study, the effect of the plasma treatment was also studied on the second generation. No changes in plant growth were characterised, suggesting that no mutations were induced. However, the expression profile of the genes was modified, showing a possible epigenetic effect in the first generation.

The effect of seed germination-enhancing plasma treatment on gene regulation, whether during stress, dormancy or vigour, deserves further study. Transcriptomic analyses would allow us to understand how the seeds would trigger gene regulation pathways following the treatment.

### 3.3.6. Summary of plasma effect on seeds

The literature review showed that cold plasma treatment can act on cellular redox homeostasis, antioxidant signalling, gene expression and biochemical content of seeds enhancing germination properties and development of resistance to biotic, abiotic and anthropogenic stresses.

Figure 22 gives an overview of the cold plasma effects on the development of seeds and plants. The properties of the plasma phase such as temperature, electromagnetic field and chemical species induce effects on seed morphology, genetics and physiology (Starič et al., 2020). Plasma treatment of seeds can modify their seed coat by improving its surface hydrophilization which, in turn, increases the water uptake and therefore promotes the germination. The plasma treatment also produces biochemical changes playing on seed physiology. Many studies report changes in the surface properties of the seed coat. Seeds become more hydrophilic through surface hydrophilization induced by the functionalization of molecules and the etching effect of plasma treatment. This implies that seeds can absorb more water, hence affecting the germination processes. Cold plasma treatment can also lead to changes in enzyme activity and hormone balance as well as gene regulation. These effects can improve seed germination, seedling development, plant growth and the ability of plants to develop resistance to stresses. For these reasons, cold plasma appears as an efficient tool for seed treatment to improve crop quality, increase yields and meet increasingly stringent environmental conditions.



**Figure 22** - Schematic overview of cold plasma treatment effects on seeds: role of plasma properties (in terms of temperature, electromagnetic field and reactive species) on seed coat, enzyme activity, hormonal balance and DNA allowing germination enhancement, plant development and stress resistance as well as crop yield increase (Starič et al., 2020).

## 4. Objectives of the Thesis

In a global context where climate change and human activities strongly impact today's agriculture, it is necessary to develop new methods and change agricultural practices to adapt to these environmental conditions. The improvement of seed germination is a fundamental issue to face these challenges. The objective of this thesis is to study the effects of an ambient air cold plasma, as a promising process for a more sustainable seed agriculture *Arabidopsis thaliana* has been chosen as a seed model and the effects of the ambient air cold plasma studied on its physiology and biological properties.

The problem of this thesis is based on preliminary experiments and results obtained at Laboratoire de Physique des Plasmas (LPP) and Laboratoire de Biologie du Développement (LBD). These results showed that the use of PAW, using a specially designed DBD for water activation, for sowing lentil seeds boosted germination and seedling development (Judée et al., 2018). The idea of using cold plasma as a treatment for seeds in a dry state then emerged. In particular, the basis of the project was whether seed cold plasmas treatment could reduce dormancy and improve germination and what are the effects of the change in physiology. It is particularly relevant to note that the effects of cold plasmas could be effective on dry seeds in anhydrobiosis and that they can induce major changes in their physiology without any addition of water. However, the molecular bases of such effects are unknown. The use of such processes could help to address a major fundamental question in biology. The study therefore focused on *Arabidopsis* seeds, a model species in seed biology. Indeed, these seeds are part of the orthodox seeds characterised by their anhydrobiosis state, i.e. they contain such a low amount of water that all metabolic activities are prevented. However, these resting, but living, organisms undergo dramatic cell reprogramming in the absence of free cellular water such as dormancy alleviation or ageing during after-ripening. In anhydrobiotic conditions, non-enzymatic mechanisms are good candidates for playing a role in seed dormancy release. Oxygen can diffuse within glasses, such as the vitreous cytoplasm at low seed moisture levels, and ultimately lead to reactive oxygen species (ROS) accumulation, as it has been shown by LBD team ((Oracz et al., 2007); (Bazin et al., 2011)). Interestingly, cold plasma processes are efficient sources of reactive oxygen and nitrogen species (ROS, RNS) as well as of charged species. With regards to their effect on seeds, such processes appear as a particularly relevant and powerful tool to modulate and investigate the mechanisms related to biology in the dry state. The major objective of the Thesis will be to better understand and decipher the effects induced by cold and ambient air plasma on seed dormancy and physiology.

In order to develop treatment methods, it is important to understand how the physico-chemical properties of cold plasma can interact with the different biological properties of seeds. To this end, the objective is to develop a DBD reactor operating in ambient air so as to treat several types of seeds, specifically *Arabidopsis* seeds. The species produced by the plasma phase will be analysed by mass spectrometry as well as by optical emission spectroscopy. This will allow the identification of the species generated by the plasma phase and that are responsible for a physiological change in the seeds. The aim is also to perform

electrical characterisations of the discharge to study the interaction mechanisms between the ambient air cold plasma and the seeds during treatment.

Investigating the biological and morphological properties of plasma-treated seeds are parts of the issues of this thesis. The effects of cold plasmas on the cellular mechanisms of germination are unknown and studying these mechanisms is mandatory to understand such effects. Conversely, the use of plasmas is also an experimental model of choice for elucidating the cellular bases of germination. One objective is to determine whether plasmas effects on seed physiology could depend on seed cytoplasmic glass properties. The question is whether treatment can affect ROS homeostasis and metabolism in dry seeds. The objective is also to elucidate the effect of the plasma treatment on the internal structure of the seed and in particular on the intercellular spaces through the tissue.

At the fundamental level, this interdisciplinary study is expected to significantly advance our understanding of the cellular mechanisms involved in response of biological systems to cold plasmas. The experimental system should also provide significant novel findings on the biology in anhydrobiosis, in particular on the biological processes triggered by the plasma treatment in the dry state and during seed imbibition leading to germination and dormancy breaking.

This study is presented in 3 chapters written in the form of articles. The first one deals with the interaction between plasma and seeds by understanding how the water content and electrical properties of Arabidopsis seeds can interplay with the properties of a cold atmospheric plasma. The second chapter shows how the breaking of dormancy of Arabidopsis seeds by ambient air cold plasma depends on the cytoplasmic glass transition. The last chapter reports on the biological processes triggered by the ambient air cold plasma to release the dormancy of Arabidopsis seeds from the dry state to imbibition leading to germination processes. These processes are related to oxidation mechanisms, anatomical changes and regulation of gene expression.

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# **Chapter 2:**

## **Interactions between Arabidopsis seeds and cold ambient air plasma: deciphering the role of seed water content, sorption sites and bioelectrical parameters**

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# Interactions between *Arabidopsis* seeds and cold ambient air plasma: deciphering the role of seed water content, sorption sites and bioelectrical parameters

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**NB:** This chapter contains 8 Figures and 1 Table.

Keywords: ambient air cold plasma, water sorption isotherm, dielectric properties, electrical characterization

## 1. Introduction

**[Context, issues]** In the current context of biodiversity loss, seeds represent a natural capital whose value is essential to address societal and environmental challenges, from restoring ecosystems and preserving species to improving quality of life through more sustainable agriculture and forestry (Breed et al., 2019), (Mattana et al., 2022). Ensuring seed germination quality, i.e. improving vigour, releasing dormancy and preserving seeds from pathogens and pests, is a cornerstone that is today limited by the conventional agricultural practices (Paparella et al., 2015). Indeed, the solutions are essentially based on the massive use of phytosanitary products, including active substances, formulants, pellicle and coating adjuvants. More generally, the use of pesticides that contribute to agricultural productivity raises major health and environmental concerns (Popp et al., 2013). Most of the chemicals used today are toxic and persistent in nature, accumulating and polluting the soils as well as rippling up and down food chains to cause trophic cascades and jeopardising human health (Boobis et al., 2008) (Aktar et al., 2009). The long-term environmental impact of these approaches is a rising problem and opens the way to disruptive processes, in particular cold plasma processes.

**[Plasma effects vs seed effects]** Cold ambient air plasmas (C2APs) are weakly ionised and drives the production of short and long lifetime reactive oxygen/nitrogen species of major interest for biological applications, whether in medicine or in agriculture [] ... They are characterised by electrical, chemical, radiative, thermal and fluid-mechanical properties whose combination can lead to desired or undesired effects. Thus, cell stimulation/proliferation is expected for wound healing applications while senescence and cell death are sought for cancer cells. For agricultural applications, plasma processes are studied in order to identify and modulate properties likely to improve seed germination and seedling growth, while controlling diseases and pest attacks and ensuring health safety. These processes could help change practices for a more sustainable and smart agriculture (Charoux et al., 2021). Indeed, numerous studies prove the effectiveness of cold plasmas for seed treatment, notably in improving germination and vigour and eliminating pathogen loads from infected seed lots of several species (Attri et al., 2021b; Li et al., 2016; Mitra et al., 2014). Cold plasma seed treatment could also induce different forms of resistance, measurable during the early stages of seedling development (Mildaziene et al., 2022). Most of the research works focus on the effects of cold plasma on seed properties, i.e. seed surface state (e.g. chemical functionalization, texturing, etching), gene regulation, seed

biochemical and physiological changes (Starič et al., 2020). However, few studies have focused on the C2AP-seed interaction and all the more on how seeds can affect C2AP owing to their material and electrical properties (Judée et al., 2020), (Dufour et al., 2021). Understanding how seed properties can influence plasma properties during their treatment is an important issue, especially for transferring C2AP technology to seed companies since a same plasma device can be utilised to treat different types of seeds that may differ in size, geometry, water content, lipid and protein reserves, etc. More specifically, we consider seed water sorption property as a cornerstone to modulate seed water content which, in turn, could change seed electrical properties and therefore mitigate the effects of cold plasma treatment.

**[Seed sorption properties]** A suitable method to describe the thermodynamic properties of seed tissues upon hydration is to plot their water content (WC) versus the relative humidity ( $R_H$ ) of the gaseous environment at given storage temperature, after a duration that is long enough (typically several days) to ensure that seeds have been completely equilibrated with water vapour (Buitink et al., 1998). The resulting sorption isotherm represents the capacity of seeds to bind water through functional sites within tissues in fixed conditions (storage temperature, relative humidity). Water sorption isotherms are classically divided into three different regions corresponding to strong binding sites, weak binding sites and multimolecular sorption sites (free water) (Vertucci and Leopold, 1987). These regions are generally analysed using a sorption fitting model to extract the values of biophysical parameters characterising the seed-water interaction as well as the physiological processes at the functional level of seeds and which depend on developmental maturity, degree of desiccation tolerance, and dormancy state (Hay et al., 2022). Furthermore, sorption isotherms are a powerful tool to: (i) Decipher seed longevity since sorption parameters strongly depend on the physiological state of the seeds and are therefore related to viability, vigour and ageing of seeds (Bazin et al., 2011); (ii) Verify the functional stability of food materials and thus contribute to the optimization of food processing (Gichau et al., 2020).

**[Seed electrical properties]** Seeds - and to a larger extent biological tissues - present both dielectric and conductive properties, in the same way as food materials (Berbert et al., 2001). The water content of biological tissues can change the electrical conductivity ( $\sigma$ ) but also the complex relative permittivity ( $\epsilon_r$ ) of the seeds (Nelson, 2015). This  $\epsilon_r$  parameter is given by the formula (Foster and Schwan, 1986):

$$\varepsilon_r = \varepsilon'_r - j\varepsilon''_r = \frac{\varepsilon'}{\varepsilon_0} - j\frac{\varepsilon''}{\varepsilon_0} \quad (1)$$

Where the real part ( $\varepsilon'_r$ ) is the dielectric constant and the imaginary part ( $\varepsilon''_r$ ) is the dielectric loss factor. The measure of the electrical properties can be determined by assimilating the seeds as a parallel-equivalent capacitance and resistance. The dielectric constant ( $\varepsilon'$ ) and the dielectric loss factor ( $\varepsilon''$ ) can be measured using inductance-capacitance-resistance (LCR) meter while the conductivity ( $\sigma$ ) and the loss tangent ( $\tan\delta$ ) can be derived. The generated electric field induces conduction and displacement currents leading to dielectric losses in the seeds. Depending on the tissue's properties, Joule effect losses may occur as well as electroporation (Piyasena et al., 2003). The dielectric constant relates the ability of the material to store energy from an external electric field and the dielectric loss factor relates the ability to dissipate energy absorbed by the material (and coming from the electric field (Sacilik and Çolak, 2010)). These parameters can be measured quickly, easily and at low cost, while serving as input data for models capable of calculating seed water content (after prior calibration). In turn, this key parameter can be used to predict seed viability (Zhu et al., 2013) (Mai et al., 2016) (Moura et al., 2013).

**[Viability, longevity, ageing]** The longevity of seeds relies on their ability to survive desiccation thanks to the biophysical properties of the intracellular glasses of the tissues strongly reducing seed metabolism in the dry state (Buitink and Leprince, 2004). On the other hand, seed quality is highly dependent on storage temperature and humidity which affects seed viability (Dickie et al., 1990). Indeed, seed ageing on the impact of biochemical stability (Ballesteros and Walters, 2019) during dry storage through lipid peroxidation, protein oxidation as well as tissue deterioration and play on viability and vigour (faculty of germination). Seed viability is strongly related to the anhydrous state within tissues (Ballesteros and Walters, 2011). In seeds, the state of the cytoplasm is modulated by seed water content and temperature which can have an effect on biochemical reactions leading to change in physiology, such as seed dormancy release or aging (Bazin et al., 2011). It is for example admitted that high relative humidities and temperature during seed storage induce rapid loss of viability, mostly because cytoplasm mobility increases and permit deleterious biochemical reactions. In contrast, maintaining cytoplasm in a highly viscous glassy state maintains seed viability during storage (Buitink and Leprince, 2004).

**[Overview]** In this study, the water sorption properties of dormant Arabidopsis seeds were studied comparing untreated seeds (Control group) and plasma-treated seeds (Plasma

group). Sorption isotherms were plotted by fitting water sorption empirical models to experimental data points. Such models highlighted the plasma conditions under which the tissue hydration parameters were changed, hence driving to the distinction of three groups: low (3 %), medium (10 %) and high (30 %) water content. The variations of the seed electrical properties were studied as a function of their water content (through equivalent resistance and capacitance measurements). These variations were also correlated with changes in the electrical properties of the ambient air cold plasma as a function of seed water content during treatment.

## 2. Materials and methods

### 2.1. Plant material

Arabidopsis (ecotype Columbia Col-0) seeds were grown and harvested dormant following the method described by Leymarie et al. (2012), and then stored at -20 °C to keep dormancy properties. All seeds have an initial moisture content of 7 %<sub>DW</sub>. Three batches were formed to carry out the following experiments: (i) plasma-seeds interactions, (ii) water sorption isotherms and (iii) LCR measurements.

### 2.2. Equilibrating seeds to specific water contents

According to the method described by Vertucci and Roos (1993), several equilibrium water contents (WC) were obtained by placing Arabidopsis seeds in different hermetically sealed jars, at 20 °C for 4 days. Seeds were placed over silica gel and saturated salt solutions of ZnCl<sub>2</sub>, LiCl, CaCl<sub>2</sub>, MgCl<sub>2</sub>, Ca(NO<sub>3</sub>)<sub>2</sub>, NaCl, KCl and KH<sub>2</sub>PO<sub>4</sub>, hence resulting to the respective values of relative humidities ( $R_H$ ): 2.5, 9.5, 17.0, 28.5, 40.0, 56.5, 75, 83.5 and 93.0 %. Seed water content, expressed on a dry weight basis (DW) in g.H<sub>2</sub>O.g DW<sup>-1</sup>, was calculated as the mass ratio of a given number of seeds equilibrated in an atmosphere of controlled relative humidity by the same number of seeds in a totally anhydrous state. Therefore, for each condition of relative humidity, three replicates of fresh seeds (approximately 15 mg of seeds per replicate) were dried during 24 h at 105 °C. The mass ratio between water-equilibrated mass to anhydrous mass resulted in the seed water content. In this work, values of WC were expressed as the mean of the three replicates with its standard deviation.

## 2.3. Water sorption empirical models and water sorption isotherms

Sorption isotherm curves were obtained by plotting WC as a function of  $R_H$ , whether for seeds from control groups or plasma groups. Sorption isotherms were fitted to experimental data points ( $R_H$ , WC) by using four types of empirical models relying on seed thermodynamic properties and providing biological and physical parameters of the water sorption phenomenon which operates on seed tissues. Each of these models was applied using the fitting tools of the Originlab software. The fitting parameters were extracted with their errors while the accuracy of the fit was given by the R-squared coefficient. In this study, several commonly used models were considered to determine such parameters from experimental data: the Brunauer-Emmett-Teller (BET) model, the Guggenheim-Anderson-de Boer (GAB) model, the D'Arcy and Watt (D&W) model and the Generalized D'Arcy and Watt (GDW) model.

### 2.3.1. The Brunauer-Emmett-Teller (BET) model

One of the first thermodynamic models describing water sorption within multimolecular layers is the BET model (Brunauer et al., 1938), described by equation (2):

$$WC = \frac{M_m C R_H}{(1 - R_H)(1 + (C - 1)R_H)} \quad (2)$$

Where WC (%<sub>DW</sub>) is the seed water content,  $R_H$  (%) is the relative humidity,  $M_m$  is the water content of the monolayer adsorbate (dimensionless quantity, normalised to 1) and C is a temperature-dependent coefficient (dimensionless). The BET model is relevant to assess WC values for  $R_H < 50\%$  and is no longer accurate beyond (Black and Pritchard, 2002). This model is very useful to estimate the quantity of strong binding sites where water is bound as a monolayer.

### 2.3.2. The Guggenheim-Anderson-de Boer (GAB) model

The GAB model was developed as an extension of the BET model. It considers that water sorption is achieved in a multilayer region, hence the appearance of a third constant (K), as reported in equation (3):

$$WC = \frac{M_m C K R_H}{(1 - K R_H)(1 - K R_H + C K R_H)} \quad (3)$$

Where  $K$  is the multilayer parameter (dimensionless) and  $C$  the temperature dependent coefficient (dimensionless). Usually, the GAB model is valid between 5 and 90 % of relative humidity (Black and Pritchard, 2002).

### 2.3.3. D'Arcy and Watt (D&W) model

The D&W model is a classical model developed by D'Arcy and Watt (D'Arcy and Watt, 1970), describing water sorption of non-homogeneous sorbents with three distinct terms related to (i) the monolayer adsorption by strongly binding sites, (ii) the monolayer adsorption by weakly binding sites and (iii) the multilayer adsorption by multimolecular sorption sites. The model is described by equation (4):

$$WC = \frac{KK'R_H}{1 + KR_H} + cR_H + \frac{kk'R_H}{1 - kR_H} \quad (4)$$

Where  $K$ ,  $K'$ ,  $c$ ,  $k$  and  $k'$  are specific parameters for calculating the numbers of sites related to the three terms. The numbers of strong sorption sites, weak sorption sites and multimolecular sites are given by  $K'N/M$ ,  $cN/(Mp_s)$  and  $k'N/M$  respectively, where  $N$  is the Avogadro number,  $M$  the molecular weight of water per sorption site (in grams) and  $p_s$  the vapour pressure at the studied temperature (2338.8 Pa at 20 °C). WC values can be accurately estimated on the full  $R_H$  range.

### 2.3.4. Generalized D'Arcy and Watt (GDW) model

As with the D&W model, the GDW model considers the existence of primary Langmuir-type adsorption sites on the inner/external walls of the seeds. Each site can adsorb one water molecule which, in turn, can become a secondary adsorption site. The GDW model relies on two assumptions: (i) each water molecule adsorbed on a primary site does not necessarily form a subsequent secondary site, (ii) a water molecule attached to a primary site can form several secondary sites. Kinetic considerations allow to write the GDW model as given by equation (5) (Furmaniak et al., 2007):

$$WC = \frac{a_{mL}p_sK_LR_H}{1 + p_sK_LR_H} \frac{1 - c(1 - \omega)R_H}{1 - cR_H} \quad (5)$$

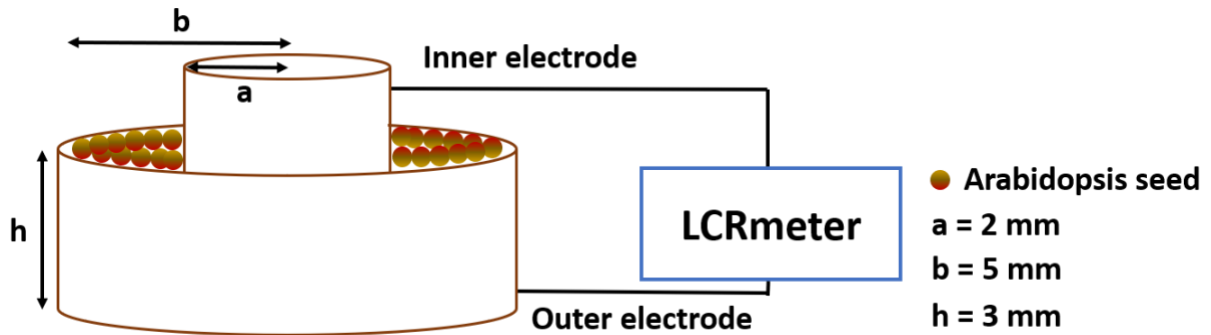
Where  $a_{mL}$  is the surface concentration of primary Langmuir-type adsorption sites ( $\text{mol.g}^{-1}$ ),  $K_L$  is the Langmuir constant characteristic of the interaction between water and tissue ( $\text{Pa}^{-1}$ ),  $c$  is a kinetic constant related to the adsorption on secondary sites (dimensionless) and  $\omega$  is the ratio of water molecules adsorbed on primary sites that are converted to secondary sites

(dimensionless). In comparison with the three previous models, the GDW model presents two major assets: (i) it describes the sorption isotherm as a whole, from 0 to 100% of relative humidity and (ii) the value of adsorption on primary sites  $a_{prim}$  (mol.g<sup>-1</sup>) can be derived from  $a_{mL}$  as given by equation (6):

$$a_{prim} = \frac{a_{mL} K_L p_s}{1 + K_L p_s} \quad (6)$$

## 2.4. LCR measurements

To perform LCR measurements, the Arabidopsis seeds were placed in a coaxial cylindrical capacitor (CCC) constituted of two copper electrodes (Figure 1) with inner (a) and outer (b) radii of 2 mm and 5 mm respectively. The two electrodes were nested in a polymer structure (polylactic acid) so that the interelectrode space was filled with seeds to a height (h) of 3 mm. Measurements were performed for frequencies ranging from 20 Hz to 200 kHz. Then, the electrical capacitance and resistance of seeds samples were measured using an LCR meter (HM-8118 model from Rohde & Schwarz company).



**Figure 1** - Sketch of the coaxial cylindrical capacitor (CCC) containing Arabidopsis seeds in its interelectrode space to achieve their LCR measurements.

First, LCR meter calibration was performed for all frequencies in the open circuit (two clips separated from each other) and then in the short circuit (two clips connected). Second, the capacitance and resistance of the CCC were measured without any seeds inside the interelectrode space. Third, 100 mg of Arabidopsis seeds was introduced so as to completely fill the interelectrode space. The parallel-equivalent capacitance and resistance of the seed amount were measured while the dielectric constant ( $\epsilon'$ ), the dielectric loss



factor ( $\epsilon''$ ), the conductivity ( $\sigma$ ) and the loss tangent ( $\tan\delta$ ) were derived (Nelson and Stetson, 1976).

The dielectric constant was calculated as the ratio of two capacitances: coaxial cylindrical capacitor with seeds (C) by coaxial cylindrical capacitor without seeds ( $C_0$ ) (Berbert et al., 2001), as proposed in equation (7):

$$\epsilon' = \frac{C}{C_0} \quad (7)$$

The dielectric loss factor was derived from the ratio of two terms: (i) a numerator corresponding to the difference of the conductance with seeds (G) and the conductance without seeds ( $G_0$ ), (ii) a denominator corresponding to the product of the angular frequency by the capacitance of CCC without seeds ( $C_0$ ) (K. C. Lawrence and S. O. Nelson, 1993), as written in equation (8):

$$\epsilon'' = \frac{G - G_0}{2\pi f C_0} \quad (8)$$

The electrical conductivity ( $\sigma$ ) of a dielectric material is expressed in  $S.m^{-1}$  and is characterised by its ability to let electrical charges pass freely through a body. It is given by equation (9) and directly depends on angular frequency and dielectric parameters (Nelson, 2015):

$$\sigma = \omega \epsilon_0 \epsilon'' \quad (9)$$

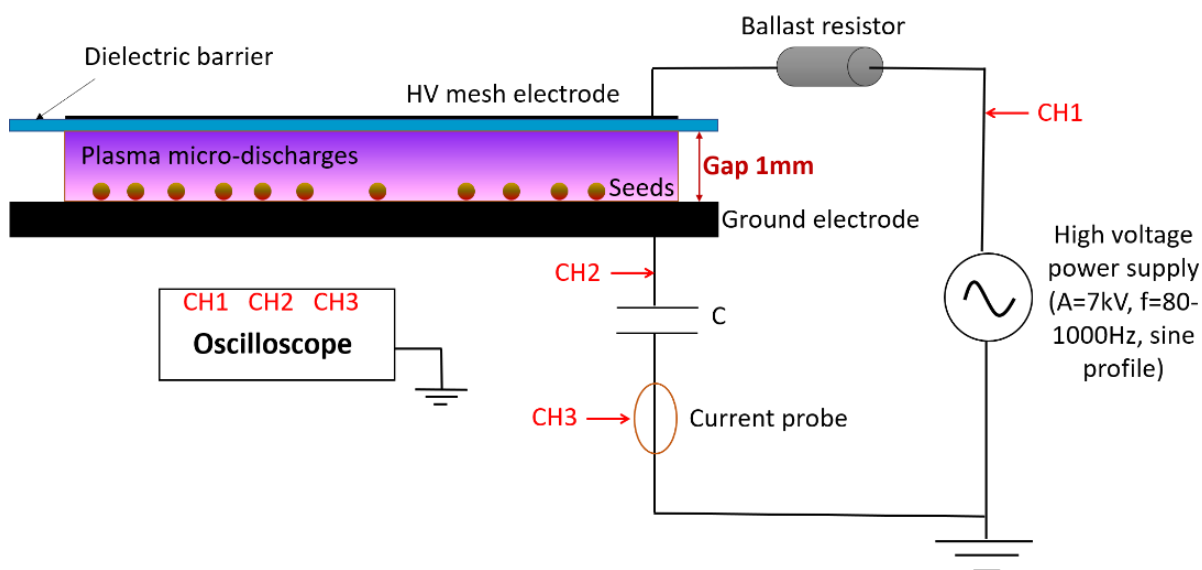
Finally, the loss tangent related to the electric power dissipation in the dielectric material can be calculated as the ratio between the dielectric loss factor and the dielectric constant, as expressed in equation (10):

$$\tan\delta = \frac{\epsilon''}{\epsilon'} \quad (10)$$

The measurements were repeated four times for three values of water contents: low water content (LW) at 3 %<sub>DW</sub>, medium water content (MW) at 10 %<sub>DW</sub> and high water content (HW) at 30 %<sub>DW</sub>. All results are expressed in nF for the capacitance and M $\Omega$  for the resistance corresponding to the mean of the 4 measurements with its standard deviation.

## 2.5. Experimental configuration of cold plasma in ambient air (source and electrical probes)

A dielectric barrier device (DBD) in a plan-to-plan configuration with a gap of 1 mm was used in this study. As shown in Figure 2, it was composed of a 2mm thick dielectric barrier and two electrodes: an electrode biased to the high voltage (stainless-steel mesh with a 100  $\mu\text{m}$  nominal opening, a wire diameter of 71  $\mu\text{m}$  and an open area of 34 %) and a counter-electrode which is grounded (bulk alumina plate). The area of the reactor was 30x40  $\text{cm}^2$ . The mesh electrode was powered with a high voltage generator composed of a function generator (ELC Annecy France, GF467AF) and a power amplifier (Crest Audio, 5500W, CC5500). The power supply was completed by a ballast resistor (250  $\text{k}\Omega$ , 600 W) to limit current.



**Figure 2** - Experimental setup of the dielectric barrier device (DBD) used to generate ambient air cold plasma and treat Arabidopsis seeds in 1 mm electrodes gap; a capacitor C and a current probe are placed in series with the DBD to characterize the micro-discharges observed with an oscilloscope.

Voltages and currents of the DBD were measured using a digital oscilloscope (model wavesurfer 3054 from Teledyne LeCroy company), with a 500 MHz bandpass and a 4GS/s sample rate. The voltage applied to the mesh electrode was measured with a high voltage probe (Tektronix P6015A 1000:1, Teledyne LeCroy PPE 20 kV 1000:1). The discharge current was monitored using a current transformer (Pearson company, model 2877) placed

between the counter-electrode and the ground. A measurement capacitor (80 nF) was inserted between the counter-electrode and the ground so as to plot Lissajous curves. Their closed contour area was calculated to deduce the energy dissipated in the DBD. Multiplying this value by the operating frequency provided the electrical power injected in the DBD.

The DBD source could process large quantities of Arabidopsis seeds, although each treatment was limited to 500 mg of Arabidopsis seeds (during 15 min) for the purposes of our experiments.

## 2.6. Statistical analysis

All the statistical analyses were performed using OriginLab Software statistical tools, including curve fitting and Mann-Whitney tests to determine significant differences on experimental data from control group and plasma group (water sorption isotherms).

## 3. Results

### 3.1. Cold plasma from ambient air significantly changes seed water content only at low and high relative humidities

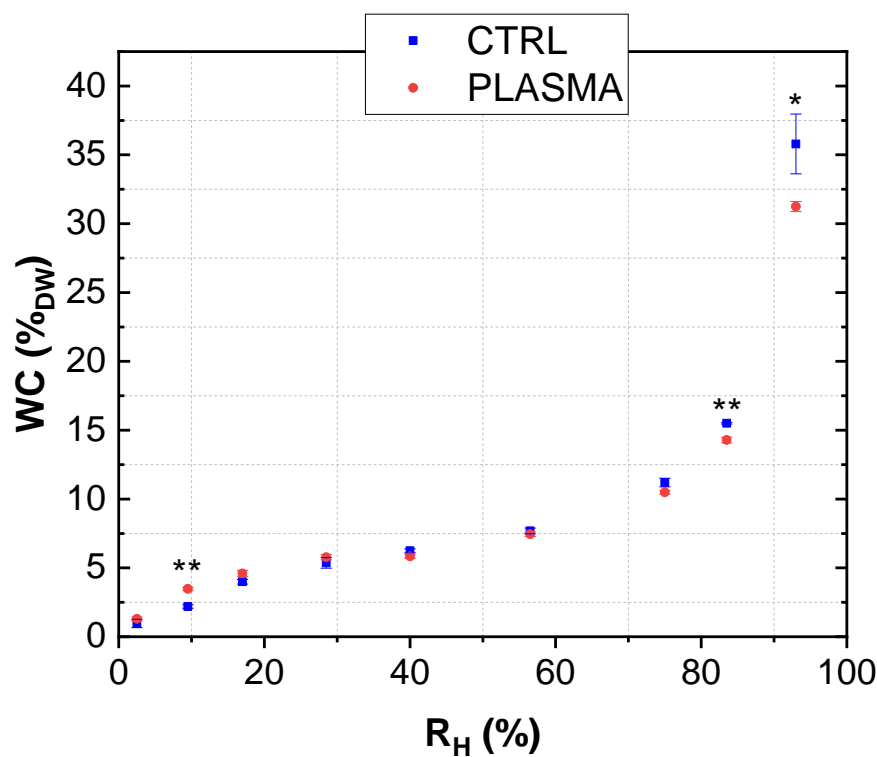
Water sorption properties in the tissues of dormant Arabidopsis seeds were evaluated by considering two groups: seeds without treatment (CTRL) and seeds exposed to the ambient air cold plasma for 15 minutes (PLASMA). To plot water sorption isotherms from the thermodynamic models presented in Section II.X, experimental data on seed water content as a function of environmental relative humidities must first be plotted, as shown in Figure 3. As already mentioned in Section 2.3, three regions can be distinguished with specific properties:

(i) The region where the relative humidity is low ( $R_H = 0-30\%$ ) and where the water contained in the seeds remains strongly bound to the sorption sites as a molecular monolayer. Hence, at  $9.5\% R_H$ , the water content of the plasma-treated seeds is significantly higher than that of the CTRL group, with values of  $3.7\%_{DW}$  vs.  $2.4\%_{DW}$  respectively.

(ii) The region where the relative humidity reaches medium values ( $R_H = 30-80\%$ ) and for which the water present in the seeds is weakly bound to the functional sites. In this region,

the two curves overlap, so that no significant difference can be evidenced between the control and plasma groups.

(iii) The region where relative humidity is high ( $R_H = 80-100\%$ ) and where the water molecules are not bound to specific sorption sites so that they can freely circulate. In this region, the cold plasma treatment significantly decreases seed water content compared to the control group, whether at 85% or 93% of relative humidity. Hence, for  $R_H = 93\%$ , water content is as high as 36 %<sub>DW</sub> for seeds belonging to the CTRL group while only 32 %<sub>DW</sub> for the PLASMA group.

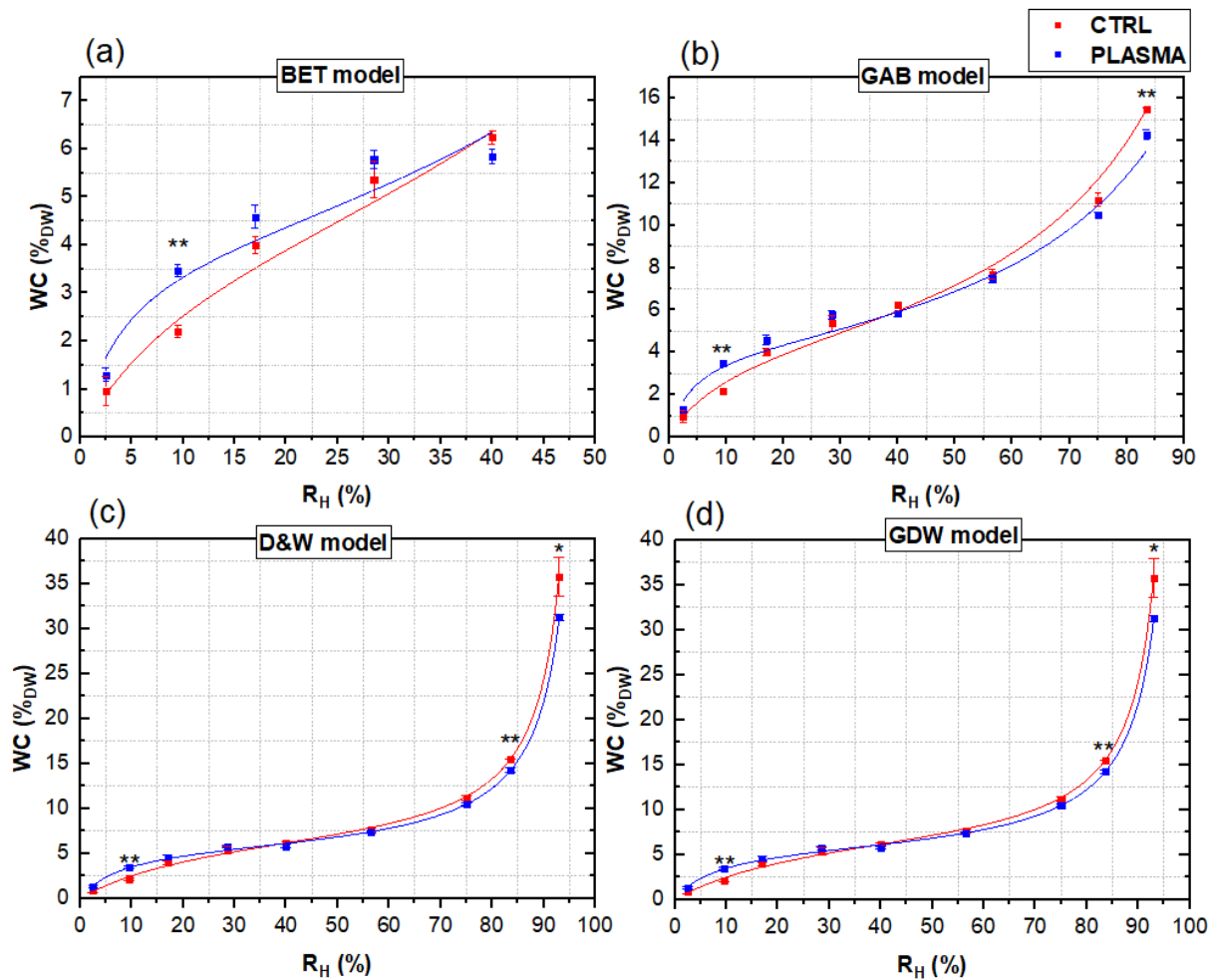


**Figure 3** - Arabidopsis seeds water content versus environmental relative humidity, considering dormant Arabidopsis seeds either non-treated (CTRL) or plasma-treated (PLASMA) at 20 °C. Means  $\pm$  SD. Mann-Whitney tests performed between control and plasma data at each relative humidity (\*:  $p$ -value < 0.05; \*\*:  $p$ -value < 0.01).

### 3.1. The D&W and GDW models reliably explain how cold plasma from ambient air enhances seed water sorption.

The experimental data of the control and plasma groups were fitted with the four water sorption empirical models introduced in Section 2.3, namely the BET, GAB, D&W and GDW

models. The fits were applied from 0 % to 40 %  $R_H$  for the BET model (Figure 4a), from 0 % to 80 %  $R_H$  for the GAB model (Figure 4b) and from 0 % to 100 %  $R_H$  for the D&W and GDW models (Figure 4c and Figure 4d respectively). Differences between the control and plasma fitted curves can be observed regardless of the model.



**Figure 4** - Water sorption isotherms plotted from experimental data points in Figure 3, considering (a) the BET model for  $R_H$ =0-40 %, (b) the GAB model for  $R_H$ =0-80 %, (c) the D&W model and (d) the GDW model. For each model, two water sorption isotherms are considered: group of non-treated seeds (CTRL) and plasma-treated seeds (PLASMA).

The biophysical parameters of the four models and the statistical coefficients assessing their fitting to the experimental data points (coefficient of determination,  $R^2$ ) are reported in Table 1, considering control and plasma groups. Several observations are noteworthy:

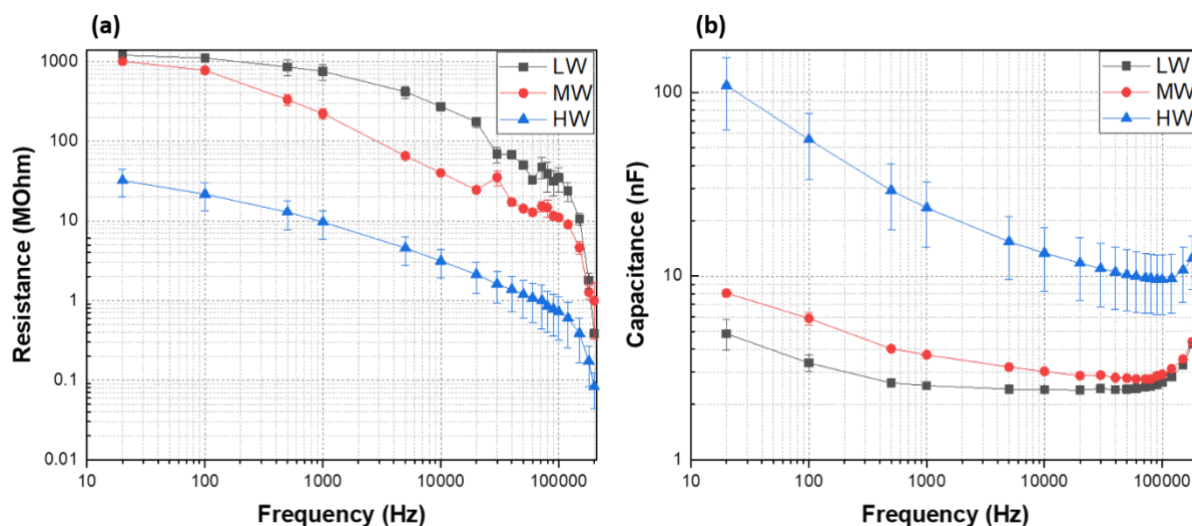
- (i) In the BET and GAB models, the  $M_m$  parameter (water content of the monolayer adsorbate) is almost the same for the control and plasma groups, hence suggesting the plasma treatment does not change the strength of the binding sites. However, the C parameter is 2.7 for the plasma group than for control group, which unambiguously indicates that at low  $R_H$ , the adsorption is stronger for the plasma-treated seeds.
- (ii) The results obtained from the BET and GAB models are consistent with the parameters extracted from the D&W model in which strong sorption sites are greater for the control seeds than the plasma-treated seeds. On the other hand, the D&W model could successfully fit to the experimental data only by setting the C parameter to 0, which forces the model to consider that the seeds contain a number of weak sorption sites equal to zero. The same issue was reported in a previous study (Hay et al., 2003), suggesting a number of too low sorption sites for detection.
- (iii) The biophysical parameters extracted from the D&W model indicate that for high values of  $R_H$  (from 80 %), more multimolecular sorption sites were calculated for the plasma-treated seeds.
- (iv) The GDW also provided interesting information and parameters regarding the water sorption within seeds tissues between the control and the plasma groups. By the same reasoning, greater adsorption values were calculated at low  $R_H$  for the plasma treated seeds (parameters  $K_L$  and  $a_{prim}$ ) presenting a lower value of primary adsorption sites ( $a_{mL}$ ). Interestingly, a greater value of  $\omega$  was found for the plasma-treated seeds, indicating that more water molecules could be adsorbed on the primary sites and then be converted into secondary adsorption sites. A similar kinetic constant (C) related to the adsorption on secondary sites was fitted for the two groups: no discrepancy was observed in terms of time scale of the adsorption mechanisms.
- (v) All these parameters determined using the fits of the different water sorption empirical models showed some difference in terms of water adsorption through the tissues of control dormant seeds and plasma treated seeds. It helps to explain the change in seed water content of the two groups at low  $R_H$  (< 30 %) where the water molecules are strongly bound and at high  $R_H$  (> 80 %) where the water molecules can circulate (free water).

**Table 1** - Biophysical parameters of BET, GAB, D&W and GDW models and their values fitted on experimental water sorption isotherms, considering two groups of Arabidopsis dormant seeds: seeds without treatment (CTRL) and seeds after plasma exposure (PLASMA). \*: dimensionless quantity; #: normalized to 0-1 range

Model	Parameter	Unit	CTRL	PLASMA	PLASMA/CTRL
<b>BET</b>	$M_m$	*, #	$4.4 \times 10^{-2}$	$4 \times 10^{-2}$	$\approx 0.9$
	C	*	9.5	26	$\approx 2.7$
	R <sup>2</sup> fit	*, #	0.97	0.94	-
<b>GAB</b>	$M_m$	*, #	$4.6 \times 10^{-2}$	$4.2 \times 10^{-2}$	$\approx 0.9$
	C	*	11.5	30.7	$\approx 2.7$
	K	*, #	$8.6 \times 10^{-3}$	$8.3 \times 10^{-3}$	$\approx 1$
	R <sup>2</sup> fit	*, #	0.99	0.98	-
<b>D&amp;W</b>	K'	*, #	$8.4 \times 10^{-2}$	$6.1 \times 10^{-2}$	$\approx 0.7$
	K	*, #	3.9	12	$\approx 3.1$
	c	*, #	0	0	-
	k'	*, #	$1.5 \times 10^{-2}$	$1.5 \times 10^{-2}$	$\approx 1.0$
	k	*, #	1.02	1.01	$\approx 1.0$
	R <sup>2</sup> fit	*, #	0.99	0.99	-
	Number of strong sorption sites	$\times 10^{20} \text{ g}^{-1}_{\text{DW}}$	28	20.4	$\approx 0.7$
	Number of weak sorption sites	$\times 10^{20} \text{ g}^{-1}_{\text{DW}}$	0	0	-
	Number of multimolecular sorption	$\times 10^{20} \text{ g}^{-1}_{\text{DW}}$	5.2	5.3	$\approx 1.0$
	$a_{\text{mL}}$	$\text{mol.g}^{-1}$	$8.8 \times 10^{-2}$	$6.2 \times 10^{-2}$	$\approx 0.7$
<b>GDW</b>	$K_L$	$\text{Pa}^{-1}$	$1.74 \times 10^{-5}$	$5.24 \times 10^{-5}$	$\approx 3.0$
	c	*, #	$1 \times 10^{-2}$	$1 \times 10^{-2}$	$\approx 1.0$
	$\omega$	*, #	$2.2 \times 10^{-1}$	$2.7 \times 10^{-1}$	$\approx 1.2$
	R <sup>2</sup> fit	*, #	0.99	0.99	-
	$a_{\text{prim}}$	$\text{mol.g}^{-1}$	$3.3 \times 10^{-3}$	$6.7 \times 10^{-3}$	$\approx 2.0$

### 3.2. LCRmetry indicates that cold plasma of ambient air does not change the bioelectrical parameters of the seeds

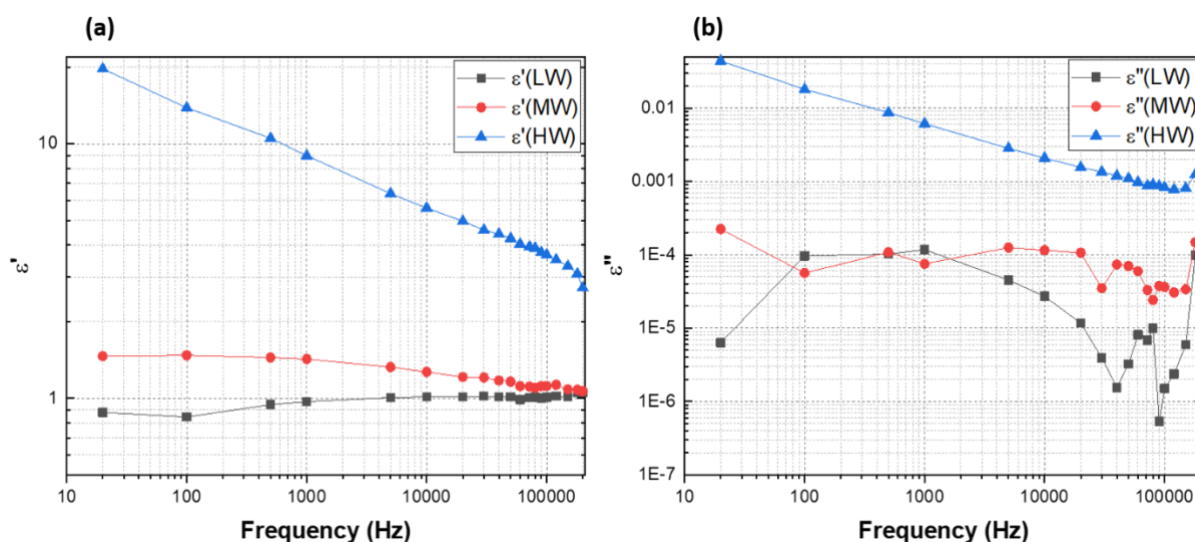
To characterise the influence of seed water content on the seed electrical parameters, LCR measurements were achieved on the Arabidopsis dormant seeds for low water content (LW group, 3 %<sub>DW</sub>), medium water content (MW group, 10 %<sub>DW</sub>) and high water content (HW group, 30 %<sub>DW</sub>). These three groups were chosen to cover the 3 water sorption zones (strong binding sites, weakly binding sites and free water) defined by the water sorption isotherm. The CCC device (Figure 1) was filled with 100 mg of Arabidopsis seeds so as to determine their equivalent resistance and capacitance by LCRmetry, over a frequency range between 20 Hz and 200 kHz. Variations in resistance and capacitance versus frequency are represented in log scale in Figure 5. The LW, MW and HW groups exhibit significant differences. If the MW group presents lower resistance and higher capacitance compared to the LW group, a gap is observed between the HW group and the two other groups. On the entire frequency range, a strong discrepancy separates the HW group (lower resistance and higher capacitance) from the two other ones.



**Figure 5** - Variation of (a) resistance and (b) capacitance as a function of frequency for the parallel equivalent circuit of the coaxial reactor filled with 100 mg of Arabidopsis non treated dormant seeds. Three groups are considered: low water content (LW, 3 %<sub>DW</sub>), medium water content (MW, 10 %<sub>DW</sub>) and high water content (HW, 30 %<sub>DW</sub>). Means of 4 replicates of 100 mg of Arabidopsis seeds +/- SD.

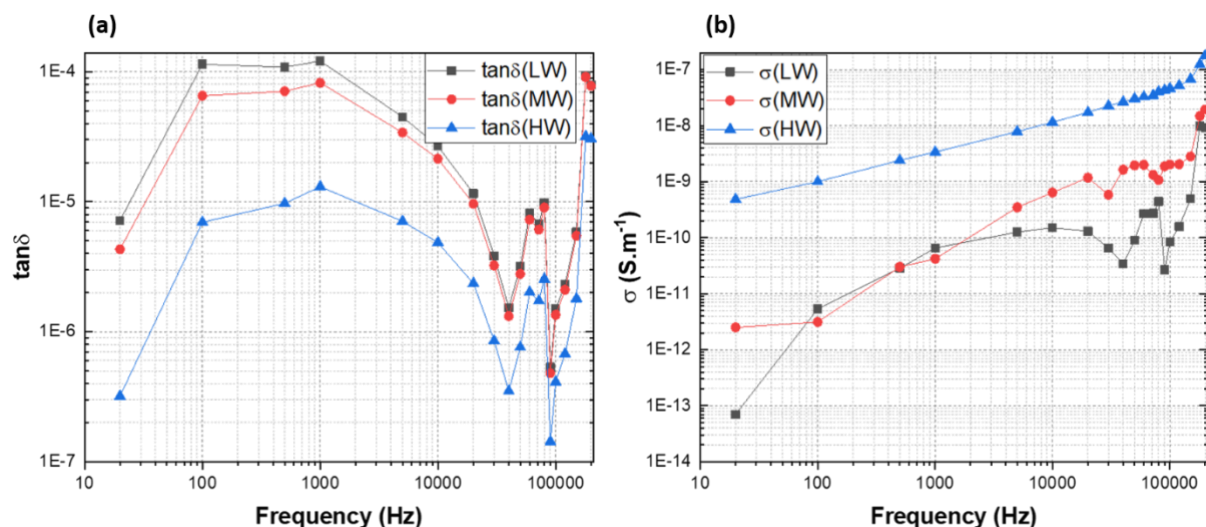


The  $\epsilon'$  (dielectric constant) and  $\epsilon''$  (dielectric loss factor) parameters can be derived through equations (7) and (8), considering the equivalent resistance and capacitance measured with and without the seeds in the CCC device. The variations of these dielectric constants are reported as a function of frequency in Figure 6, considering the LW, MW and HW groups. Unsurprisingly, the HW group is separated from the two other groups. These LCR measurements provide valuable information on the ability of a seed to behave as a capacitive material whose dielectric permittivity is directly controlled by its water content: the higher the water content, the higher the seed's dielectric constants. It is noticed that resistance and capacitance values decrease with frequency, which shows the specificities of the electrical behaviour as a function of the frequency of the external electric field.



**Figure 6** - The dielectric constant  $\epsilon'$  (a) and the dielectric loss factor  $\epsilon''$  (b) as a function of frequency (from 20 Hz to 200 kHz) for 3 seed water content; low water content in black (LW, 3 %<sub>DW</sub>), medium water content in red (MW, 10 %<sub>DW</sub>) and high water content in blue (HW, 30 %<sub>DW</sub>).

From equation (9) and (10), the conductivity ( $\sigma$ ) and the loss tangent ( $\tan \delta$ ) were also determined and are presented in Figure 7. The same trends were found, with lower loss tangent values and higher conductivity values for the HW group, regardless the frequency value. This indicates that at high water content, the seeds allow electrical charges to spread and facilitate electric current.



**Figure 7** - The tangent delta  $\tan\delta$  (a) and the conductivity  $\sigma$  in  $S.m^{-1}$  (b) as a function of frequency (from 20 Hz to 200 kHz) for 3 seed water content; low water content in black (LW, 3 %DW), medium water content in red (MW, 10 %DW) and high water content in blue (HW, 30 %DW).

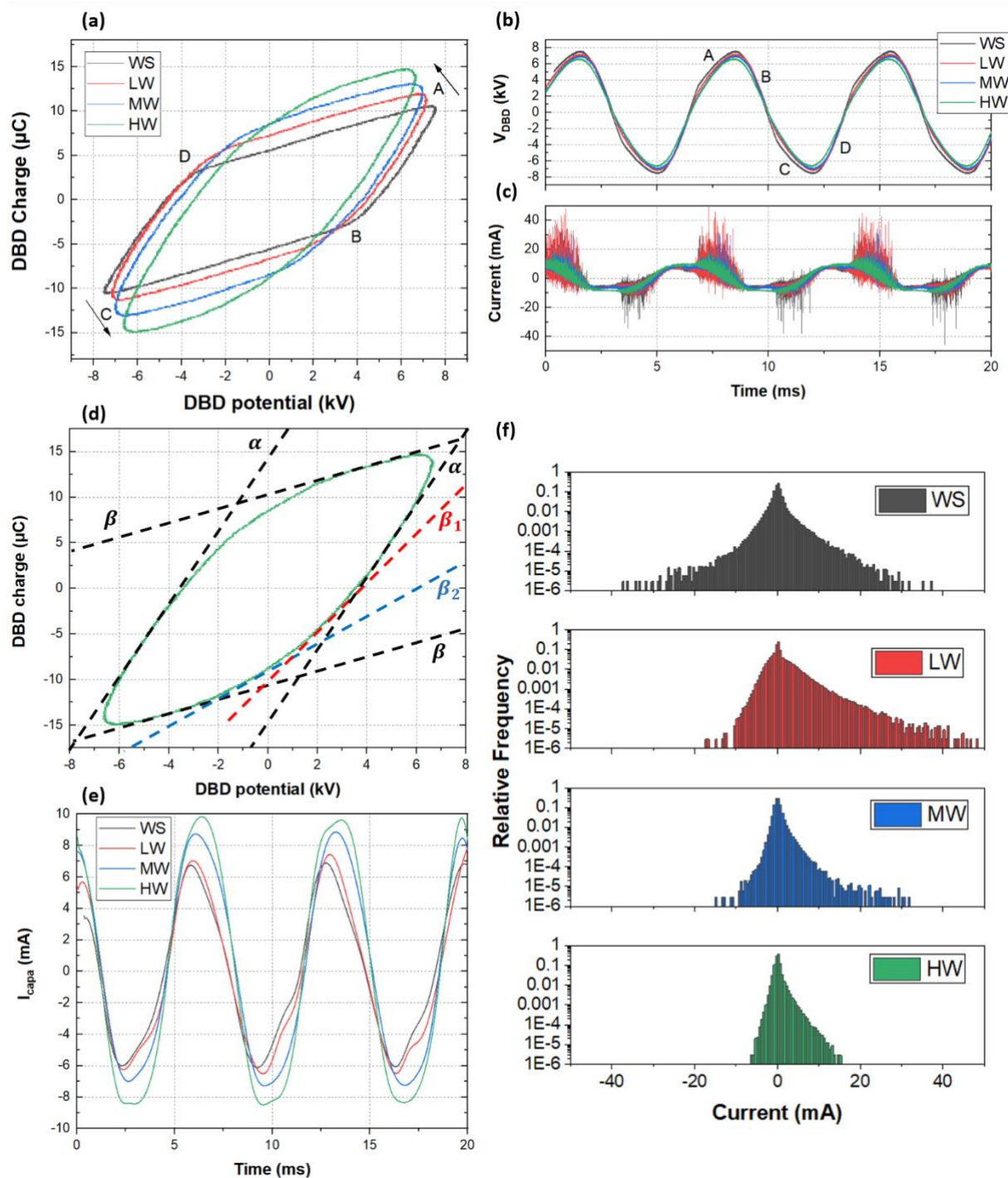
### 3.3. Seed water content modifies the distribution of the current peaks from cold plasma generated in ambient air

Electrical characterization of the ambient air plasma has been achieved considering four cases: DBD without the seeds (WS) and with Arabidopsis seeds of low water content (LW, 3 %<sub>DW</sub>), medium water content (MW, 10 %<sub>DW</sub>) and high water content (HW, 30 %<sub>DW</sub>) correlated with the variation of the seed dielectric properties. For each of the three later cases, 500 mg of seeds were spread all over the counter-electrode surface to guarantee an optimal interplay with the cold plasma of ambient air.

The Lissajous curves plotted in Figure 8(a) indicate that the power dissipated in the DBD is always 20 W. However, the typical form of the parallelogram changes in presence of the seeds and as a function of seed water content. The slope of the [BC] edge corresponds to the capacitances of the air gap, dielectric barrier and seeds sample when the plasma is off. Variations of this slope value indicate an increase in the equivalent capacitance of the system when seeds are present for a given water content. At high water content, it is clearly observed (Figure 8(d)) that the form of the Lissajous curve is radically changed, from a parallelogram to an almond shape. The Lissajous curve at HW condition is characterised by the slopes  $\alpha$  (0.4 nF) and  $\beta$  (8 pF) which correspond to the slopes the plasma-on phase and

plasma-off phase respectively. In the plasma-off phase (between points B and C), the capacitance of the system slides. We can extract 2 other slopes  $\beta_1$  (0.25 nF) and  $\beta_2$  (0.15 nF) characterising this phenomenon. These two slopes decrease with time before returning to the seedless form (slope  $\beta$ ).

One can observe that the plasma voltage seems to decrease slightly with seed water content, as shown in Figure 8(b). Moreover, the temporal profiles of current exhibit strong differences, clearly visible in Figure 8(c). The current presents a dielectric component proportional to the derivative of the applied voltage and a conduction component composed of current peaks forming micro-discharges operating between the breakdown and extinction voltages. The current distribution removing the capacitive part on 2 periods shown in Figure 8(f) allows us to analyse the amplitude and distribution of the current peaks. If the DBD contains no seeds, values range from -40 to 40 mA while the profile changes strongly with seed and water content. For LW, values range from -20 to 50 mA, for MW from -15 to 30 mA and for HW from -8 to 15 mA. Figure 8(e) completes the curve giving the capacitive current for the 4 conditions. Interestingly, the dielectric component seems to increase with the presence of the seeds and with the water content.



**Figure 8** - Electrical characterizations of the plasma discharge without seeds (WS) and with the seeds as a function of water content for low water content (LW, 3 %<sub>DW</sub>), medium water content (MW, 10 %<sub>DW</sub>) and high water content (HW, 30 %<sub>DW</sub>). Lissajous figures (a), waveforms of plasma potential (b), discharge current measured in the DBD (c), Lissajous figures for the HW case (d), capacitive current (e) and current distribution of the plasma phase (f).

## 4. Discussion

The water sorption isotherms revealed three distinct zones where water molecules are strongly, weakly or not bound on surfaces/tissues and described with the help of the water sorption empirical models: the BET and GAB models describing the monolayer adsorbed at low  $R_H$  and the D&W and GDW models on the whole isotherm allowing extraction of biophysical parameters. It was found that cold plasma of ambient air changed the sorption properties of seed tissues since the two sorption isotherms show discrepancies only in the regions of low and high  $R_H$ . At low  $R_H$ , the plasma-treated seeds bound more water than the control seeds. In this zone, water molecules are bound on polar protein or starch surface groups (Vertucci and Leopold, 1984). Plasma treatment could act on the organisation of these groups and the water adsorption strength. Indeed, the results of the 4<sup>th</sup> model fits have given a lower number of strong binding sites but with a higher adsorption value. The control seeds had more strong adsorption sites than the treated one calculated with the D&W model ( $28$  vs  $20.4 \times 10^{20} \text{ g}^{-1} \text{ dw}$ ) and a lower adsorption value ( $3.9$  vs  $12$ ). Moreover, the BET monolayer and the GAB monolayer corresponded to a WC of  $4.4 \%_{\text{DW}}$  and  $4.6 \%_{\text{DW}}$ , respectively, for untreated seeds compared to  $4 \%_{\text{DW}}$  and  $4.2 \%_{\text{DW}}$  for treated seeds. The D&W and the GDW models showed that there were more multimolecular sites for the plasma treated seeds, especially at high  $R_H$ . One can wonder if the treatment can change seed storability, since seed WC is a major component of seed viability loss during storage. Our results showed that when  $R_H$  is higher than  $50 \%$  (Figure 4) plasma treated seeds bind less water, which could have a positive effect on their longevity. For practical purposes this is also suggesting that plasma treated seeds probably store as well as untreated seeds, but this will have to be addressed properly. However, these slight differences open the question of a plasma-induced structural change through the seed tissue, both intracellular and extracellular (Nagarajan et al., 2005). Further studies on water sorption isotherms need to be carried out at several storage temperatures, allowing for example sorption enthalpy calculation and study the effect of plasma treatment on the water binding properties and oxidative processes, which in turn could influence seed physiology (Bazin et al., 2011).

Changing seed WC on the three zones of water sorption isotherm induces strong differences in seeds electrical properties as observed with the change in the dielectric constant and the dielectric loss factor in Figure 6. The values of these parameters increased with water content, which allows us to conclude that increasing WC induces a stronger degree of polarisation of the seeds with an external electric field (Berbert et al., 2001). This

assertion might be correlated with the electrical characterization of the discharge in presence of seeds with various water contents. As shown in Figure 8(e), the discharge current at high water content has a larger capacitive component. This is reflected in the fact that the seeds allow the external electric field current to pass more easily as the conductivity of the seeds increases with water content (Figure S1; Jha et al., 2011). In the presence of the seeds, the micro-discharges could be therefore concentrated and attracted to the seeds which are more capable of accumulating charges. The higher the water content, the less intense the micro-discharges (Figure 8(f)), but the more concentrated they become on the seeds. The electrical energy from the applied voltage and the plasma phase would dissipate more easily as heat in seeds with a high water content.

The seeds introduced in the DBD can be modelled as a non-homogeneous dielectric layer whose properties change with water content and affect their interactions with the applied voltage and the discharge. The effective capacitance of the reactor is not only composed of the dielectric barrier and the gap (air) but also of the seeds whose value will change according to the water content. This could explain the change in the shape of the Lissajous curves showing an almond shape for the HW group. This shape is similar to the ones already observed in the case of packed-bed reactors with high dielectric constant materials (Butterworth et al., 2016). The slopes observed on the different parts of the Lissajous curve depend on the effective capacitance. The seeds could vary the value of the effective capacitance as a function of time (Peeters et al., 2018). As shown in Figure 8(d), the Lissajous part corresponding to the plasma-off time is modulated by the water content of the seeds on which different slopes can be associated ( $\beta_1$  and  $\beta_2$  for HW) showing a capacitive change over time. The higher the water content, the more the dielectric properties increase and therefore the more this phenomenon is accentuated, characterised by an increase in the capacitive current (Figure 8(e)). During a plasma phase, the seeds have accumulated residual charges that take some time to disperse before the next plasma phase.

## 5. Conclusion

In this study, it was shown that the plasma treatment modified the seed water sorption properties. Indeed, the plasma treatment increased the amount of strongly bound water at low  $R_H$  and decreased the amount of free water at high  $R_H$ . Furthermore, considering three groups of seeds characterising the three zones of the water sorption isotherm, it was shown



that the water content had a strong effect on the dielectric properties of the seeds by a significant increase in the dielectric constants at high water content. The presence of free water could be related to these dielectric changes and to the effects on the electrical properties of the plasma discharge suggesting a higher charge accumulation during seed treatment. This raises the question of whether the physiological response of Arabidopsis seeds to plasma treatment can vary with water content, particularly at high water content, and what the associated mechanisms might be.

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## **Chapter 3:**

**Release of Arabidopsis seed  
dormancy by cold ambient air  
plasmas relies on cytoplasmic  
glass transition**

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# Release of *Arabidopsis* seed dormancy by cold ambient air plasmas relies on cytoplasmic glass transition

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## **ABSTRACT**

When mature Arabidopsis seeds are dormant, their germination is prevented in apparently favourable conditions. Seed dormancy release occurs through a process called after-ripening that can last several weeks or months, depending on storage conditions. Cold Ambient Air (C2AP) are weakly ionized gases and they have been shown to stimulate germination of seeds of various species. However, their mode of action on seed physiology is largely unknown and whether their effect could depend on seed water status is unclear. Here we have investigated the effect of C2AP, produced using a dielectric barrier device, on alleviation of Arabidopsis seed dormancy in relation with seed cytoplasmic viscosity. Seed exposure to C2AP triggered a substantial alleviation of dormancy but this effect was more pronounced when seeds were equilibrated at high relative humidity before C2AP or when the temperature increased up to 60 °C during C2AP. We designed a phase diagram of Arabidopsis seeds that shows that these conditions were associated with a glass to rubber transition, thus demonstrating the role of cellular viscosity in the efficiency of C2AP on seed dormancy release. We propose that lower cytoplasmic viscosity could promote the oxidative signaling induced by C2AP treatment and in turn improve the germination process.

## 1. Introduction

According to the Food and Agriculture Organization (FAO) of the United Nations, the world population is projected to reach more than 9 billion people by the year 2050, accompanied by a mechanical increase of 60 % in food supplies (FAO, 2020). In addition, modern agriculture faces considerable challenges owing to the impact of global warming and reduction of arable lands with drought and soil pollution resulting from intensive use of phytochemicals products. Synthetic nitrogen fertilizers in agriculture are yearly estimated at 110 Mt with negative impacts on climate change and biodiversity (Foyer et al., 2016). If seeds provide around 70 % of our food, finding complementary and innovative solutions to increase crop yields appears more than ever imperative while subscribing to a logic of sustainable development (Tester and Langridge, 2010).

While cold ambient air plasmas (C2AP) have already shown efficiency in a large diversity of applications (bio-decontamination, material micro-processing, surface modifications, medicine, ... (Brandenburg et al., 2019), (Misra et al., 2016), they appear today as a green option for sustainable agriculture, food or seed decontamination, seed germination and agricultural productivity (Randeniya and Groot, 2015), (Starič et al., 2020). C2AP generated in ambient air are weakly ionized gases composed of reactive species like oxygen and nitrogen species (RO(N)S), free energetic electrons, metastables, photons from UV and visible spectral ranges. Owing to their electrical, physical, radiative and chemical properties, C2AP appears as a particularly promising technological solution to face the aforementioned issues and has proven to be an alternative to face agriculture issues (Lieberman and Lichtenberg, 2005), (Samukawa et al., 2012). Dielectric barrier devices (DBD) supplied with green electricity could be a sustainable seed treatment to replace conventional phytosanitary approaches.

Since the early 21<sup>st</sup> century, many plasma processes have been developed to improve the quantitative parameters of seed germination such as vigour, homogeneity and germination rate as well as seedlings and plants' growth. Two main approaches can be distinguished: (i) the dry plasma approach (also called direct approach or single-step approach) which consists to expose seeds, seedlings or plants directly to a discharge or post-discharge and (ii) the wet plasma approach (also called indirect approach or two-steps approach) which consists to plasma-activate a liquid (water, liquid fertilizer, ...) subsequently utilized to imbibe seeds or irrigate plants. A wide variety of seed species has already been studied as

oilseed rape, bean, soybean, wheat, tomato, radish, mulungu, lentil... (Ling et al., 2015; Bormashenko et al., 2012; Zahoranová et al., 2016; Zhou et al., 2016; Jiang et al., 2014; Lu et al., 2014; Kitazaki et al., 2014; Alves Junior et al., 2016; Zhang et al., 2017). Both approaches are relatively efficient even though the dry one seems easier to transfer to industrial applications. Besides, seeds/seedlings treated following the wet approach can only benefit from the indirect effects of plasma, i.e. the long lifespan reactive species present in the liquid medium. On the contrary, the seeds treated following a dry approach benefit all the aforementioned plasma properties (electric field, reactive chemistry, radiation, ...). Among the various devices offering such dry plasma operation, DBD appears as quite relevant, especially thanks to their operation at atmospheric pressure and in ambient air, hence reducing investment costs from usual low-pressure devices. For example, these devices enable to reduce osmotic and saline stresses of *Arabidopsis* (Bafail et al., 2019) or to improve the size of crop plants from sowing to harvest (Koga et al., 2016).

Here, we assess the potential of C2AP to release the dormancy of *Arabidopsis* seeds, a genus of herbaceous plants from the Brassicaceae family. Seed dormancy can be characterized by the inability of seeds to germinate although all the necessary environmental conditions are apparently fulfilled (Bewley, 1997). Dormancy depends on intrinsic characteristics of the species but also on habitats and development conditions. It is an adaptive process which allows the survival of species in their ecosystems waiting for the favourable season to trigger seed germination and plant establishment (Baskin and Baskin, 1998). Dormancy of *Arabidopsis* dormant seeds is a physiological non-deep dormancy that blocks the germination of seeds (Baskin and Baskin, 2004) after maturation on the mother plants and in which the plant hormones abscisic acid (ABA) and gibberellins (GA) play antagonist roles. ABA, an inhibitor of germination, is produced in the embryo during seed development and induces dormancy whereas GA stimulates seed germination (Bewley, 1997). For *Arabidopsis*, the highest expression of dormancy is usually obtained when dormant seeds are germinated above 20 °C (Leymarie et al., 2012).

*Arabidopsis* seed dormancy is typically released during after-ripening which occurs while the dry seeds are stored over a prolonged period. The storing conditions, in particular the temperature and the relative humidity, play a crucial role in regulating the breaking of dormancy (Weitbrecht et al., 2011; Basbous-Serhal et al., 2016). An interesting assumption is that the combined effect of water content and storing temperature can change intracellular viscosity which, in turn, can release dormancy. From the point of view of

condensed matter physics, dry seeds can indeed be assimilated to amorphous solid materials owing to their ability to exist either in a glassy state or in a rubbery state depending on their temperature and water content. After the maturation on the mother plant, orthodox seeds will survive from a severe desiccation by entering into a glassy quiescent state (Buitink and Leprince, 2008). In an amorphous glassy state, no enzymatic reaction can theoretically occur other than reactive oxygen species (ROS) related oxidations or peroxidations (Ballesteros et al., 2020). When seed WC or temperature increases, the cytoplasm enters an amorphous rubbery state which allows higher mobility (Buitink and Leprince, 2008). The mechanisms of seed dormancy release in the dry state, i.e. when seeds are in a glassy state, are largely unknown. Several studies show that during after-ripening, non-enzymatic ROS production within the seeds could participate in their dormancy alleviation (El-Maarouf-Bouteau et al., 2013). ROS production is assumed to promote oxidative signalling during subsequent seed imbibition, thus modifying hormone signalling, gene expression, redox regulation and protein oxidation, which in turn increase the germination potential (Bailly, 2019). Conversely, an excessive accumulation of ROS results in a lower germinative potential and a shorter viability of the seeds. In consequence, the ROS content should remain within an 'oxidative window' allowing both dormancy release and germination completion. The generation of ROS is therefore a key issue to induce the transition from a quiescent state of a dormant dry seed to a non-dormant status. However, the effect of molecular mobility on dormancy release, oxygen diffusion and related non-enzymatic ROS production through autoxidation reactions within desiccated seed tissues are still unknown.

C2AP is a source of reactive oxygen and nitrogen species and can therefore be a relevant technique to break seed dormancy in dry seeds. So far and whatever the dry/wet approach, few studies have investigated whether cold plasma can significantly release the dormancy of seeds. Gómez-Ramírez et al. (2017) have demonstrated how an atmospheric air plasma can release the dormancy of Quinoa seeds with a DBD reactor operating in air at 500 mbar, with germination rates increasing from 60% to almost 100% (Gómez-Ramírez et al., 2017). Complementary works carried out with seeds of *Mimosa Caesalpinia Folia* presenting integument physical dormancy, increase the germination rate from 6% to 50% (da Silva et al., 2017). Here we postulate that the C2AP effect on seed dormancy breaking in the dry state depends on molecular mobility and cellular status, such features altering in turn oxygen diffusion and ROS generation within seed tissues. In consequence we assume that

seed water content, which directly controls glass to rubber transition, is a key determinant in the efficiency of C2AP for releasing seed dormancy.

The objective of this work is to investigate the effect of C2AP operating in ambient air on Arabidopsis seed dormancy release, in relation with water and glass properties within seed tissue. First, C2AP generated in a DBD is characterized by electrical measurements, optical emission spectroscopy and mass spectrometry. Then the physico-chemical properties of intracellular glasses are changed either by equilibrating seeds in a range of various relative humidities, or by modifying temperature. We assess the efficiency of C2AP on seed dormancy release under these conditions. Finally, glass transitions are determined using differential scanning microcalorimetry (DSC) measurements which provide information on cellular viscosity and cytoplasmic diffusion. Our results demonstrate that the effect of C2AP on seed dormancy release relies on seed cytoplasmic viscosity.

## 2. Material & Methods

### 2.1. Plant material and germination assay

*Arabidopsis thaliana*, ecotype Col-0, seeds are grown and harvested dormant, following the method described by *Leymarie et al.* (2012). Two independent seed batches are used in this study. For germination assays, 4 replicates of 100 seeds each are distributed on a layer of cotton wool moistened with demineralized water in glass Petri dishes (9 cm in diameter) and placed at 25 °C in darkness, conditions in which Arabidopsis seed dormancy is strongly expressed (*Leymarie et al.*, 2012). Seed germination is scored twice a day for approximately 12 days. A seed is considered as germinated once the radicle protrudes the envelope. Each germination curve corresponds to the average value of the 4x100 seeds with standard deviation.

### 2.2. Seed water content equilibration and measurement.

Seed water content (WC) is expressed on a dry weight (dw) basis (expressed as g.H<sub>2</sub>O.g dw<sup>-1</sup>) which is determined after oven drying for 24 h at 105 °C. Seeds are placed in tightly closed jars at 20°C and equilibrated for 4 days at various WC over Silica Gel or over saturated saline solutions of ZnCl<sub>2</sub>, LiCl, CaCl<sub>2</sub>, MgCl<sub>2</sub>, Ca(NO<sub>3</sub>)<sub>2</sub>, NaCl, KCl and KH<sub>2</sub>PO<sub>4</sub> giving relative humidities of 2.5, 9.5, 17, 28.5, 40, 56.5, 75, 83.5 and 93 %, respectively, according to the method described by *Vertucci et al.* (1993).

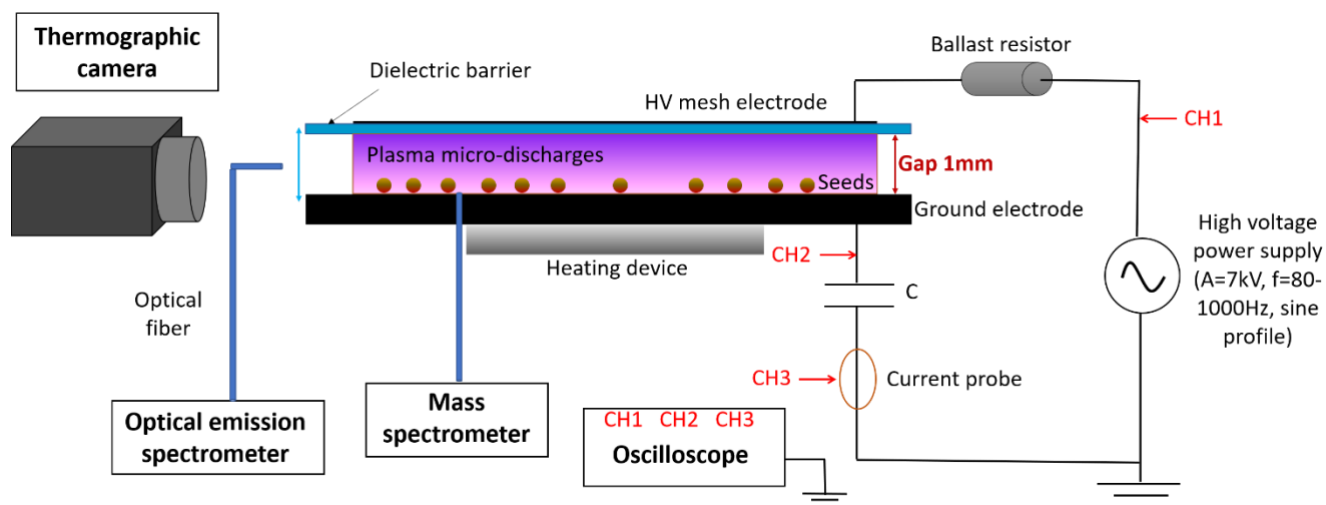
### 2.3. Plasma process.

Seeds are treated using a dielectric barrier device (DBD) in a plan-to-plan configuration with a gap of 1 mm, i.e. 3 times larger than the average diameter of Arabidopsis seeds. The DBD has two electrodes: (i) an electrode biased to high voltage, 30x40 cm<sup>2</sup> in area, composed of a stainless-steel mesh with a 100 µm nominal opening, a wire diameter of 71 µm and an open area of 34 % and (ii) a counter-electrode which is grounded, 30x40 cm<sup>2</sup> in area, bulk alumina plate. The dielectric barrier is a 2mm thick glass plate on which the mesh electrode is fixed. Optionally, a heating plate device (Froilabo EM130 Hotplate Magnetic Stirrer) is in contact with the counter-electrode to control temperature in the gap of the DBD between 30 °C and 80 °C with an accuracy of  $\pm 2$  °C.

The mesh electrode is powered with a high voltage generator composed of a function generator (ELC Annecy France, GF467AF) and a power amplifier (Crest Audio, 5500W, CC5500). The power supply is electrically protected by a ballast resistor (250 kΩ, 600 W) presented in Figure 1. As shown in Fig. 2, the peak amplitude of the voltage applied on the mesh electrode reached 7 kV at 145 Hz (for an optimized plasma power, see below). Typically, 1000 seeds of *A. Thaliana* can be placed on the grounded electrode, occupying a volume as low as  $V_{\text{seeds}} = 1000 \times V_{\text{seed}} = 14 \text{ mm}^3$ . Since the gap volume is  $V_{\text{GAP}} = 30 \times 40 \times 0,1 \text{ cm}^3 = 120 \text{ cm}^3$ , the volumetric filling rate of the device is only 0.01 %.

### 2.4. Electrical characterization

Voltages and currents of the DBD are measured using a digital oscilloscope (model wavesurfer 3054 from Teledyne LeCroy company), with a 500 MHz bandpass and a 4GS/s sample rate. The voltage applied to the mesh electrode is measured with high voltage probes (Tektronix P6015A 1000:1, Teledyne LeCroy PPE 20 kV 1000:1, Teledyne LeCroy PP020 10). The discharge current is monitored using a current transformer placed between the metal plate counter-electrode and the ground and enables to rebuild the distribution of plasma current peaks. A measurement C2APacitor ( $C_m = 80 \text{ nF}$ ) is inserted between the counter-electrode and the ground to measure the voltage ( $V_{\text{DBD}}$ ) and the charge ( $Q_{\text{DBD}}$ ) of DBD. Then, Lissajous curves are plotted to measure the energy dissipated in the DBD. Multiplying this value by the operating frequency provides the electrical power injected in the DBD.



**Figure 1** - Sketch diagram of the DBD utilized to treat seeds by cold ambient air plasma.

## 2.5. Optical emission spectroscopy (OES)

Optical emission spectroscopy is achieved to identify the radiative species emitted by plasma. The spectrometer (Andor, SR-750- B1-R) is used in the Czerny Turner configuration with a focal length of 750 mm over a 200-850 nm spectral range. The device includes an optical fiber (Leoni fiber optics SR-OPT-8014, 100  $\mu\text{m}$  diameter), a diffraction grating (1200 grooves. $\text{mm}^{-1}$ ) blazed at 500 nm and an ICCD camera (Andor Istar, 2048  $\times$  512 imaging array of 13.5  $\mu\text{m} \times 13.5 \mu\text{m}$  pixels). To collect a maximum of plasma emission with a 1 mm spatial resolution, a converging lens (ThorLabs, LA4380-UV,  $f = 100\text{mm}$ ) is placed between plasma and the optical fiber.

## 2.6. Mass spectrometry

Mass spectrometry is performed to analyse the gas phase and identify the relevant species from the plasma phase. The mass spectrometer used in this study is a quadrupole-based mass spectrometer (Model HPR-20 from Hiden Analytical Ltd). Air plasma chemical species are collected by a quartz C2APillary whose inlet is fixed onto a 2-axis stages plate placed in the middle-left of the plasma reactor (see Fig. 1). The 1 m long C2APillary is flexible, chemically inert and heated at 200  $^{\circ}\text{C}$  to prevent chemisorption. Then, a three-stage differentially pumped inlet system separated by aligned skimmer cones and turbo molecular pump, enables a pressure gradient from  $10^5$  bar to  $10^{-7}$  bar at the entrance of the



70-eV energy ionization chamber. The species detected by mass spectrometry are quantified by measuring their intensities when the gas is ionized (plasma) and then subtracting the corresponding intensities when the gas is non-ionized (background). In both cases, the measurements are performed in steady state, obtained once the pressure is stabilized after the ignition of the filament (about 1h).

## 2.7. Thermographic infrared imaging

A thermographic camera (Infrared camera Jenoptik VarioCAM HD head 900, InfraTec) is used to assess the temperature of the material surfaces in contact with plasma, especially the counter electrode and the dielectric barrier. Temperature survey is carried out in real time when plasma operates with/without the heating plate device.

## 2.8. Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter (DSC 30, Mettler Toledo) is used to measure enthalpy variations of the seeds and deduce the glass to rubber transition temperature (i.e. glass transition temperature,  $T_g$ ). All the DSC measurements are carried out on samples composed of approximately 20 mg of Arabidopsis seeds. Each sample is placed into a 40  $\mu$ L aluminium crucible which is hermetically sealed to suppress the endothermic evaporation, vaporization or sublimation of volatile substances in the furnace. The seeds are exposed to a 10 °C/min ramp between 0 °C and 100 °C, so that the glassy-rubbery transition is highlighted by a discontinuity of the seeds sample thermal capacity upon the 10 °C/min rate. The  $T_g$  is estimated as the mid-point of the temperature range associated with the shift in specific heat. Between 8 and 12 DSC scans are performed with seeds equilibrated at each relative humidity used in this study.

## 2.9. Statistical analyses

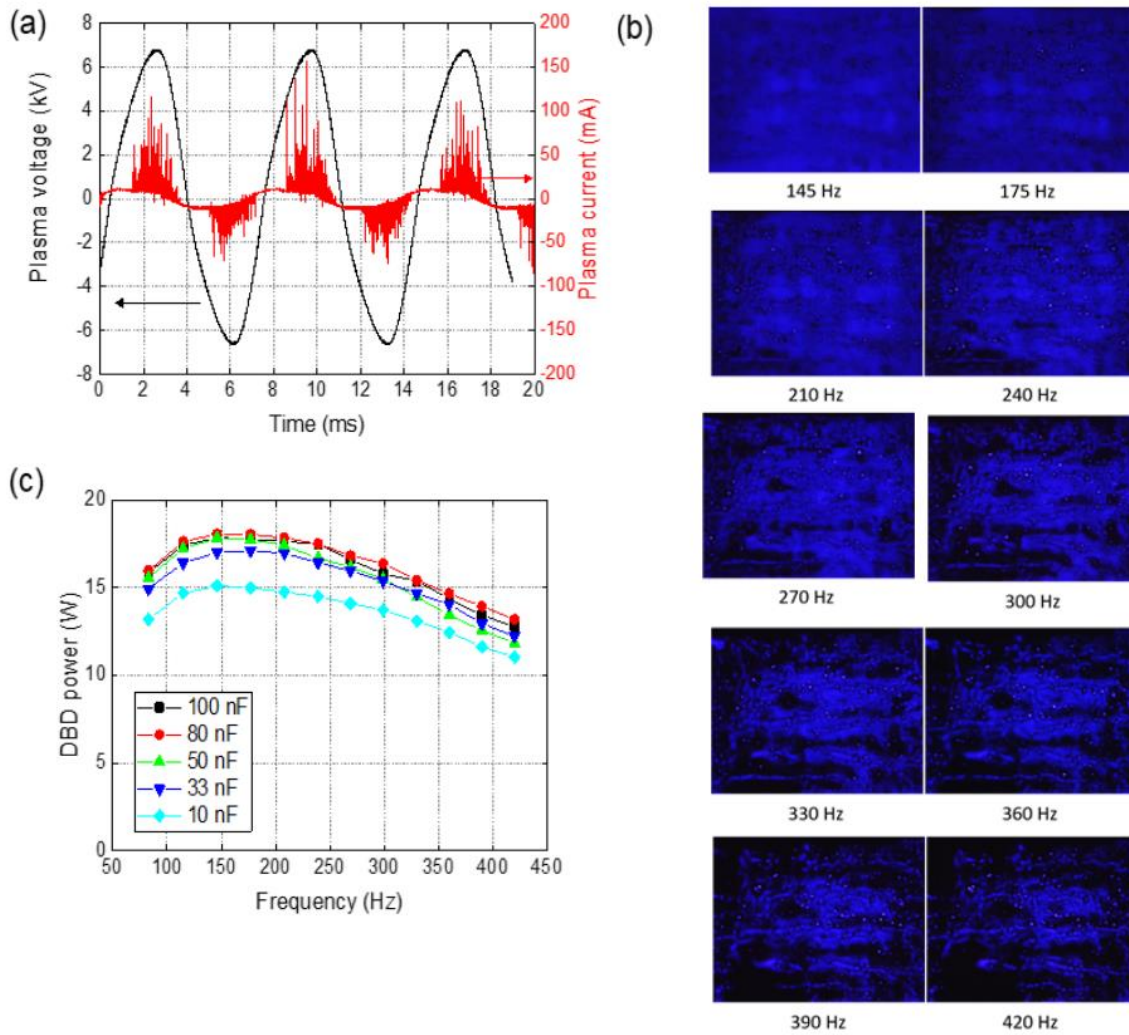
All the statistical analyses are performed using OriginLab Software statistical tools, including curve fitting, Mann-Whitney and ANOVA tests to determine significant differences between control group and plasma group on the total germination rates.

## 3. Results

### 3.1. Plasma characterization

#### 3.1.1. Electrical characterizations

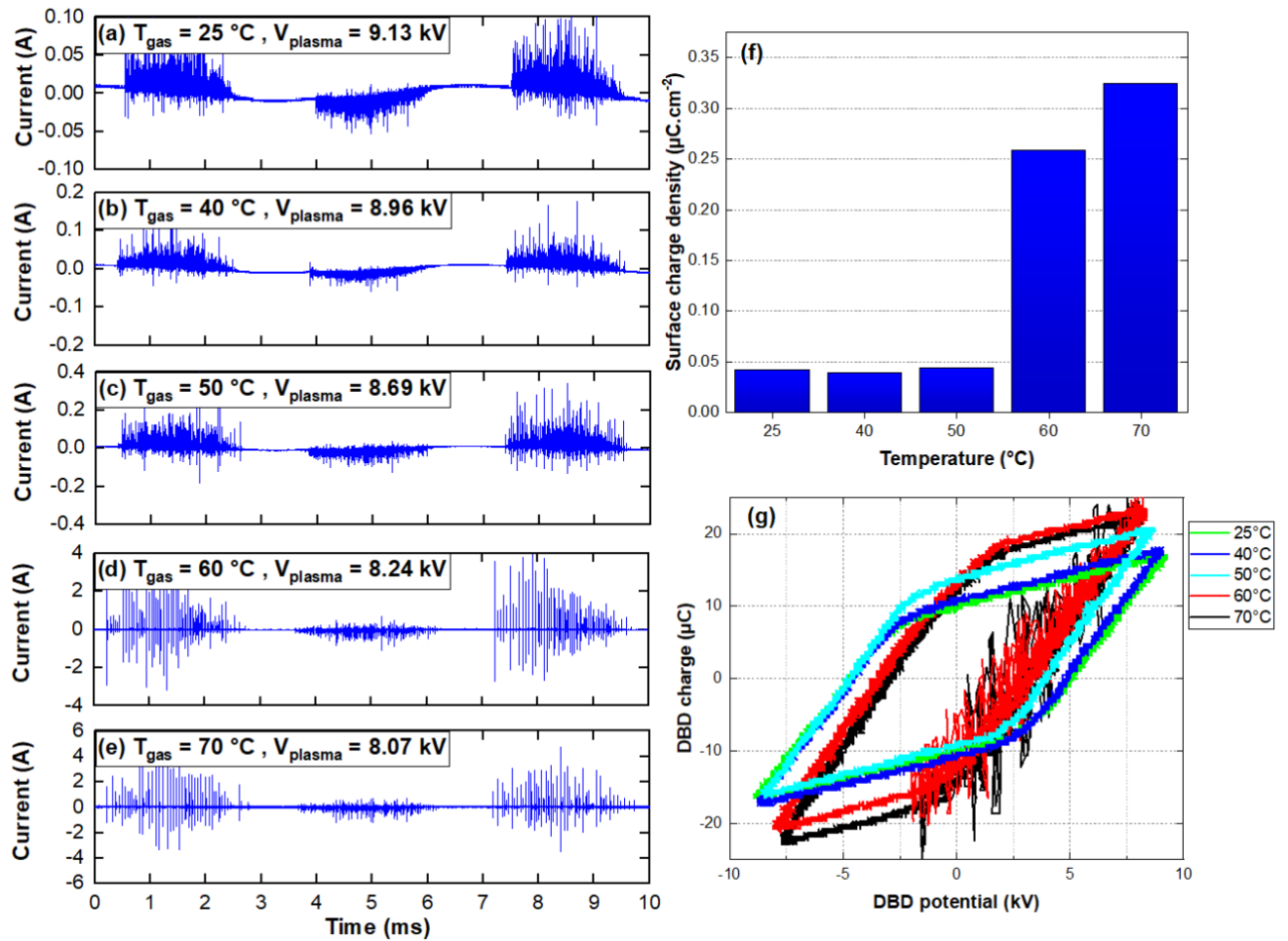
Since the volumetric filling rate of the device is only 0.01 %, the action of the seeds on the plasma electrical properties can be neglected. As shown in Figure 2(a), the DBD voltage has a sine waveform, 7 kV in magnitude, while the DBD current shows two components: (i) a dielectric component proportional to the derivative of the applied voltage and (ii) a conduction component composed of current peaks (typically a few tens of nanoseconds) that represent the micro-discharges randomly distributed over a time window delimited by the breakdown and extinction voltages. At first sight, the spatial distribution of these micro-discharges is uniform in the DBD, hence ensuring a homogenous treatment of the seeds. However, owing to the large size of the electrodes ( $S_{\text{elec}} = 30 \times 40 \text{ cm}^2$ ), the plasma spreading area does not match necessarily to  $S_{\text{elec}}$ , especially because of a frequency dependence. As indicated in Figure 2(b), while the plasma is homogeneous over the entire electrode surface for  $f < 200 \text{ Hz}$ , its spreading area gradually decreases for higher frequencies, leaving dead zones where the plasma never forms throughout processing. Besides, the coupling of the measurement C2APacitance ( $C_m$ ) with the DBD can influence the plasma electrical properties. Usually, the value of  $C_m$  is chosen to be non-intrusive, i.e. at least 10 times higher than that of the DBD. Since this latter value is estimated to be 3.7 nF,  $C_m$  has to be in the range of a few tens nF. The Figure 2(c) represents the electrical power deposited in the DBD as a function of the applied frequency for different values of  $C_m$ . It turns out that a maximum power (18 W) is deposited for  $f = 145 \text{ Hz}$  and  $C_m = 80 \text{ nF}$ : two values that are fixed for all the subsequent experiments carried out on the seeds.



**Figure 2** - Electrical characterizations of the DBD supplied with ambient air and a gap of 1 mm. (a) Typical waveform of the plasma voltage and current measured in the DBD at  $C_m = 80$  nF,  $f = 145$  Hz. (b) Images of the plasma observed through the HV mesh electrode for 10 values of frequency at  $C_m = 80$  nF. (c) Electrical power of the DBD reactor for different values of frequency and measurement C2APacitors.

The influence of an external heating source on the current peaks is clearly highlighted from Figure 3(a) to Figure 3(e) with values of 25 °C, 40 °C, 50 °C, 60 °C and 70 °C. A rise in temperature simultaneously leads to an increase in the amplitude of the peaks and a decrease in their number, resulting in a higher charge-per-filament. Knowing the area covered by the micro-discharges within the DBD, a surface charge density can be deduced, as reported in Figure 3(f). For temperatures lower or close to 50 °C, the surface charge density is always lower than  $0.05 \mu\text{C}.\text{cm}^{-2}$  whereas its value drastically increases to  $0.25 \mu\text{C}.\text{cm}^{-2}$  at 60°C and  $0.32 \mu\text{C}.\text{cm}^{-2}$  at 70°C. To verify the effects of the temperature on the

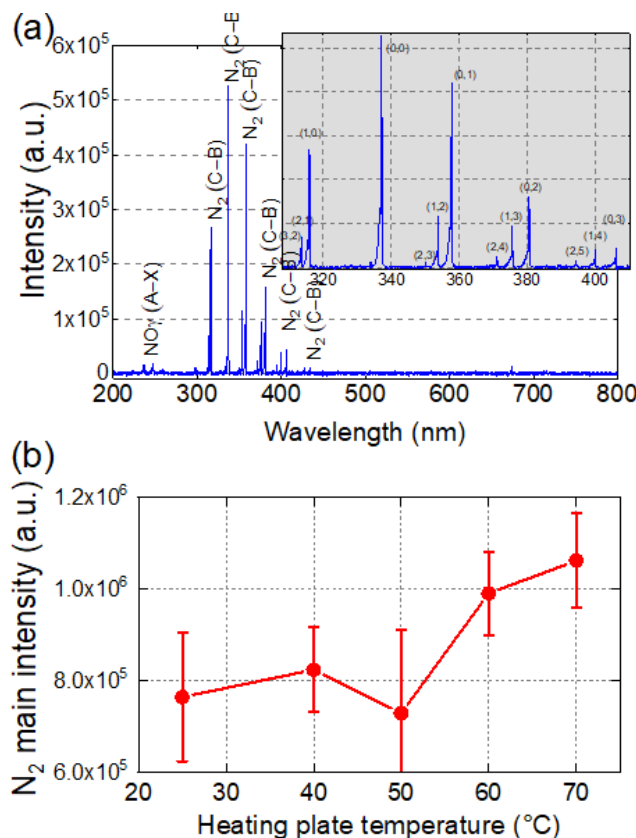
electrical power, the charge  $Q_{\text{DBD}}$  is plotted as a function of the DBD voltage in Figure 3(g). Each resulting Lissajous figure looks like a parallelogram whose upper and lower edges correspond to the capacitance of the dielectric barrier ( $C_{\text{bd}}$ ) while the left and right edges correspond to  $C_{\text{bd}}$  in series with the gas capacitance ( $C_{\text{g}}$ ). The area covered by each Lissajous parallelogram corresponds to the electrical energy from which the electrical power is deduced by multiplying the energy by the frequency. Surprisingly and whatever the temperature, the electrical power is quite constant with a value close to 20 W.



**Figure 3** - Electrical characterizations of the DBD as a function of temperature. Waveforms of discharge current measured in the DBD (80 nF, 145 Hz) (a) at 25 °C, (b) 40 °C, (c) 50 °C, (d) 60 °C, (e) 70 °C. (f) Surface charge density estimated through 9 periods vs gas temperature. (g) Lissajous figures.

### 3.1.2. Optical emission spectroscopy

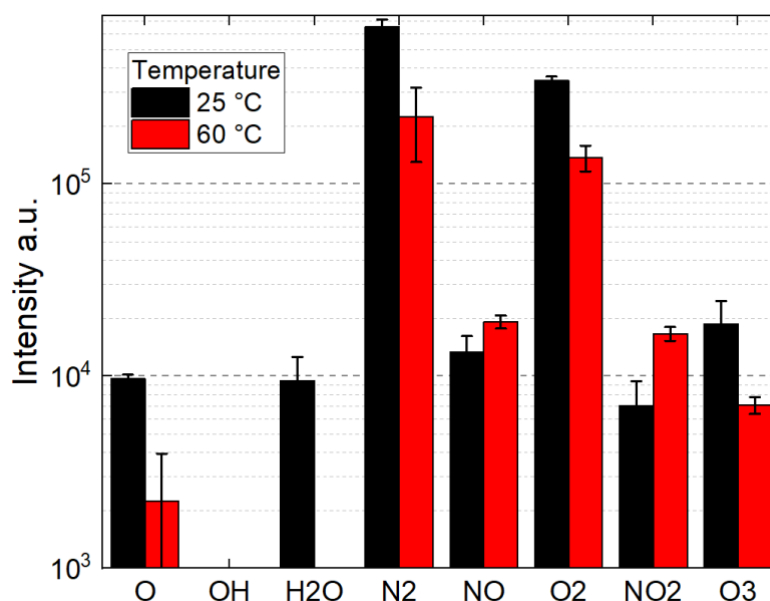
Figure 4(a) presents the optical emission spectrum of the air plasma generated in the DBD. The emission lines and bands are part of the UV-VIS range (200-800 nm) and are derived from the energetic processes producing reactive oxygen and nitrogen species. If one can clearly observe the vibrational bands of the Second Positive System (SPS) of molecular nitrogen ( $C^3\Pi_u - B^3\Pi_g$ ) (Walsh et al., 2010; Misra et al., 2013; Feizollahi et al., 2020), it is worth notifying the absence of oxygen line at 777 nm. It can be explained by the fact that in air atmospheric discharges, the  $O(^5P)$  and  $O(^3P)$  lines are quenched by  $N_2$  and  $O_2$  before being detected by OES (Walsh et al., 2010). A large part of atomic oxygen could also be involved in fast kinetic reactions, i.e. recombination into molecular oxygen or impinging with molecular oxygen to form ozone. Interestingly, emission bands of NO are detected (Machala et al., 2007) in the 220-260 nm region while no emissive band of OH radicals is evidenced at 309 nm. Finally, just as the temperature influences the electrical properties of the plasma, it influences its optical properties as well. As observed in Figure 4(b), the maximum emission of the  $N_2$  band at 337 nm increases with temperature in a range between 25 and 70 °C.



**Figure 4** - Optical emission spectra (a) and N<sub>2</sub> main lines intensities integration as a function of temperature (b) of DBD operating in air, 7 kV, 145 Hz, 1 mm gap.

### 3.1.3. Mass spectrometry

Figure 5 represents the intensity of the chemical species detected by mass spectrometry in the plasma phase considering the DBD supplied with air at 25 °C (ambient temperature) or air at 60 °C (heating plate device). At room temperature, the air plasma is composed of non-reactive stable molecules (N<sub>2</sub>, O<sub>2</sub>, H<sub>2</sub>O) as well as reactive species of short lifespan (O, NO and NO<sub>2</sub>) and long lifespan (O<sub>3</sub>). Although it is common to measure OH radicals in air plasmas, their amount remains undetectable by mass spectrometry, which is consistent with the optical emission spectroscopy results presented in Figure 4a. Gas heating of the air plasma leads to a strong decrease in native molecular nitrogen and oxygen as well as to the total disappearance of water vapour. This results into the production of NO<sub>x</sub> species and to the simultaneous consumption of the reactive oxygen species, namely O and O<sub>3</sub>.



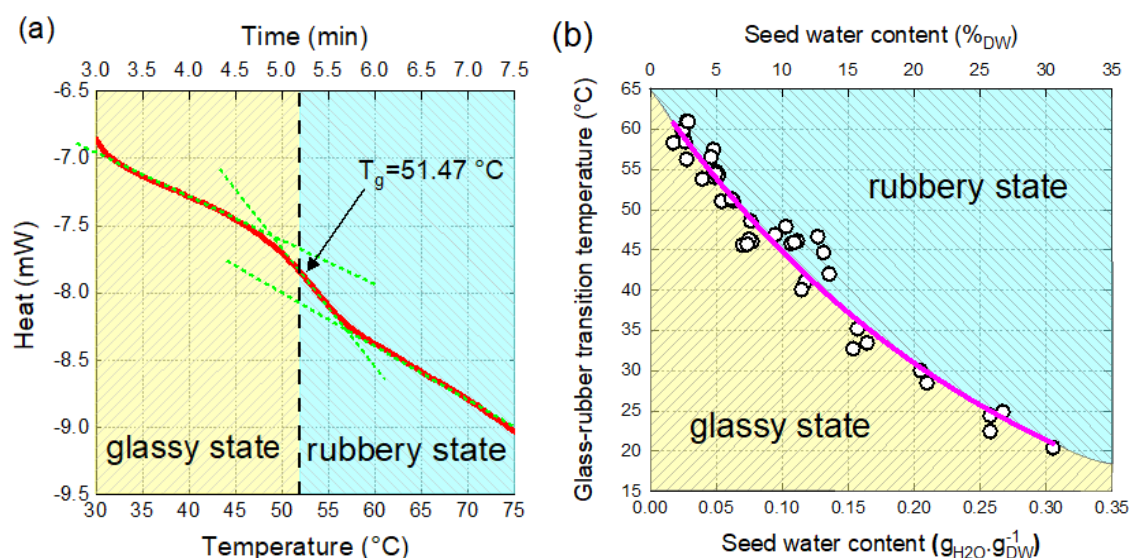
**Figure 5** - Variation of the main chemical species detected by mass spectrometry in air plasma treatment operating either at 25 °C or 60 °C (air, 7 kV, 145 Hz, 1 mm gap).

## 3.2. Deciphering the effects of cold plasma on seeds properties

### 3.2.1. Phase diagram of Arabidopsis seeds

The characterization of the seed amorphous solid state described by either a cytoplasmic glassy state or a cytoplasmic rubbery state is investigated by determining the glass transition temperature. The relation between seed WC and glass transition temperature ( $T_g$ ) is investigated using DSC measurements which allow drawing phase diagram of Arabidopsis seeds. As shown in Figure 6(a), the glass transition is characterized by a second-order transition, i.e. a shift in the baseline of the DSC thermogram. Multiple DSC measurements are carried out with seeds equilibrated at various WC ranging from 0.0173 to 0.31 g.H<sub>2</sub>O.g<sup>-1</sup> to determine  $T_g$  in order to design the state diagram shown Figure 6(b). Within the range of seed WC,  $T_g$  decreases from 61 °C to 20 °C following an exponential decay whose fitting equation is given by  $T_g = 64.8e^{-3.7WC}$ . The accuracy of the fit is validated by the R-square coefficient as high as 0.95. Therefore, using the state diagram, we can unambiguously delineate glassy and rubbery state regions versus both temperature and WC in Arabidopsis seeds.



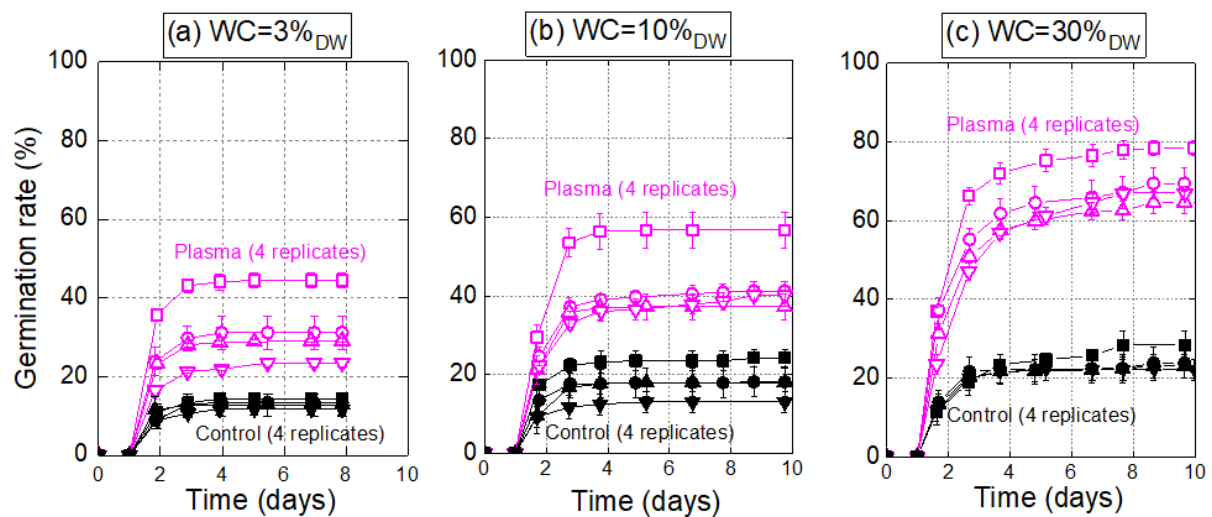


**Figure 6** - Seed glass transition characterization in *Arabidopsis* seeds. (a) a DSC thermogram of 20 mg *Arabidopsis* seeds of  $\text{WC} = 6.5\%_{\text{DW}}$  and (b) phase diagram of *Arabidopsis* seeds showing glass transition temperature (purple line) vs WC; each white circle being an experimental data extracted from a DSC thermogram.

### 3.2.2. Role of seed WC on seed dormancy release by plasma treatment

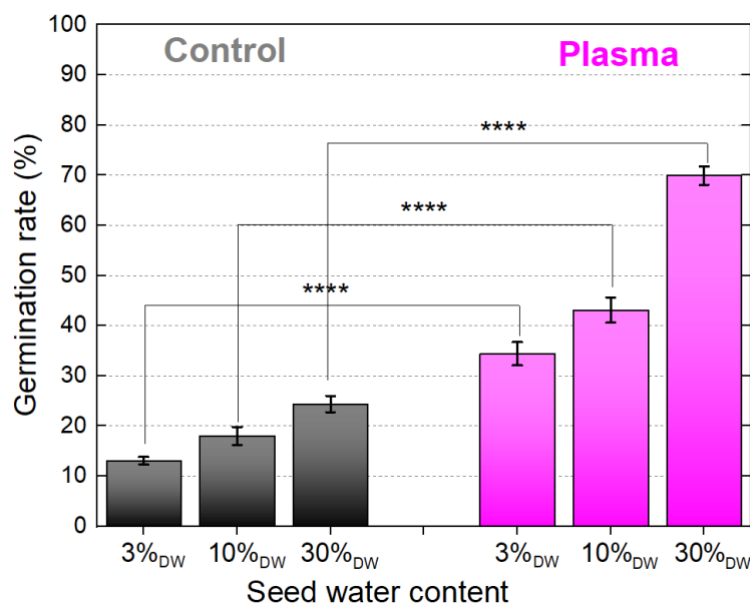
To determine whether the effects of cold plasma on seed dormancy release could depend on cytoplasmic viscosity, we apply the C2AP treatment on freshly harvested dormant seeds previously equilibrated at 3 values of WC, i.e. low water content ( $3\%_{\text{DW}}$ ), medium water content ( $10\%_{\text{DW}}$ ) and high water content ( $30\%_{\text{DW}}$ ). Considering that the plasma gas temperature is around  $30^\circ\text{C}$ , the state diagram Figure 6(b) shows that the seeds of the two first groups are in a glassy state while those from the third group are in a rubbery state. Each group is individually placed in the DBD where an air plasma is generated at  $V = 7\text{ kV}$ ,  $f = 145\text{ Hz}$  for 15 min. Germination of treated seeds is reported in Figure 7(a), (b) and (c) for the low, medium and high WC conditions, respectively. Untreated seeds are dormant since their germination does not exceed 20 %, whatever the replicate (Figure 7). Plasma treatment enhances the germination of dormant seeds at  $25^\circ\text{C}$ ; the higher the initial seed WC, the higher the final germination percentage (Figure 7). While plasma increases the germination rate from 10% to 30% for seeds in a glassy state ( $\text{WC} = 3\%_{\text{DW}}$ ), it increases the germination rate to a value as high as 70% for seeds in a rubbery state ( $\text{WC} = 30\%_{\text{DW}}$ ) (Figure 7).





**Figure 7** - Germination at 25 °C of seeds equilibrated for 4 days at (a) 3 %<sub>DW</sub>, (b) 10 %<sub>DW</sub> and (c) 30 %<sub>DW</sub> water contents (WC, dry weight basis) and exposed to C2AP (plasma) for 15 minutes or not (control). Means  $\pm$  SD of 4 replicates (around 100 seeds per replicate).

The trend of efficiency of plasma treatment with WC is highlighted in Figure 8. Mann-Whitney tests have been performed between control and plasma groups for each water content condition to characterize significant statistical differences. It is found that for the three WC groups, the control and the plasma distributions are significantly different.



**Figure 8**. Final germination rate after 10 days at 25 °C of seeds equilibrated for 4 days at (a) 3 %<sub>DW</sub>, (b) 10 %<sub>DW</sub> and (c) 30 %<sub>DW</sub> water contents (WC, dry weight basis) and exposed to C2AP (plasma) for 15 minutes or not (control). Means  $\pm$  SD of 4x4 replicates (\*\*\*\*:  $p$  value  $< 0.0001$ ).

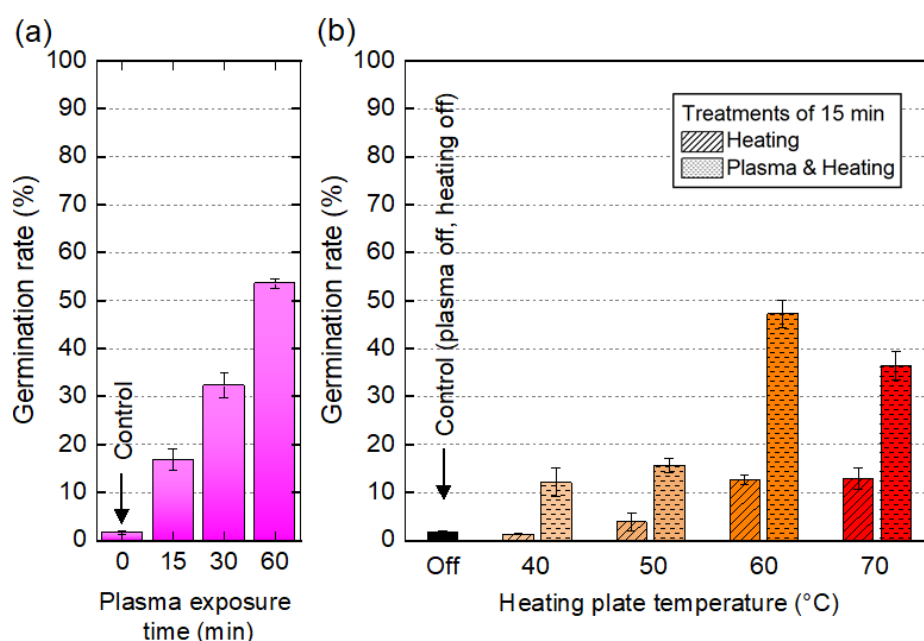
### 3.2.3. Effect of temperature on seed dormancy release by plasma

To demonstrate the relationship between cellular viscosity and plasma efficiency on seed dormancy release, the glass transition is triggered at a constant seed WC by increasing seed temperature upon plasma exposure. By knowing seed WC and referring to the state diagram Figure 6(b), one can unambiguously predict the physical state of the seeds upon their plasma exposure at a determined temperature. As depicted in Figure 1, a heating plate device is in contact with the DBD counter electrode, transferring heat by thermal conduction to the gas temperature inside the DBD. For each of the experiments described below, the gas heating procedure requires a period of 30 to 45 minutes in order to reach a permanent regime, i.e. without significant temperature variation ( $< 2^{\circ}\text{C}$ ). The seeds are placed into plasma only when this regime is reached. Temperature of the whole system, including seeds, is monitored in real time with IR imaging techniques (thermographic camera and laser beam sensor).

First, the effect of the plasma exposure duration is studied on seed germination at  $20^{\circ}\text{C}$  (Figure 9(a)). Fifteen min, 30 min and 1 h of treatment are performed using seeds at  $7.0\%_{\text{DW}}$  while controlling the gas temperature to ensure that its value remains lower than the glass transition temperature. At this WC, glass transition temperature occurs at values slightly higher than  $50^{\circ}\text{C}$  (Figure 6(b)). In this experiment, the temperature is initially  $20^{\circ}\text{C}$  ( $\pm 1^{\circ}\text{C}$ ) and gradually increases until reaching a maximum value of  $30^{\circ}\text{C}$  after 1 h of treatment, which nevertheless remains lower than  $T_g$  (Figure 6(b)). The longer treatment increases germination from 2 % to 50 % but a short treatment (15-30 min) has only a slight beneficial effect on seed dormancy release (Figure 9(a)). In order to assess a potential synergy resulting from plasma treatment and thermally-controlled glass transition, we consider two groups of seeds: (i) seeds placed in the DBD during 20 min while plasma is off but temperature set from 40 to  $70^{\circ}\text{C}$  and (ii) seeds placed in the DBD where they are heated for 5 min while plasma is off and then 15 min while plasma is on.

The heating procedure slightly stimulates seed germination, but it never exceeds 15 % (Figure 9(b)) suggesting the heat treatment only does not release seed dormancy. At  $7.0\%_{\text{DW}}$ , plasma treatment has a significant beneficial effect on seed dormancy release when temperature is higher than  $50^{\circ}\text{C}$  (Figure 9(b)). A germination rate as high as 48 % is obtained at  $60^{\circ}\text{C}$  which underlines the spectacular effect resulting from the combination of plasma & thermal procedures, since the germination rate is multiplied by 24 compared to control.

At 70°C, however, the germination rates are lower than the one obtained at 60 °C. This confirms that the positive effect of plasma on seed dormancy alleviation requires a cytoplasmic rubbery state.



**Figure 9** - Final germination rate after 10 days at 25 °C of dry seeds (WC = 7 %<sub>DW</sub>) (a) exposed at room temperature to C2AP for various durations and (b) of dry seeds heated for 15 min without (Heating) or with C2AP (Plasma & Heating) (b). Means +/- SD of 4x4 replicates.

## 4. Discussion

### 4.1. C2AP generated in ambient air as a tool to break physiological seed dormancy

We use a DBD reactor in ambient air to treat seed and the results about seed dormancy alleviation are significantly obtained for treatments as short as 15 min, making C2AP a relevant method to release seed dormancy. If an electrical optimization is achieved in order to maximize the plasma power during the treatments obtained at  $f = 145$  Hz and for  $C_m = 80$  nF as shown in Figure 2, it is noteworthy that the electrical behaviour of the discharge completely changes with the increasing temperature. As shown in Figure 3, we observe a

shift of electrical properties above 50 °C which remains misunderstood. The current waveforms are clearly modified from 60 °C; the intensity of the current peaks increase radically until values of ampere as well as the surface charge density that is multiplied by a factor of 7. Increasing the temperature reduces the relative humidity in the interelectrode gap and could explain variations in terms of current peak amplitudes and time spreading of the micro-discharges (Abdelaziz et al., 2018). The dielectric barrier is also affected by the rise of temperature as evidenced in Figure 3(g). The change in the slope of the upper/lower edges of the Lissajous curves indicate a slight increase in  $C_{db}$  due to temperature. This may result from an increase in the relative permittivity of the glass, as already evidenced in other experiments (Yadav et al., 2015). Mass spectrometry also attests the increase in NO and NO<sub>2</sub> intensity (Figure 5) when increasing the gaseous temperature of the air plasma.

Whatever the method used in this study (playing on the seed water content or the temperature during the treatment), the results demonstrate the real ability of C2AP to release seed dormancy. Generally, the effect of cold plasma on seed dormancy is limited to physical dormancy with integument or coat features that limit the water uptake which, in turn, reduces the germination rate (Gómez-Ramírez et al., 2017; da Silva et al., 2017). A physiological study was also achieved about the potential of C2AP to break physiological dormancy regarding the time of storage after the harvest of radish seed (Degutytė-Fomins et al., 2020). It is clearly demonstrated that an atmospheric air DBD device could improve germination rate after different periods of storage after harvest expressed by the change in the balance of phytohormones measuring the rate of ABA and GA after plasma treatment. In our study, we significantly improve the germination rate of strongly dormant Arabidopsis seeds without changing time of storage but by playing on the seeds' amorphous solid state, hence proving that C2AP is an appropriate method to release dormancy associated with the seed glass transition.

#### 4.2. Glass transition temperature is a pinpoint for seed dormancy release by C2AP

One of the major findings of this work is the demonstration that C2AP effect on seed dormancy release depends upon intrinsic physicochemical properties of cytoplasm. This first required establishing a state diagram of Arabidopsis seeds which, to our knowledge, has never been performed before. The state diagram Figure 6(b) shows that  $T_g$ , which is

measurable when seed WC ranged from 0.017 to 0.31 g H<sub>2</sub>O.g<sup>-1</sup>, classically decreased when seed WC increased, as already observed for other seed species (e.g. for axes of bean seeds (Buitink and Leprince, 2004) or for sunflower seeds (Lehner et al., 2006)) and which can be explain because water is a plasticizer of glasses that loosen molecular connections within the glassy matrix (Walters, 1998).

Here we have taken advantage of the knowledge provided by the state diagram of *Arabidopsis* seeds to alter cytoplasmic viscosity prior to the C2AP treatment by modulating seed WC or temperature. We show that whatever the means to ensure glass transition from a glassy to a rubbery state, C2AP treatments become very effective in releasing seed dormancy when cytoplasm is in a rubbery state. Practically this is reached when seed WC is 30 %<sub>DW</sub> at ambient temperature (~25 °C) or when seed temperature is 60 °C at 7 %<sub>DW</sub> (see state diagram Figure 6(b)). This indicates that the reduction of cellular viscosity promotes C2AP effect on seed dormancy release. Glasses are known to dramatically restrict molecular mobility and therefore a wide range of enzymatic and non-enzymatic reactions, and this status is generally associated with seed survival in the dry state (Ballesteros et al., 2020). However, in *Pinus densiflora* seeds, gaseous oxygen has been demonstrated to diffuse more easily in glassy state than in rubbery state and to participate in oxidative reactions leading to seed ageing (Gerna et al., 2022). The shift to a rubbery state that increases the beneficial effect of plasmas on dormancy release, suggests that efficient C2AP treatments require some cytoplasmic mobility. Interestingly, the fact that C2AP is efficient both when increasing seed WC to 30 %<sub>DW</sub> and when increasing temperature of dry seeds allow to rule-out that the effect of plasmas strictly depends on an active metabolism. At 30 %<sub>DW</sub>, indeed, seeds contain free water which allows metabolism, including respiration, but at 7 %<sub>DW</sub> (seed moisture content when seeds are treated at 60°C) seeds contain only bound water that is not available for metabolic activity, as described by water sorption isotherms (Buitink and Leprince, 2008). Since it is known that seed dormancy release relies on oxidative processes (Bailly, 2019), we therefore postulate that the stronger effect of plasma when seeds are in a rubbery state might be related to a better diffusion of ionized gases containing active species like ROS and RNS. Thus, in such conditions, the interaction between C2AP and seeds will be enhanced, which would favour oxidative reactions within seed tissues, and in turn favour seed dormancy alleviation. It has indeed been demonstrated that alleviation of seed dormancy in the dry state can be explained by a selective oxidation of negative regulators of seed germination, that results from auto-oxidative reactions and ROS

generation (El-Maarouf-Bouteau et al., 2013; Bailly, 2019) . To confirm this hypothesis, further studies will have to investigate whether C2AP treatments can trigger ROS generation within dry seeds.

## 5. Conclusion

In this study, we have identified two approaches to improve the germinative properties of Arabidopsis seeds by plasma treatment through a modification of their cytoplasmic viscosity: either by increasing the air plasma temperature to 60°C or by increasing seed water content to 30%<sub>DW</sub>. That C2AP effect relies on gas diffusion during heating or on diffusion of ROS and RNS for seeds with high water content; a lower cytoplasmic viscosity seems to enhance the effect of the treatment on the release of Arabidopsis seed dormancy. Ensuring a transition from a glassy to the rubbery state after seed harvest is therefore a prerequisite for improving C2AP dormancy breaking processes. Our findings should open novel areas of C2AP utilization to rapidly release seed dormancy of crop species.



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**Chapter 4: Deciphering the  
biological mechanisms and  
morphological changes  
induced by ambient air cold  
plasmas to release the  
dormancy of Arabidopsis seeds**

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# Deciphering the biological mechanisms and morphological changes induced by ambient air cold plasma to release the dormancy of *Arabidopsis* seeds

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**NB:** Supporting figures for this chapter are provided in the Annex Chapter 4 and the lists of up and down regulated genes and present in SeedNet are available on google drive using the following link: [https://drive.google.com/drive/folders/1ZuOU-r7Mx6FWMR6lbb74Pe\\_rRP9qKt8c](https://drive.google.com/drive/folders/1ZuOU-r7Mx6FWMR6lbb74Pe_rRP9qKt8c)

**NB:** This chapter contains 15 Figures, 4 Tables, 9 Supplemental figures and 3 Supplemental tables in Annex, and 4 Supplemental lists on Google Drive.

Keywords: ambient air cold plasma, dormancy release, X-ray microtomography, synchrotron, transcriptomic analysis

## 1. Introduction

### 1.1. Cold ambient air plasmas: a new priming approach to release dormancy

Cold ambient air plasmas (C2APs) turn out to be an effective technique to release physiological Arabidopsis seed dormancy (Chapter 2), (Degutytė-Fomins et al., 2020). They also appear as a promising approach to replace agrochemicals by improving germination and avoiding proliferation of pathogens (Mravlje et al., 2021). Using C2APs to treat seeds before sowing could reduce – or even replace – the use of phytopharmaceuticals. While this eco-friendly and cost-effective seed priming method would ensure higher crop yields and substantial benefits (Shelar et al., 2022) (Holubová et al., 2020), applied research is a mandatory to answer key issues related to food safety. Furthermore, fundamental research is crucial to understand (i) the seed modifications at structural, physiological and molecular levels, (ii) the biological mechanisms of anhydrobiosis implemented by seeds from C2APs exposure to germination (Bourke et al., 2018).

Generally, physiological seed dormancy is released by after-ripening occurring upon seed dry storage or by stratification (Ali-Rachedi et al., 2004). Release of seed dormancy and germination processes depend on oxidative processes that arise during seed imbibition through oxidative and hormonal signalling (Bailly, 2019). These phenomena are regulated by reactive oxygen species (ROS) that operate either on redox signalling with positive changes in the cell redox status (El-Maarouf-Bouteau and Bailly, 2008) or in the selective oxidation of stored mRNAs (Bazin et al., 2011) and proteins (Holdsworth et al., 2008a). Production of hydrogen peroxide ( $H_2O_2$ ) has been associated with germination of non-dormant Arabidopsis (Leymarie et al., 2012). Accumulation of ROS in the dry state also modifies the hormonal crosstalk between abscisic acid (ABA) and gibberellins (GA). Upon subsequent imbibition, this ROS accumulation works in favour of GA, leading to seed germination (Bahin et al., 2011). Nevertheless, ROS content exceeding the oxidative window can have deleterious effects on seed germination (Baek et al., 2015). Whether seeds are in the dry state or imbibed, the oxidative processes must remain enclosed within the oxidative window to ensure the oxidative processes to have positive effects on germination (Bailly et al., 2008). The ROS produced by C2APs could play on this oxidative window and allow faster release of seed dormancy. Pauzaite et al. (2018) have proposed that cold plasma

treatment of Norway spruce seeds could involve ROS metabolism. Until now, experiments showing C2APs-induced modifications of the redox homeostasis have only involved growing seedlings (Adhikari et al., 2020). This means that even today, no information is available on the putative effects of C2APs on the ROS content of dry seeds.

## 1.2. Seeds surface properties and inner architecture

C2APs is an effective method for modifying the physico-chemical properties of various material surfaces, especially in the food packaging industry where public health issues and food safety regulations are becoming increasingly important. (Bourke et al., 2017), (Chizoba Ekezie et al., 2017). C2APs treatments can modify the outer surface properties of seeds, either physically (i.e. texturing, etching, smoothing) or chemically (i.e. chemical functionalization driving to the creation of sorption sites). These physico-chemical surface modifications can both change seed coat wettability, and more specifically hydrophilize the coating surface, as already highlighted by water contact angle measurements, correlated with water uptake measurements, both driving to enhanced germinative parameters (median germination time and/or germination rate) (Zahoranová et al., 2016), (Dufour and Gutierrez, 2021). In addition, permeability to O<sub>2</sub> and CO<sub>2</sub> may increase oxidative processes, as hypothesized by Bormashenko et al. (2012). Many studies relying on scanning electron microscopy (SEM) observations highlighted morphological discrepancies between control and plasma-treated seeds (Stolárik et al., 2015), (Dauwe et al., 2021), (Shapira et al., 2017). From a material and structural point of view, a seed corresponds to an amorphous and porous system, containing hollow cavities (called "intercellular spaces"), interconnected the ones to the others. These intercellular spaces form a complex network of pores containing gas which can either remain confined in hermetic cavities or on the contrary communicate via tortuous channels with the ambient air. This finding has been highlighted by quantitative phase tomography on Arabidopsis seeds (Cloetens et al., 2006). If the low permeability of a seed coat can prevent gas exchanges with the environment, it is assumed that this air space network supports gas exchange and oxygen diffusion through seed tissues, which is essential for germination with an oxidative component.

The study of pore network parameters (e.g. tissues porosity, pore network tortuosity, ...) point out the gas diffusion and the oxidation kinetics (Nicholson, 2001), (Wang and Yang, 1998). Porosity is defined as the ratio between the volume of all pores, namely void spaces and the volume of the material. Tortuosity can be defined as the ratio of the average length

of the geometric flow paths through the medium to the straight-line length (Ghanbarian et al., 2013). The definition of this geometrical parameter can also be expressed as the ratio of the shortest pathway to the straight-line length. Porosity-Tortuosity can be related to gas and even electrical diffusive properties (Matyka et al., 2008). The seeds can be assimilated to grain/pore structures in which granular material exists as tissue compounds (lipids, proteins, membrane, ...). To evaluate whether seed porosity is a relevant parameter to understand the effect of C2APs on seed germination, X-ray synchrotron microtomography is a relevant technique to explore the pore network without inducing degradation of seed tissues.

### 1.3. Influence of gene regulation and transcriptional changes induced by C2APs

So far, only a limited number of studies have demonstrated that C2APs treatment could trigger molecular response in seeds. During seed dormancy release and germination of Arabidopsis seeds, gene regulation has been reported in many studies; it has provided insights on how transcriptional changes can control germination during seed imbibition (Cadman et al., 2006), (Ponnaiah et al., 2019). The study of transcriptional changes in dormant and non-dormant seeds has led to generate gene regulatory networks that revealed developmental modules and key regulators related to seed germination (Bassel et al., 2011). Different functional classes of up-regulated genes have been associated with germination such as the ones related to protein degradation, cell wall modification, or translation whereas functional groups associated with dormancy included genes associated with ABA, seed maturation or heat shock proteins (Holdsworth et al., 2008b). A few studies have shown the effect of C2APs on gene regulation. For wheat seed, a DBD treatment induced regulation of functional genes LEA 1, SnRK2 and P5CS in the wheat seedlings assuming the enhancement of oxidation resistance (Guo et al., 2017). Oxygen plasma has been shown to affect the expression of genes associated with cell growth, stress response, photosynthesis and hormone response in Arabidopsis seeds (Hayashi et al., 2016). Ambient air C2APs induced gene regulation in rice seeds initially exposed to heat stress and genes associated with ABA biosynthesis (OsNCED2 and OsNCED5) were downregulated although genes associated with ABA catabolism (OsABA8'OH1 and OsABA8'OH3) and  $\alpha$ -amylase (OsAmy1A, OsAmy1C, OsAmy3B, and OsAmy3E) were up-regulated (Suriyasak et al., 2021). Overall, cold plasma effects on the whole transcriptome of seeds is poorly known

and needs to be investigated to better understand the molecular basis of this treatment on seed germination.

Previously, we demonstrated and explained how short treatments with ambient air cold plasma could release *Arabidopsis* seed dormancy (Chapter 2). Here, the present study aims to investigate whether C2APs can modify seed internal architecture which, in turn, could facilitate gas diffusion, particularly ROS penetration. C2APs could thus reorganise the complex three-dimensional network of intercellular spaces in the seed and modify gas circulation and its exchange with the atmosphere (ambient air). To this end, synchrotron X-ray microtomography and transcriptomic experiments were achieved to decipher some C2APs-triggered biological mechanisms that are related to seed dormancy release.

## 2. Materials and methods

### 2.1. Plant Material

*Arabidopsis* (*Arabidopsis thaliana*, ecotype Columbia Col-0) seeds were obtained from plants grown in growth chambers under controlled conditions with a temperature of 21°C and a photoperiod of 16 h per day (light intensity of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). At harvest, seeds were stored at -20 °C to keep dormancy properties until C2APs treatment.

### 2.2. Germination assays

To evaluate *Arabidopsis* seed germination, 4 replicates of 100 seeds each were sown in 9 cm Petri dishes on a layer of cotton wool moistened with demineralized water and placed at 25 °C in darkness. Seed germination was scored daily for approximately 10 days. A seed was considered as germinated when the radicle broke the seed coat.

In addition, germination under hypoxia (4 % oxygen) was assessed by controlling the air atmosphere during germination, as described by (Gay et al., 1991). The germination rate was evaluated after 5 days considering 4 replicates of 100 seeds each, placed at 15 °C in darkness. Each experimental condition (plasma treatment) was repeated four times, bringing the total number of seeds to 1600.

## 2.3. Plasma treatments

The plasma reactor was a dielectric barrier device (DBD) in a plan-to-plan configuration, with a gap of 1 mm and two electrodes: the mesh electrode (30x40 cm<sup>2</sup> in area, composed of a stainless-steel mesh with a 100 µm nominal opening, a wire diameter of 71 µm and an open area of 34 %) And the counter-electrode (bulk alumina plate). The dielectric barrier (2 mm thick glass plate) was placed below and in contact with the high voltage electrode. To ensure a fixed process temperature of 60 °C, a heating plate device (Froilabo EM130 Hotplate Magnetic Stirrer) was placed in contact with the grounded electrode.

The mesh electrode was connected to a high voltage generator composed of a function generator (ELC Annecy France, GF467AF) and a power amplifier (Crest Audio, 5500W, CC5500). The power supply was electrically protected by a ballast resistor (250 kΩ, 600 W). The peak amplitude of the voltage applied on the mesh electrode can reach 10 kV at 145 Hz. The counter electrode was grounded and Arabidopsis seeds were directly placed on it during the plasma treatment.

The study was initially carried out with dormant seeds (CTRL, 7 %<sub>DW</sub>), treated for 15 min (P-15'), 30 min (P-30') or 1h (P-1h) seeds heated at 60°C for 15 min (C-H) and (P-H) low water content (C-LW, 3 %<sub>DW</sub>) for 15 min (P-LW), seeds with high water content (C-HW, 30 %<sub>DW</sub>) and treated for 15 min (P-HW) as shown in Table 1.

**Table 1** - Experimental seed conditions in terms of water content, plasma treatment and heating used in this study.

Water content	Heating process	C2AP treatment	Notation
CTRL (7 % <sub>DW</sub> )	No	No	CTRL
		15 min	P-15'
		30 min	P-30'
		1 h	P-1h
	60 °C	No	C-H
		15 min	P-H
Low (1 % <sub>DW</sub> )	No	No	C-LW, 3 % <sub>DW</sub>
		15 min	P-LW
High (30 % <sub>DW</sub> )		No	C-HW, 30 % <sub>DW</sub>
		15 min	P-HW

## 2.4. Hydrogen peroxide measurements

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content of dry seeds was determined using the method described in Leymarie et al. (2012). Hydrogen peroxide was extracted from 30 mg of Arabidopsis seeds in 2 mL of perchloric acid (0.2 N). The amount of hydrogen peroxide was determined spectrophotometrically using DMAB (3-dimethylaminobenzoic acid) and MBTH (3-methyl-2-benzothiazolinone hydrazine). Results correspond to the mean and standard deviation of 3 replicates and are expressed in  $\mu\text{mol.g}^{-1}$  FW (Fresh Weight).

## 2.5. Malondialdehyde Assay

Malondialdehyde (MDA) content was determined using the method previously described in Oracz et al. (2007). MDA was extracted from 30 mg of Arabidopsis seeds in a solution with an equal volume of 0.5 % thiobarbituric acid (TBA) in 20 % trichloroacetic acid (TCA). MDA content was determined spectrophotometrically. Results correspond to the mean and standard deviation of 3 replicates and are expressed in  $\mu\text{mol.g}^{-1}$  FW (Fresh Weight).

## 2.6. Reactive oxygen species imaging

Seeds were soaked in water for 2 h before removing their seed coat under a binocular loupe. Embryos were then prudently incubated for 30 min in the dark at 20 °C in 2 mL of K-phosphate buffer (20 mM, pH 6) containing 25  $\mu\text{M}$  DCFH-DA (2',7'-Dichlorodihydrofluorescein diacetate, 25 mM) (Leymarie et al., 2012). Embryos were consequently washed for 15 min with buffer 2 times and mounted in water on a microscope slide for observation.

The fluorescence following ROS reaction with DCFH-DA within seed tissues was visualised by confocal microscopy imaging using a Leica TCS SP5 confocal microscope upon excitation at 488 nm with an Argon laser. Dye emission was recorded using a 498-530 nm band pass filter. Images were analysed using Fiji software by segmenting embryo volume and calculating the sum of pixel values over the embryo volume with Fiji analyse tools. Results are expressed as integrated intensity over embryo volume of approximately 50 embryos with its standard deviation.

## 2.7. Oxygen consumption measurement during imbibition

To evaluate the oxygen rate consumption by the seeds, 15 mg of Arabidopsis seeds were pre-soaked in a Petri dish on a layer of cotton wool moistened with demineralized water placed at 25 °C in darkness. After 1 h, all the seeds were thoroughly removed from the Petri dish and placed in a tightly closed box (cylinder a cylinder of height 2.5 cm and diameter 1 cm) containing an oxygen probe (Fixed & Protected Tip fiber Oxygen Minisensors, Pyroscience), that measured oxygen content in the box every 2 min during 24 h at 15 °C in darkness. The results were extracted from the software FireStingGO2\_Manager. Measurements were repeated 3 times for each condition. Results are expressed in amount of O<sub>2</sub> consumed in the air per hour and per seed mass (mol.h<sup>-1</sup>.mg<sup>-1</sup> FW).

## 2.8. Electrolyte leakage measurement

Arabidopsis seeds (30 mg) were placed in 12 mL of deionized water at ambient temperature (23 °C) and conductivity of the solution was measured with a conductimeter (Jenway 4010, Dunmow, UK) during 24 h. The electrolyte leakage kinetic was monitored during 24 h considering control seeds and treated seeds in triplicates each. The experiment was repeated 3 times. Results are expressed in % of total leakage obtained after boiling the seeds for 30 min in water.

## 2.9. High Resolution Transmission X-ray $\mu$ -tomography measurements

### 2.9.1. X-ray microtomography experiment

Seeds were prepared in such a way as to retain the hydric, biological and processing properties before the experiments. The highly hydrated seeds were prepared the same morning. Ten seeds per condition were mounted and glued onto toothpicks for scanning and tomographic imaging.

#### 2.9.2.1. Image acquisition

The X-ray microtomography acquisitions were carried out on the ANATOMIX beamline at the Synchrotron SOLEIL (France). The system was operated with a slightly filtered polychromatic ("white") beam at a central photon energy of 18 keV using 600  $\mu$ m of chemical vapour deposition diamond. The source was a cryogenic undulator U18 setting



with a gap equal to 11 mm. The detector used an objective of 20x combined with a Hamamatsu camera Orca Flash 4 giving a final voxel size of 0.325  $\mu\text{m}$ . Two thousand projections were taken in-between rotation angles from 0° to 180° with a 100 ms of exposure time for a total scan time of about 3.3 min. A distance close to 10 mm was used in-between the sample (Arabidopsis seed) and the detector. For each sample, one scan was acquired giving a scan height of 0.65 mm for a diameter of about 0.65 mm.

#### *2.9.2.2. Images processing*

The volumes were reconstructed using the High-Performance Computing (HPC) resources of the Anatomix beamline and the PyHST2 software. The 3D volumes were composed of  $2048 \times 2048 \times 2048$  voxels (for a region of interest of  $0.65 \times 0.65 \times 0.65 \text{ mm}^3$ ). A phase contrast correction was applied with a Paganin filter value equal to 20 pixels (Paganin et al. 2002). The images were then segmented using the Dragonfly software.

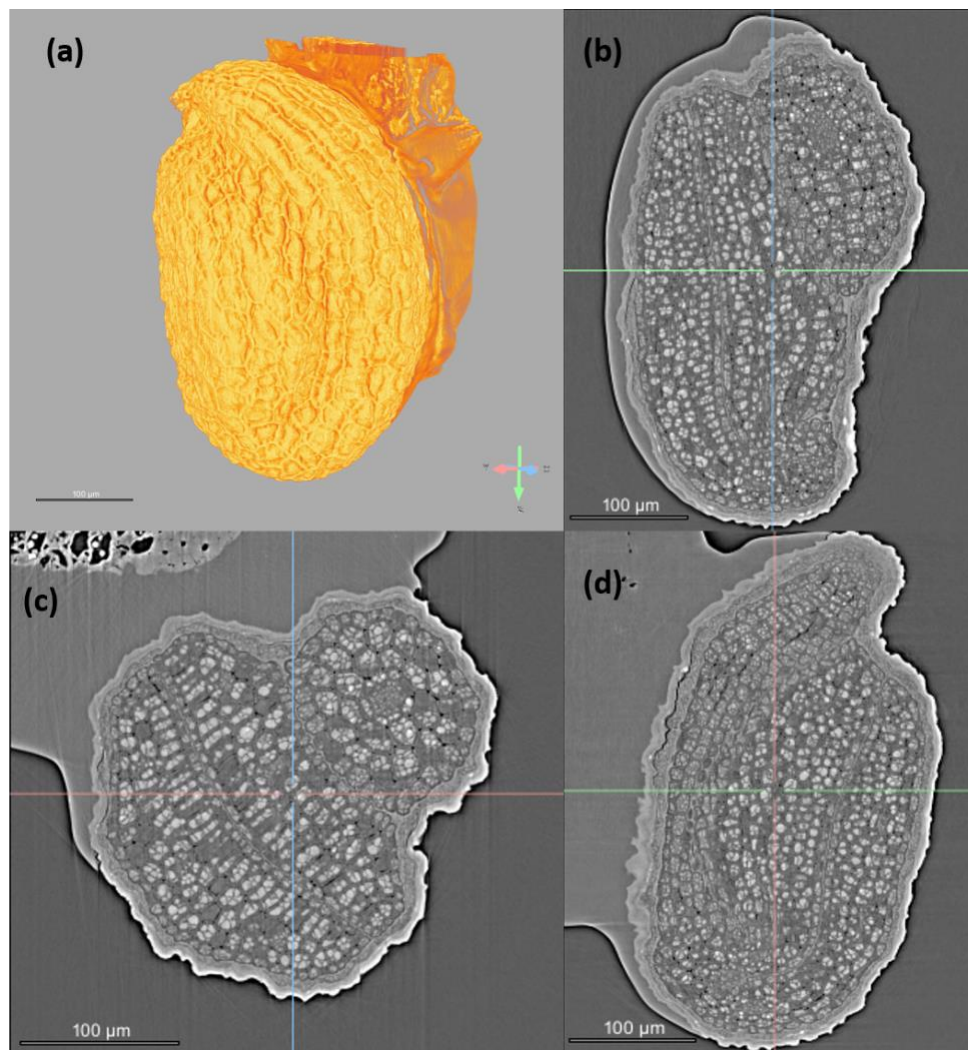
#### *2.9.2.3. 3D image analysis*

Images were generated and analysed using Dragonfly software, Version 2022.1.0.1259 for Windows. Object Research Systems (ORS) Inc, Montreal, Canada; (software available at <http://www.theobjects.com/dragonfly>) is free for academic users and has a wide range of image analysis and 3D reconstruction capabilities to extract parameters for the analysis of CT images.

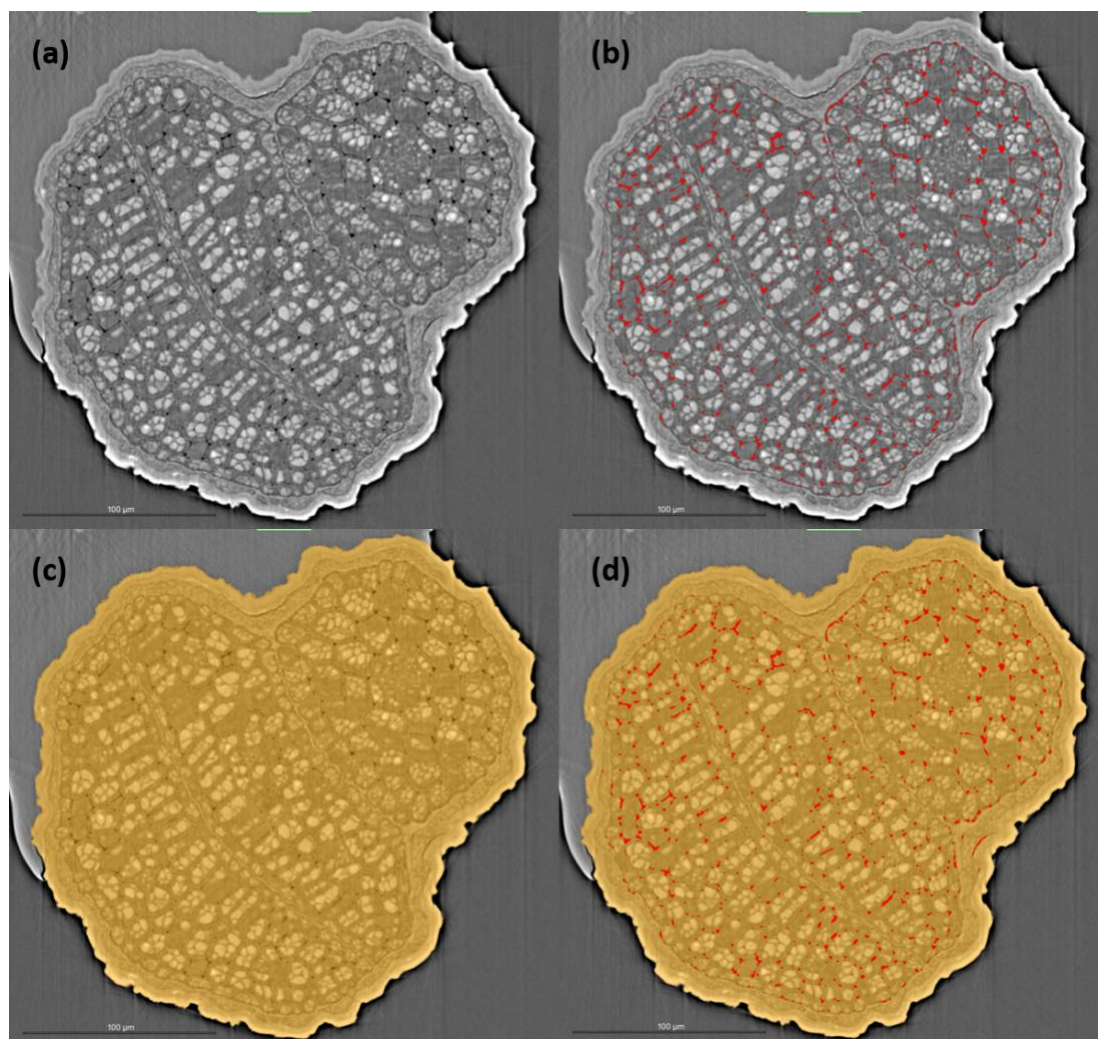
A thorough understanding of the software using videos featuring in-depth lessons on Dragonfly's tools and advanced capabilities ([Dragonfly | In-Depth Lessons | Playlist \(theobjects.com\)](http://www.theobjects.com/dragonfly)) allowed to determine a protocol (Figure S1) with several steps to extract the pores (intercellular spaces in which gases circulate in the seed tissues) and the seed volume. From these segmentations, porosity, pore density and 3D pore network analysis of seeds for each biological and treatment condition were determined.

- (i) The whole reconstructed scan was visualised using the Dragonfly environment to observe properties of the seed tissues in 2D in X, Y or Z axes (Figure 1) and in 3D. Slices were cropped to work on the seeds and minimise the size of images. Seed tissues, seed coat, seed embryo (cotyledons and radicle) and endosperm were discerned and examined as grayscale images. The images were coloured by applying Look-Up Table functions and edit colour as a function of images intensity.

- (ii) Segmentation operation was achieved and applied to define the regions of interest (ROIs) of the pores and seed volume (Figure 2). The method was applied over all slices using the Lower and Upper Otsu thresholding method implemented in Dragonfly. This thresholding was edited and enhanced manually using Dragonfly segment ROI 2D and 3D tools to add or remove parts of segmented objects that had been incorrectly highlighted by the thresholding method.
- (iii) The pores and the seed volume classes were considered from the segmentation results and statistical properties to deduce seed porosity and seed pore density calculation. For remind, porosity is defined as the ratio of the volume of all pores to the total analysed tissue volume which is the seed volume. Pore density is defined as the ratio of the number of segmented pores to the total analysed tissue which is here the seed volume. From these considerations and pores segmentations, 3D pores were observed (Figure 3) and analysed by calculating pores surface, pores volume as well as Minimum, Maximum and Mean Feret diameter, known also as Caliper diameter (distance between any two points along each pore's boundary), for each sample.
- (iv) Once the seed volume and pores were segmented, Dragonfly graphing functions of ROI were used to analyse the pores. Computing graphs from ROI allowed us to have access to pores properties and characterise such data as connected areas and tortuous pore space with spheres and lines representing pores and connections with edges and vertices. Dense graphs and sparse graphs were generated (Figure 3). Edges statistics were determined from these graphs: number of edges, edges total length, segment Euclidean length, segment length and segment tortuosity (segment length divided by segment Euclidean length). Moreover, vertex statistics were also determined: number of vertices and connectivity (number of edges connected to the vertex).

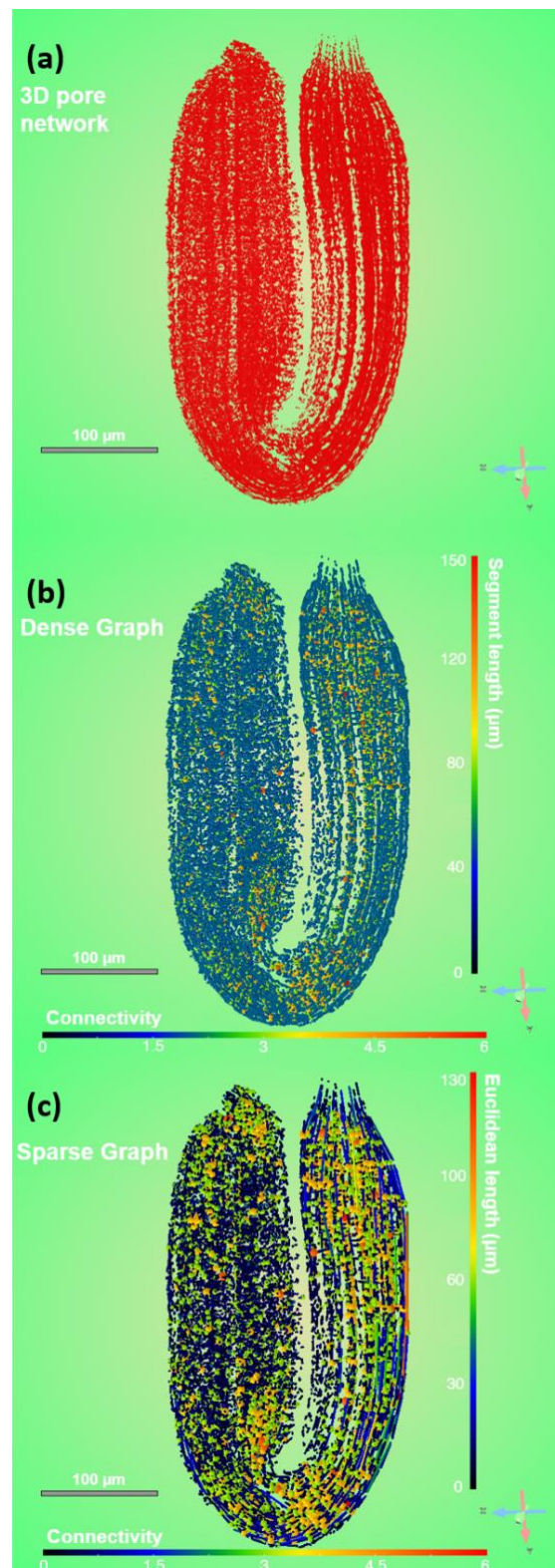


**Figure 1** - Seed micro-tomogram observation of Arabidopsis seed with Dragonfly Software. 3D reconstruction of slices (a), 2D slices XZ direction (b), XY direction (c), YZ direction (d).



**Figure 2** - Images segmentation of Arabidopsis seed with Dragonfly software tools: seed without segmentation (a), pores segmentation in red (b), seed segmentation in yellow (c), pores and seed segmentation (d).





**Figure 3** - Images of the pore network segmentation and graph modelling characterized by vertices and edges of an Arabidopsis dormant seed obtained with Dragonfly software. 3D pore reconstruction in red (a), Dense graph of pores (b), Sparse graph of pores (c). Edges are scaled with segment length in  $\mu\text{m}$  (Dense graph) and Euclidean length in  $\mu\text{m}$  (Sparse graph). Vertices are scaled with connectivity (both graphs).

## 2.10. Transcriptomic analysis

Total RNA was extracted from 50 mg of Arabidopsis seeds following the method developed by (Hellemans et al., 2007) and adapted by (Zhang et al., 2019), as already described (Leymarie et al., 2012). RNA extracted samples were purified using the RNA Clean & Concentrator Kits (Zymo Research®, California, U.S.A.). RNA-seq libraries were constructed by the POPS platform (IPS2) using the QuantSeq 3' mRNA-Seq library prep kit (LeXogen®, for Illumina) with unique molecular identifier (UMI) according to the supplier's instructions. Libraries were sequenced on a NextSeq500 instrument (Illumina) with 75 base single-end sequences.

UMIs were removed and added to the read identifier with the extract command of UMI-tools (v1.0.1, (Smith et al., 2017)). Reads with a basic UMI quality score of less than 10 were removed. Besides, the removal of adapter sequences, poly(A), poly(G) sequences and low-quality nucleotides was achieved by trimming reads with BBduk from the BBmap suite (v38.84, Bushnell, 2014) with the options `k=13 ktrim=r useshortkmers=t mink=5 qtrim=r trimq=10 min length=30`. Then, trimmed reads were mapped and counted using STAR (v2.7.3a, Dobin et al., 2013), with the following parameters `--alignIntronMin 5 --alignIntronMax 60000 --alignMatesGapMax 6000 --alignEndsType Local--outFilterMultimapNmax 20 --outFilterMultimapScoreRange 0 --outSAMprimaryFlag AllBestScore -mismatchNoverLmax 0,6` on the Arabidopsis thaliana genome reference (TAIR10+ARAPORT11+MtCol0). Reads with identical mapping coordinates and UMI sequences were collapsed to remove PCR duplicates using the dedup command of UMI-tools with the default directional method parameter. RSeQC (v2.6.6, Wang et al., 2012) was used to evaluate deduplicated mapped reads distribution. Deduplicated reads were counted using HTSeq version v0.12.4. (Anders et al., 2015) (htseq-count mode intersection-nonempty) based on the A. thaliana TAIR10-ARAPORT11 annotation. Between 83.4 and 89.2% of the reads were associated with annotated genes. Statistical analyses were conducted on R v3.6.2 (R Core Team, 2020) using the script-based tool DiCoExpress ((Lambert et al., 2020); (Baudry et al., 2022)). DiCoexpress used the Bioconductor package edgeR (v 3.28.0, (Robinson et al., 2010); (McCarthy et al., 2012)). Three separate statistical analyses were conducted on samples from (i) Dry and H6, (ii) Dry and H16 and (iii) Dry and H24. Since quality control with all the samples identified the sample H16\_Plasma\_rep3 as an outlier, the sample H16\_Plasma\_rep3 was removed from the analyses Dry and H16.

For all analyses, low counts genes were filtered using the “FilterByExpression” feature available through the edgeR package. The minimum count threshold was determined for each analysis: 3 for the analyses (i) Dry and H6, and 25 for the analyses (ii) Dry and H16 and (iii) Dry and H24. For the three analysis, raw counts were normalised with the trimmed mean of M values (TMM) method implemented in the edgeR package.

The differential analysis was based on a negative binomial generalised linear model. For the three analyses, the log2 of the average normalised gene expression is an additive function of a time effect (2 modalities), a treatment effect (2 modalities) and an interaction between the time and the treatment. To make the processing of these multifactorial designs easier, Differential Expression (DE) analyses were performed using the DiffAnalysis\_edgeR function of DiCoExpress to automatically generate the contrasts. In each analysis, we considered the difference between the two treatments at each time and averaged on the two times, the difference between two times given a treatment and averaged on the two treatments and the interaction effect defined as the difference between the two treatments at a given time point minus the difference between the same two treatments at the second time point. It led to a total of 7 contrasts per analysis and only biologically meaningful contrasts were interpreted. For each contrast, a likelihood ratio test was applied and raw p-values were adjusted with the Benjamini-Hochberg (Benjamini and Hochberg, 1995) procedure to control the false discovery rate. The distribution of the resulting p-values followed the quality criterion described by Rigaiil et al. (2018). A gene was declared differentially expressed if its adjusted p-value was lower than 0.05 and the fold change absolute value higher than 2.

In this study, the Venn diagrams were generated using the online software Bioinformatics & Evolutionary Genomics: <http://bioinformatics.psb.ugent.be/webtools/Venn/>.

The gene ontology classification was obtained using the graphical gene-set enrichment tool for animals and plants ShinyGO (Ge et al., 2020). Only significantly represented categories with a false discovery rate (FDR) value less than 0.05 were represented. Categories were selected and sorted by Fold Enrichment and False Discovery Rate.

RNAseq project was deposited (Gene Expression Omnibus, (Edgar et al., 2002)): <http://www.ncbi.nlm.nih.gov/geo/> (submission to GEO in progress). All steps of the experiment, from growth conditions to bioinformatic analyses, were detailed in CATdb (Gagnot et al.,

2008): <http://tools.ips2.u-psud.fr.fr/CATdb/>; Project: 2022\_01\_QS\_ColdPlasma according to the MINSEQE 'minimum information about a high throughput sequencing experiment'.

## 2.11. Statistical analysis

All the statistical analyses were performed using OriginLab Software statistical tools, including curve fitting, Mann-Whitney and ANOVA tests to determine significant differences between control group and plasma group on the amount of ROS, MDA, oxygen rate consumption and porosity calculations.

## 3. Results

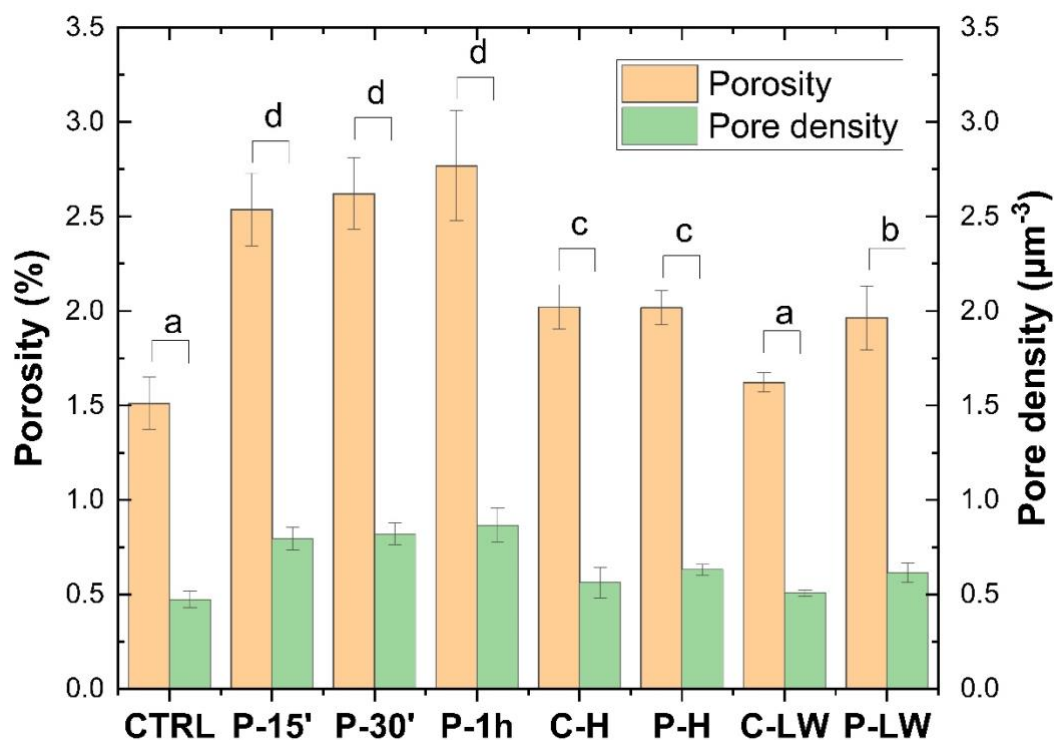
### 3.1. Effects of C2APs on the internal architecture of the seeds

Micro-tomography experiments were carried out to examine the morphology and internal structure of dormant seeds considering two situations: (i) seeds treated or not by C2APs, (ii) seeds with low/medium/high water content. However, the tomography experiments achieved on seeds at high water content provided no observable data due to the strong interaction between the X-rays and the hydrated tissues. Therefore, the C-HW and P-HW groups were excluded from the analyses. Some reconstructed tomograms could not be analysed due to technical problems and movement of the seeds during the measurements. The parameters measured in the following sections refer to 10 seeds for the CTRL group, 9 seeds for the P-15' group, 10 seeds for the p-30' group, 7 seeds for the P-1h group, 8 seeds for the C-H group, 9 seeds for the P-H group, 10 seeds for the C-LW group and 9 seeds for the P-LW group.

#### 3.1.1. Pore density and porosity

Figure 4 shows the effect of C2AP on seed porosity and pore density. In all cases, except for the heated seeds, C2AP treatment induced a significant increase of porosity. More specifically, 15, 30 and 60 min of plasma exposure increased significantly the porosity of the seeds passing from 1.5 % for the CTRL group to more than 2.5 % for the treatment time groups. For the seeds at low water content, the plasma treatment also induced a significant increase in porosity but no statistical difference was measured when the seeds were heated to 60 °C. Similar trends were observed when estimating pore density (Figure 4).





**Figure 4** - Porosity (%) and pore density ( $\mu\text{m}^{-3}$ ) of the segmented image groups: control seeds (CTRL), plasma-treated seeds during 15 min (P-15'), plasma-treated seeds during 30 min (P-30'), plasma-treated seeds during 1h (P-1h), seeds heated at 60 °C during 15 minutes without plasma exposure (C-H), seeds heated at 60 °C and plasma-treated during 15 minutes (P-H), seeds with low water content at 3 %<sub>DW</sub> (LW) without any heating: plasma treatment and seeds with low water content at 3 %<sub>DW</sub> and only plasma-treated during 15 min (P-LW). Means over the number of segmented tomograms (between 7 and 10)  $\pm$  SD (a, b, c, d: p-Value < 0.05). Mann-Whitney tests performed between all groups in pairs. 4 statistical groups were found: a (CTRL and C-LW), b (P-LW), c (C-H and P-H) and d (P-15', P-30' and P-1h).

A 3D analysis of the segmented pores of all conditions was also performed (Table 2). Volume and Surface parameters are the volume and the interpolated surface of the segmented pores respectively. Statistical differences were found between the CTRL group and the P-15', P-30', P-1h groups and between the C-LW group and the P-LW group. However, no difference was found between the C-H and the P-H group which are both different from the CTRL group. If no statistical differences were found, an increasing trend in Feret diameters after plasma treatment was evidenced when separating CTRL, P-15', P-30', P-1h groups from the C-H, P-H groups and LW, P-LW groups. However, this increase was not observed for the P-15' group compared to the CTRL group for the mean Feret diameter and the maximum Feret diameter.

**Table 2** - Parameters of 3D pores analysis from segmented tomograms of all groups: control seeds (CTRL), seeds plasma-treated during 15 min (P-15'), seeds treated plasma-during 30 min (P-30'), seeds plasma-treated during 1h (P-1h), seeds heated at 60 °C during 15 minutes (C-H), seeds simultaneously heated at 60 °C and plasma-treated during 15 minutes (P-H), seeds with low water content at 3 %<sub>DW</sub> without treatment (LW) and seeds with low water content at 3 %<sub>DW</sub> and plasma-treated during 15 min (P-LW). Means +/- SD. Mann-Whitney tests performed between all groups in pairs and statistical differences are shown in bold between the CTRL group and the P-15', P-30', P-1h groups, between the C-H group and the P-H group and between the C-LW group and the P-LW group.

3D parameters	CTRL	P-15'	P-30'	P-1h	C-H	P-H	C-LW	P-LW
Volume (x10 <sup>4</sup> μm <sup>3</sup> )	24.3	<b>40</b>	<b>37.5</b>	<b>43.4</b>	29.4	29.4	24.7	<b>33.7</b>
	±	±	±	±	±	±	±	±
	2.5	<b>3.6</b>	<b>3.4</b>	<b>4.3</b>	2.1	2.4	7.6	<b>3.8</b>
Surface (x10 <sup>4</sup> μm <sup>2</sup> )	74.6	<b>119.4</b>	<b>102.7</b>	<b>120.3</b>	97.6	92.7	78.4	<b>105.8</b>
	±	±	±	±	±	±	±	±
	7.9	<b>10.5</b>	<b>5.1</b>	<b>7.2</b>	5.9	8.1	2.2	<b>10.2</b>
Min Feret Diameter (μm)	233.1	236.1	240.4	246	228.3	231.4	235.7	245.7
	±	±	±	±	±	±	±	±
	4.5	6.2	6	7.1	3.8	6.9	4.2	5.5
Mean Feret Diameter (μm)	298.7	293.9	317.6	327.3	286.4	299.9	303.9	310
	±	±	±	±	±	±	±	±
	9.8	7.3	12.8	17.7	5.6	9.4	7	9.7
Max Feret Diameter (μm)	398.7	397.5	410.9	422.5	412.7	426.6	417.4	416.6
	±	±	±	±	±	±	±	±
	11.8	8.3	15.3	10.1	8.5	13.4	11.4	7.1

### 3.1.2. Dense graphs analysis

Dense graphs were generated, as described in Section 2, which gave access to vertices and edges. A significant increase in number of vertices, number of edges as well as edges total length, edges total length density (edges total length divided by seed volume), vertex density (number of vertices divided by seed volume) and edges density (number of edges divided by seed volume) were observed between the CTRL group and the P-15', P-30', P-1h groups as well as between the C-LW group and the P-LW group (Table 3). No significant increase was found between the C-H group and the P-H group, although the two groups were significantly different from the control group.

**Table 3** - Dense graph parameters (number of vertices, number of edges, edges total length, edges total length density, vertex density, edge density) of all groups: control dormant seeds (CTRL), seeds treated during 15 min (P-15'), seeds treated during 30 min (P-30'), seeds treated during 1h (P-1h), seeds heated at 60 °C during 15 minutes (C-H), seeds heated at 60 °C and treated during 15 minutes (P-H), seeds with low water content at 3 %<sub>DW</sub> (C-LW) and seeds with low water content at 3 %<sub>DW</sub> and treated during 15 min (P-LW). Means +/- SD. Mann-Whitney tests performed between all groups in pairs and statistical differences are shown in bold between the CTRL group and the P-15', P-30', P-1h groups, between the C-H group and the P-H group and between the C-LW group and the P-LW group.

Dense graph parameters	CTRL	P-15'	P-30'	P-1h	C-H	P-H	LW	P-LW
Number of vertices (x10 <sup>4</sup> )	38.9	<b>59.1</b>	<b>50.2</b>	<b>61.3</b>	50	47.7	40.6	<b>54.1</b>
	±	±	±	±	±	±	±	±
	4.8	<b>5.5</b>	<b>2.7</b>	<b>4.4</b>	3.1	4.5	1.4	<b>5.4</b>
Number of edges (x10 <sup>4</sup> )	35.4	<b>52.7</b>	<b>45.1</b>	<b>54.9</b>	43.9	42.9	36.8	<b>48.7</b>
	±	±	±	±	±	±	±	±
	4.3	<b>4.7</b>	<b>2.6</b>	<b>4</b>	2.9	4	1.3	<b>5.2</b>
Edges total length (mm)	167.2	<b>255.6</b>	<b>219.3</b>	<b>259.3</b>	211.4	205.8	173.6	<b>232.9</b>
	±	±	±	±	±	±	±	±
	20.9	<b>23.8</b>	<b>12.7</b>	<b>20.2</b>	14.5	19.9	5.6	<b>25.8</b>
Edges total length density (mm <sup>-1</sup> )	10.7	<b>16.2</b>	<b>15.5</b>	<b>16.4</b>	14.5	14	11.5	<b>13.6</b>
	±	±	±	±	±	±	±	±
	1.2	<b>1.2</b>	<b>0.8</b>	<b>0.8</b>	0.8	0.8	0.3	<b>1.3</b>
Vertex density (x10 <sup>6</sup> mm <sup>-3</sup> )	25	<b>37.3</b>	<b>35.4</b>	<b>38.7</b>	34.4	32.4	26.8	<b>31.7</b>
	±	±	±	±	±	±	±	±
	2.6	<b>2.5</b>	<b>1.8</b>	<b>1.9</b>	1.5	1.8	0.6	<b>2.5</b>
Edge density (x10 <sup>6</sup> mm <sup>-3</sup> )	22.6	<b>33.3</b>	<b>31.8</b>	<b>34.7</b>	30.2	29.2	24.3	28.4
	±	±	±	±	±	±	±	±
	2.4	<b>2.2</b>	<b>1.7</b>	<b>1.6</b>	1.5	1.5	0.6	2.3

In addition, edge and vertex statistical properties were generated from the dense graphs and edge parameters such as segment Euclidean length, segment length and segment tortuosity as well as vertex connectivity were measured for all groups with maximum and mean values (Table 4). Significant increases of edge maximum segment tortuosity and vertex maximum connectivity were observed between the CTRL group and the P-15', P-30', P-1h groups. If no statistical difference was found, an increasing trend of edge maximum segment tortuosity was also observed between the C-H and P-H groups, as well as between the LW and P-LW group (Table 4).

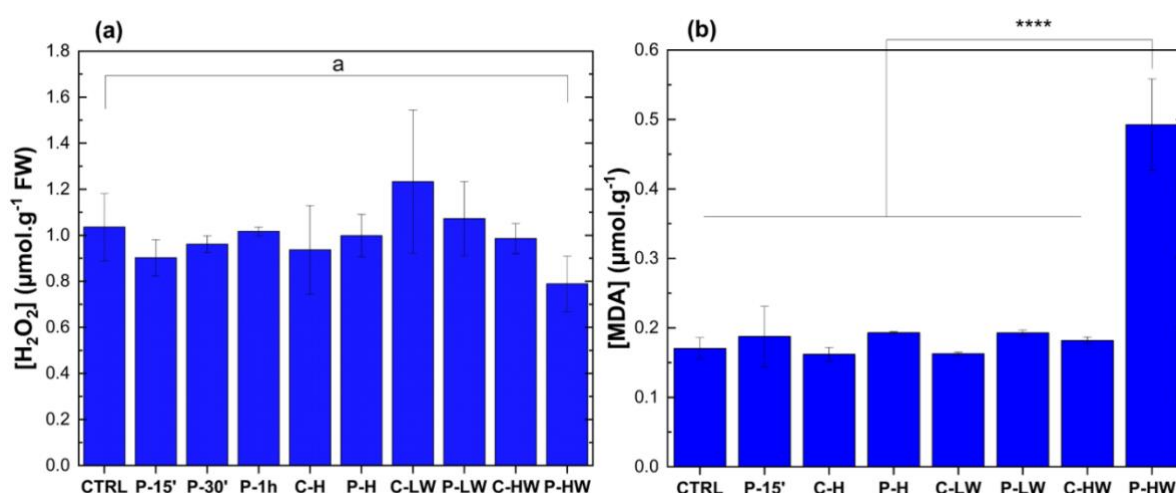
**Table 4** - Edge and Vertex statistical properties of dense graphs for all groups: control dormant seeds (CTRL), seeds treated during 15 min (P-15'), seeds treated during 30 min (P-30'), seeds treated during 1h (P-1h), seeds heated at 60 °C during 15 minutes (C-H), seeds heated at 60 °C and treated during 15 minutes (P-H), seeds with low water content at 3 %<sub>DW</sub> (C-LW) and seeds with low water content at 3 %<sub>DW</sub> and treated during 15 min (P-LW). Means +/- SD. Mann-Whitney tests performed between all groups in pairs and statistical differences are shown in bold between the CTRL group and the P-15', P-30', P-1h groups, between the C-H group and the P-H group and between the C-LW group and the P-LW group.

			CTRL	P-15'	P-30'	P-1h	C-H	P-H	LW	P-LW
Edge	Segment Euclidean length (µm)		112.4	96.8	112.9	97	79.6	112	110.7	96.5
		Max	±	±	±	±	±	±	±	±
			6	9.6	7.6	13.7	5.8	12.5	6.7	5.2
			9.6	8	8.2	8.2	7.5	8.2	9.6	8.8
		Mean	±	±	±	±	±	±	±	±
	Segment tortuosity		0.4	0.5	0.4	0.3	0.3	0.4	0.2	0.5
			14.9	<b>16</b>	<b>16.3</b>	<b>18.4</b>	18.8	20.9	16.3	17.9
		Max	±	±	±	±	±	±	±	±
			1.3	<b>1.5</b>	<b>1.2</b>	<b>3.6</b>	1.8	2.1	1.6	1.3
			1.286	1.279	1.278	1.276	1.306	1.278	1.303	1.279
		Mean	±	±	±	±	±	±	±	±
	Segment length (µm)		0.012	0.007	0.006	0.007	0.010	0.005	0.011	0.008
			136.1	117.6	139	121.8	96.7	<b>136</b>	136.4	116.7
		Max	±	±	±	±	±	±	±	±
			7.4	11.8	9.7	14.6	7.4	<b>15.2</b>	7	7.2
			11.85	10.18	10.46	10.4	9.67	10.53	12.26	10.88
Vertex	Connectivity	Mean	±	±	±	±	±	±	±	±
			0.58	0.69	0.51	0.39	0.43	0.53	0.23	0.58
			6.8	<b>8.4</b>	<b>9.9</b>	<b>8.3</b>	8.6	7.4	8	7.6
		Max	±	±	±	±	±	±	±	±
			0.5	<b>1.2</b>	<b>1.1</b>	<b>0.6</b>	0.7	0.3	0.4	0.4
			1.8	1.59	1.8	1.79	1.75	1.8	1.81	1.79
		Mean	±	±	±	±	±	±	±	±
			0.04	0.2	0.02	0.02	0.02	0.02	0.01	0.02

Similar studies have been performed for sparse graphs giving the same trends. Results are given in the supplementary data (Table S2 and Table S3).

### 3.2. Effect of ambient air cold plasma on seed oxidative processes

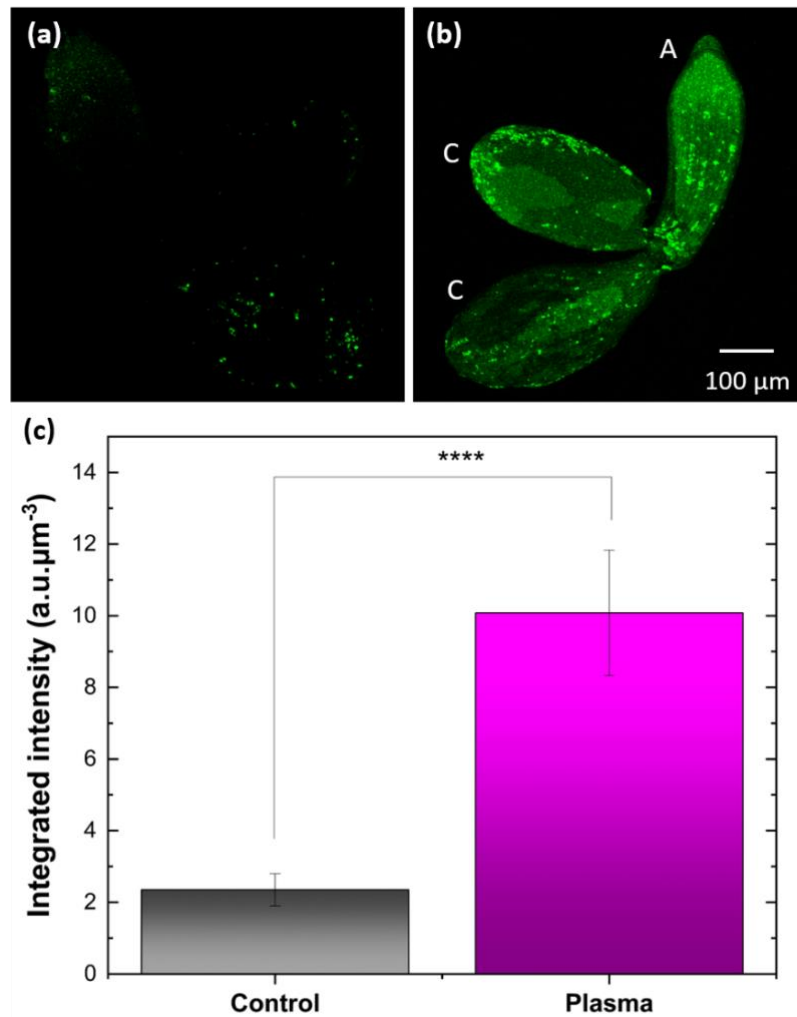
First, the effects of C2APs treatments were highlighted on the hydrogen peroxide content of seeds, using whole seeds (Figure 5(a)). No significant difference of  $H_2O_2$  content was found between all conditions, thus suggesting that C2AP did not induce a global increase in  $H_2O_2$  in dry seeds even for increasing treatment times up to 1h or for seed water contents as high as 30 %<sub>DW</sub> (C-HW). Second, the effects of C2AP treatments were assessed on the MDA contents. Similarly, no significant amount of MDA was found in all the conditions, excepted for the seeds of the P-HW group (Figure 5(b)).



**Figure 5** - Seed  $H_2O_2$  content (a) and MDA content (b): control seeds (CTRL), plasma-treated seeds for 15 min (P-15'), plasma-treated seeds for 30 min (P-30'), plasma-treated seeds for 1h (P-1h), seeds heated at 60 °C during 15 minutes (C-H), seeds heated at 60 °C and treated during 15 minutes (P-H), seeds with low water content at 3 %<sub>DW</sub> (C-LW), seeds with low water content at 3 %<sub>DW</sub> and treated during 15 min (P-LW), seeds with high water content at 30 %<sub>DW</sub> (C-HW), seeds with high water content at 30 %<sub>DW</sub> and treated during 15 min (P-HW). Means on 30 mg of seeds  $\pm$  SD of 3 replicates (\*\*\*\*:  $p$ -value < 0.0001). Mann-Whitney tests have been performed between all groups in pairs.

Because the previous analysis was performed on whole seeds and without spatial resolution, ROS may remain undetectable if they are region-localized to specific locations in the seed. For this reason, we performed confocal microscopy experiments coupled with DCF-DA. This study was only performed on two groups: "Control" (dormant seeds without any treatment) and "Plasma" (dormant seeds plasma-treated for 15 minutes). Fluorescence pictures of Arabidopsis embryos are shown in Figure 6 (a) and (b) for Control and Plasma group respectively. While the untreated seed shows only regions of very low fluorescence, the seed exposed to PAC shows high levels of ROS as evidenced by bright green

fluorescent foci in the seed tissue. Interestingly, the detected ROS do not have a preferred location, i.e. they are produced uniformly along the embryonic axis and in the cotyledons (Figure 6).



**Figure 6** - Fluorescence pictures of Arabidopsis seed embryos of dormant seed (a) and plasma treated seed (b) with the same confocal microscope parameters (C: cotyledon, A: embryonic axis), integrated intensity of fluorescence normalised by seed embryo volume for the control group and plasma group (c). Mean over 50 embryos  $\pm$  SD (\*\*\*\*:  $p$ -value  $< 0.0001$ ). Mann-Whitney tests have been performed between the 50 embryos of the control and plasma groups.

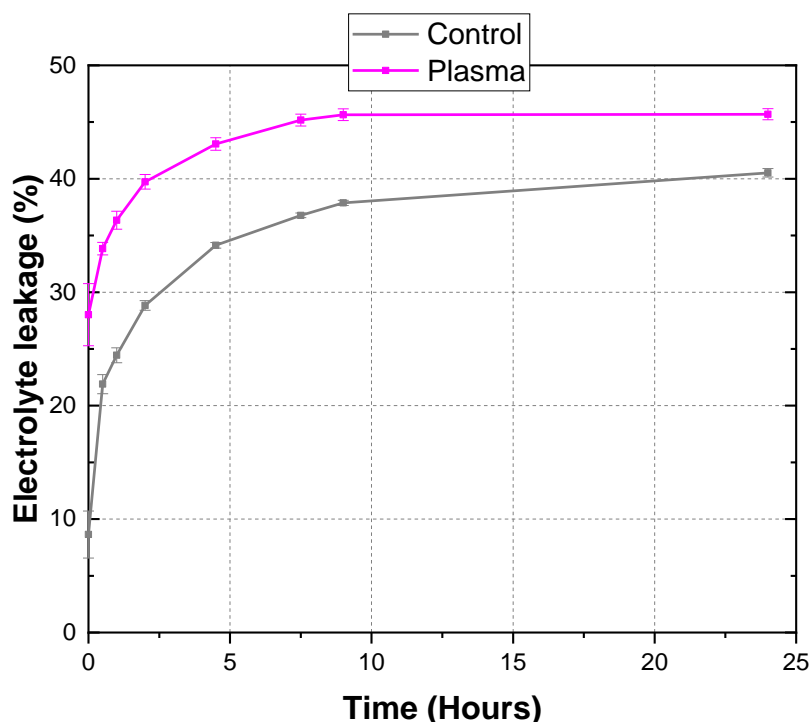
For the sake of accuracy, experiments were repeated and achieved on 50 individual embryos from each group. Integrated intensities were measured using Fiji software tools. For each embryo, the fluorescence intensity was integrated and normalised over the

embryo volume. The normalised fluorescence intensity for control and plasma groups are plotted in Figure 6 (c). The fluorescence of the plasma-treated appears almost 5 times higher than that of the control seeds.

### 3.3. Effect of ambient air cold plasma on seed physiology

#### 3.3.1. Effects on the electrolyte leakage

Selectivity and permeability of seed cell membranes are often associated with seed germinability while seed electrolyte is generally considered as a reliable indicator of seed vigour. For this reason, the electrolyte leakage of plasma-treated seeds was measured during 24 h at 25°C (Figure 7). At the very first time point of seed imbibition, electrolyte leakage of the plasma-treated seeds was much higher than that of the control seeds; this difference was maintained throughout the experiments, hence suggesting that C2AP had an effect on membrane properties. The experiment was repeated twice (Figure S2 and Figure S3).

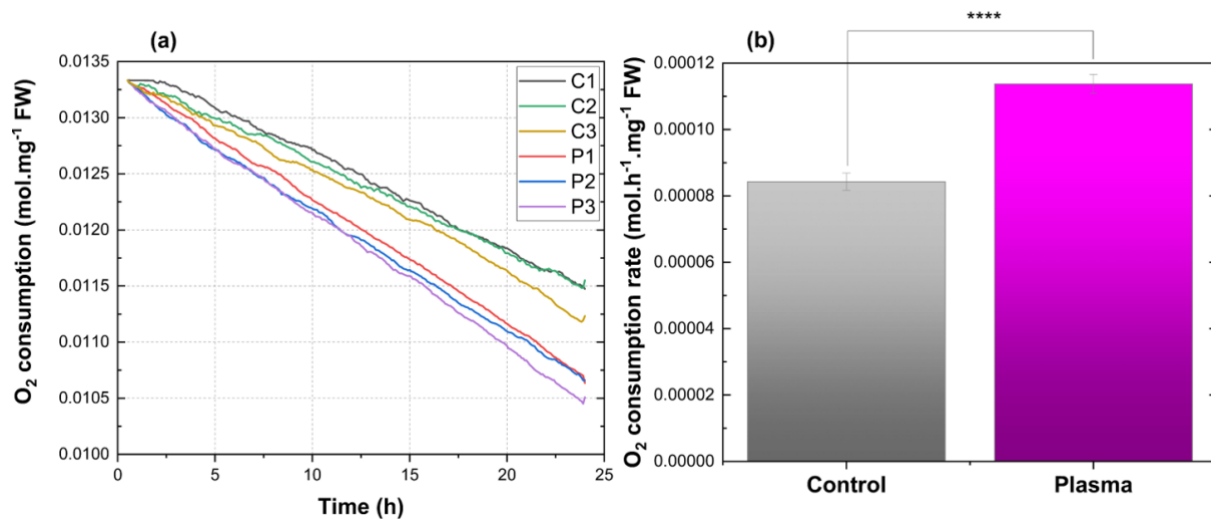


**Figure 7** - Kinetics of electrolyte leakage (%) for 30 mg of control dormant *Arabidopsis* seeds (Control) and plasma-treated seeds (Plasma) over 24 h. Means over 3 replicates, each replicate (or group) containing 30 mg of *Arabidopsis* seeds with SD.

### 3.3.2. Effects on seed respiration and germination in hypoxia conditions

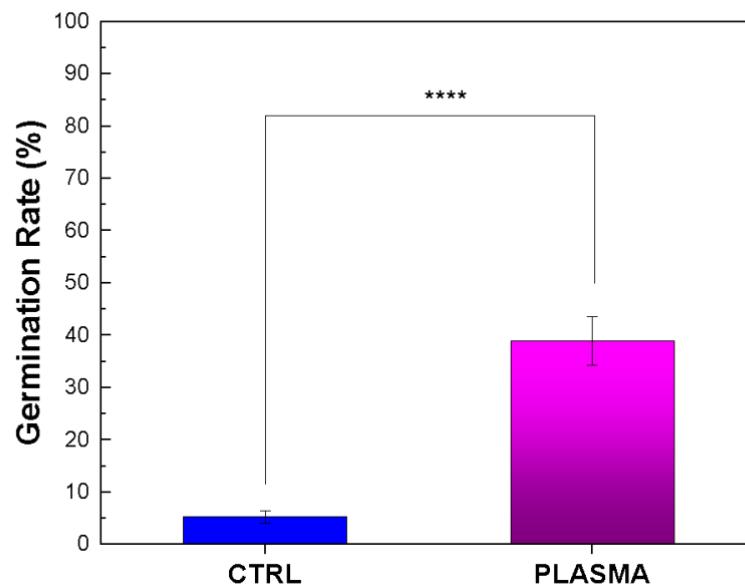
We monitored oxygen consumption of control and plasma-treated seeds during their imbibition with water at 15 °C for 24 h (Figure 8). When seeds were imbibed in a confined atmosphere, the O<sub>2</sub> content regularly decreased from 21 % to 18 % for the control seeds and to 16.5 % for the plasma-treated seeds within 24 h. C2AP treatment enhanced respiratory activity of *Arabidopsis* seeds, as shown by the slopes of O<sub>2</sub> concentration consumption shown in Figure 8(a). Analysis of the slopes allowed to determine the respiratory activity of the plasma-treated and the control seeds which were 0.057 g.mol<sup>-1</sup>.h<sup>-1</sup> and 0.042 g.mol<sup>-1</sup>.h<sup>-1</sup> respectively (Figure 8(b)).





**Figure 8** - Oxygen consumption (mol.mg<sup>-1</sup>FW) as a function of time for 3 replicates of 15 mg control seeds (C1, C2, C3) and 3 replicates of 15 mg plasma-treated seeds (P1, P2, P3) (a), Oxygen consumption rate (mol.h<sup>-1</sup>.mg<sup>-1</sup>FW) between control dormant seeds group (Control) and plasma treated seeds group (Plasma) (b): mean of 3 replicates +/- SD (\*\*\*\*: p-Value<0.0001). Mann-Whitney tests have been performed between control and plasma groups on the 6 replicates of the 6 experiments.

In addition, the effects of ambient air C2APs on germination in hypoxia conditions was assessed by evaluating the germination of freshly harvested Arabidopsis seeds at 15 °C in the dark under 4 % oxygen conditions. Here, C2APs treatment was applied on freshly harvested dormant seeds with their native water content. Interestingly, the seed germination rate dramatically increased from 7.5 % for control seeds to 42.5 % for plasma-treated seeds (Figure S4).



**Figure 9** - Germination after 4 d of imbibition at 15 °C under hypoxia (4 % of oxygen available) of control seeds (CTRL) and of 15 min plasma treated seeds (PLASMA). Means  $\pm$  SD of 4x4 replicates (\*\*\*\*:  $p$ -value < 0.0001). Mann-Whitney tests were performed between control and plasma groups on the 16 replicates of the 4 experiments.

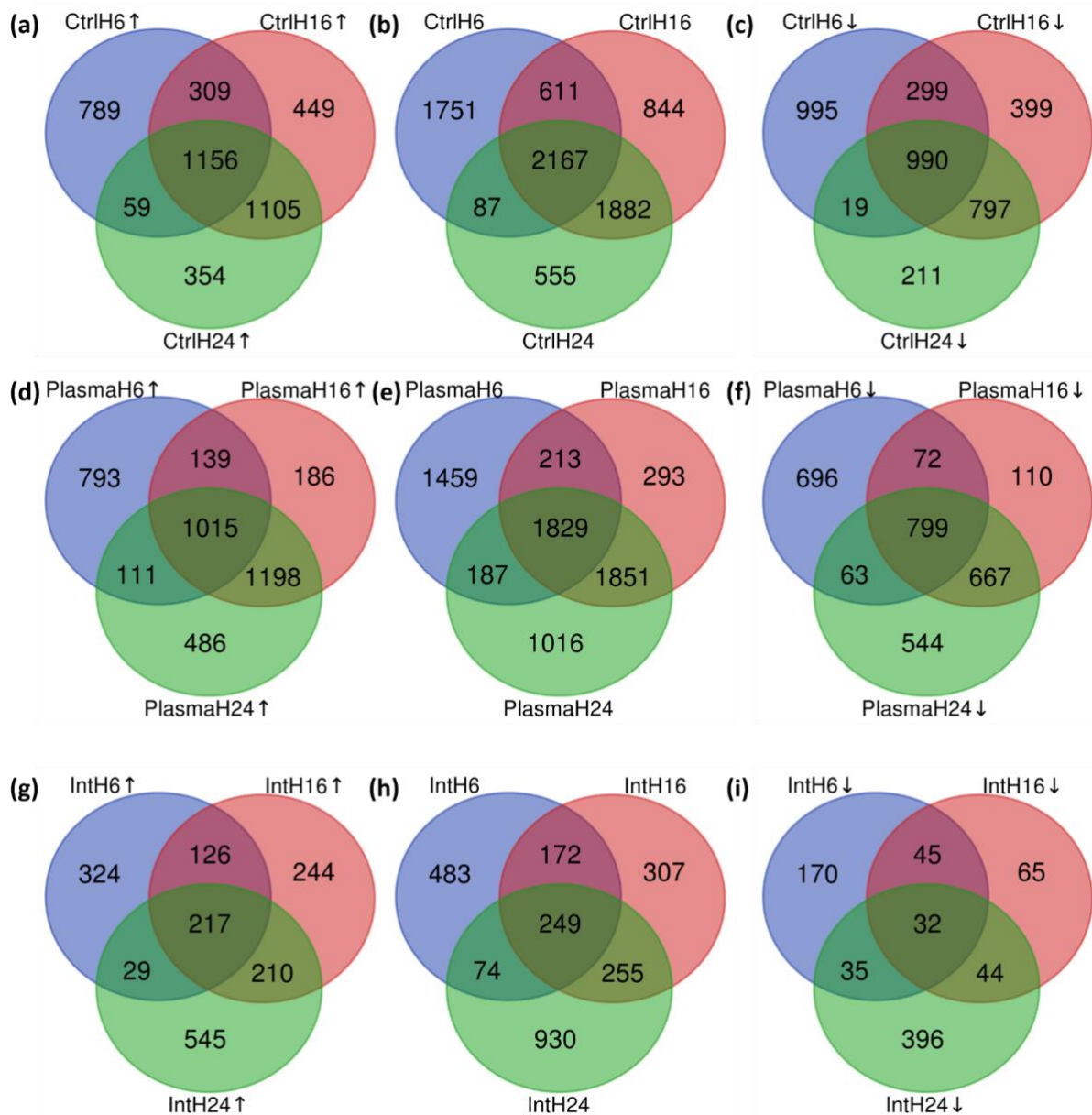
### 3.4. Effect of ambient air cold plasma on seed transcriptome

We also performed a transcriptomic analysis to determine whether C2APs treatment could have an effect on transcript abundance. RNA sequencing was performed in dry seeds and in seeds imbibed for 6, 16 and 24 h at 25°C. C2APs treatment (15 min) was performed after equilibrating seed WC at 20 %<sub>DW</sub>, since we previously demonstrated that the effect of C2APs was magnified when increasing seed WC. Freshly harvested seeds were deeply dormant since their germination did not exceed 10 % but 15 min C2APs treatment strongly stimulated germination and more than 40 % of seed became able to germinate (Figure S4). The PCA diagram (Figure S5) clearly shows the effect of C2APs treatment on seed transcriptome. In dry seeds, all samples were grouped together suggesting that C2APs treatment has little effect on transcript abundance in the dry seeds. In contrast, during seed imbibition, plasma-treated seeds gradually segregated in a distinct group of non-treated seeds (Figure S5).

Venn diagrams (Figure 10) show the dynamics of the transcriptome during seed imbibition. They demonstrate that C2APs treatment had a significant effect on the transcriptome. This

effect was very pronounced at 24 h of imbibition since twice more transcripts were identified in plasma-treated seeds than in untreated seeds (Figure 10(b) and (e)). These genes were either up or down regulated (Figure 10(d) and (f)). These observations were confirmed when studying the interaction (Int) between the time, defined as the difference between the plasma and the control at 24 h imbibition compared to the difference between the plasma and the control in the dry state (1), which shows the effect of plasma on transcript abundance.

$$Interaction \equiv [DryCTRL - DryPLASMA] - [H24CTRL - H24PLASMA] \quad (1)$$

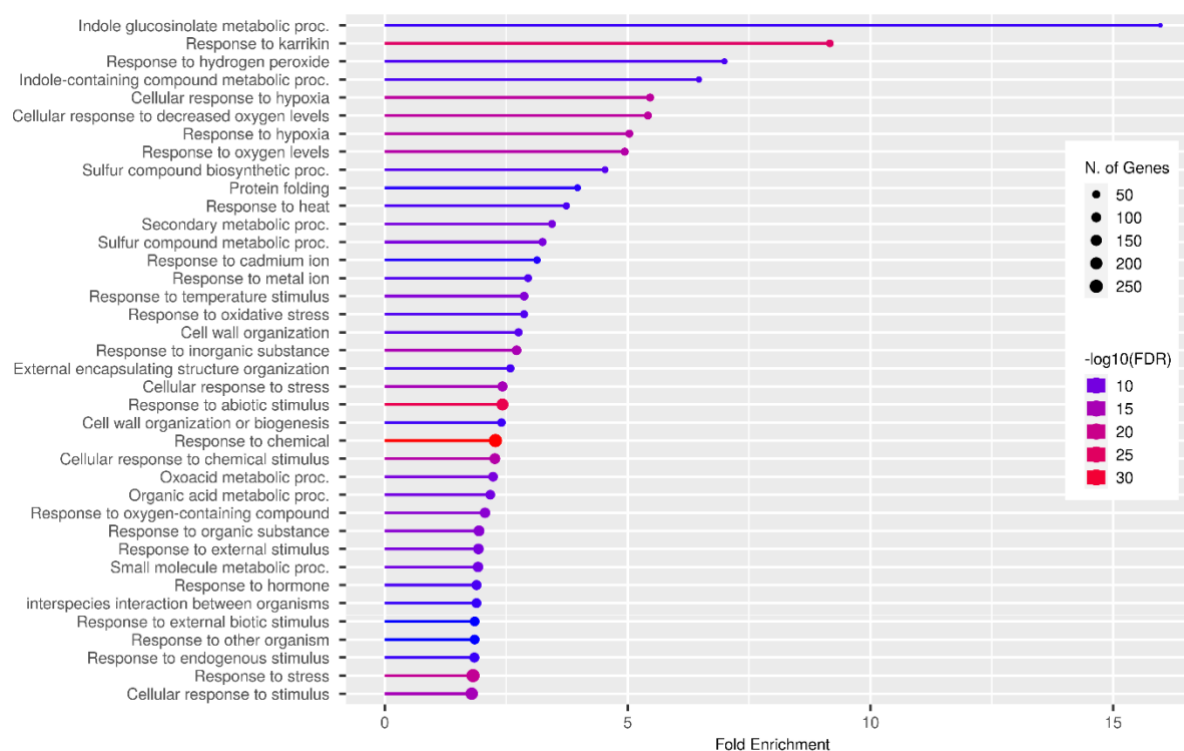


**Figure 10** - Venn diagrams showing kinetics change in the transcriptome: for control dormant seeds up regulated genes (a), total differentially expressed genes (b), down regulated genes (c); for plasma treated seeds up regulated genes (d), total differentially expressed genes (e), down regulated genes (f); for interaction up regulated genes (g), differentially expressed genes (h), down regulated genes (i). Abs(Fold Change) > 2, p-Value < 0.05.

Since this analysis revealed that the transcriptome was dramatically modified in plasma treated seeds after 24 h of imbibition, we further classified the differentially expressed genes (DEGs) by gene ontology classification with the ShinyGO database of Arabidopsis.

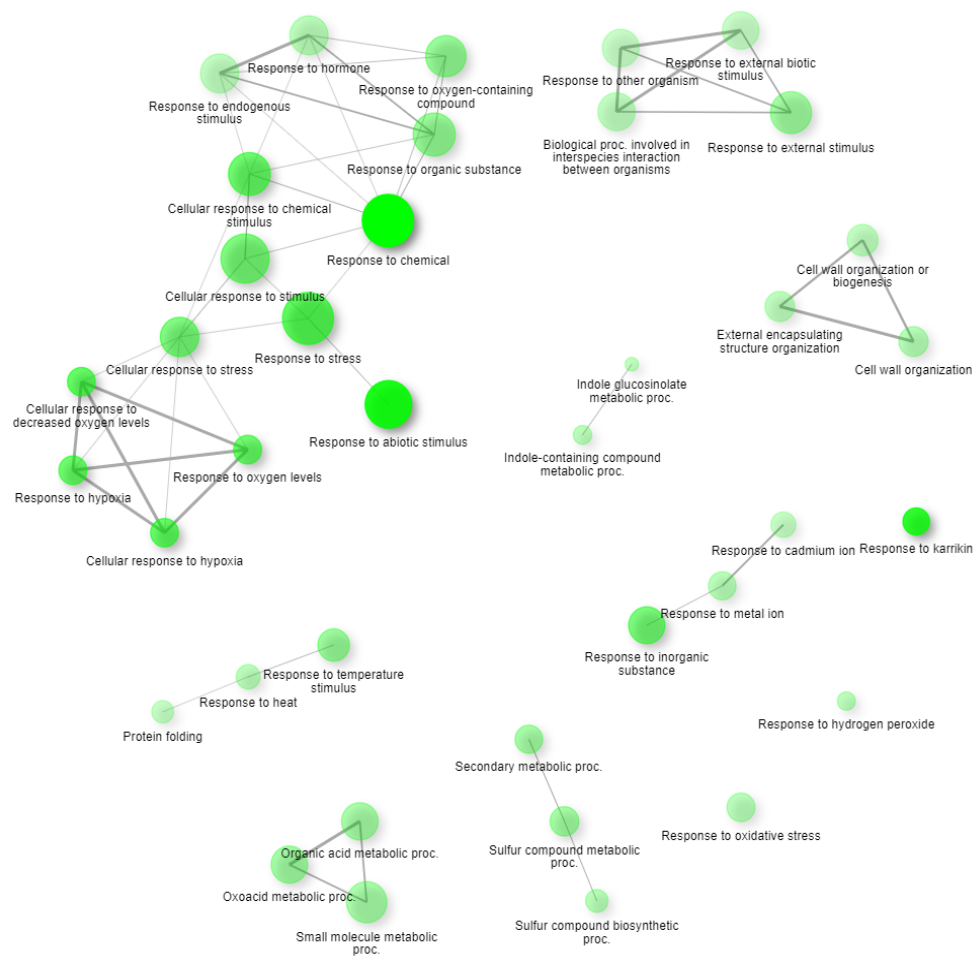
## Chapter 4: Deciphering the biological mechanisms and morphological changes induced by ambient air cold plasmas to release the dormancy of Arabidopsis seeds

This analysis revealed that biological processes related to oxygen (response to hypoxia, response to oxygen levels, cellular response to oxygen levels) and ROS (response to oxidative stress, response to hydrogen peroxide) were trustworthy represented (Figure 11 and 12) among the up-regulated genes. Responses to various stimuli including hormones such as karrikins were also identified by the GO analysis (Figure 11 and 12).



**Figure 11** - Chart Gene Ontology classification of Biological Processes of 24 hours of imbibition up regulated interaction DEGs (1001 genes) selected by False Discovery Rate (FDR cutoff of 0.05) and sorted by Fold Enrichment.

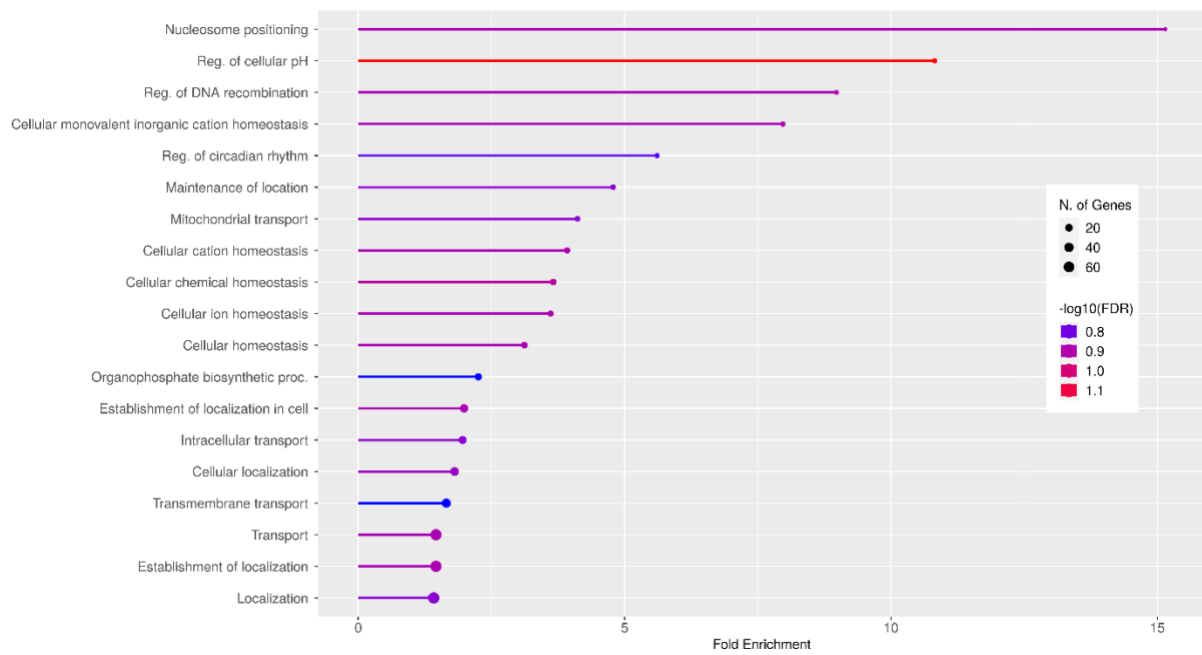
## Chapter 4: Deciphering the biological mechanisms and morphological changes induced by ambient air cold plasmas to release the dormancy of Arabidopsis seeds



**Figure 12** - Network Gene Ontology classification of Biological Processes of 24 h of imbibition up regulated interaction DEGs (1001 genes) selected by False Discovery Rate (FDR cutoff of 0.05) and sorted by Fold Enrichment.

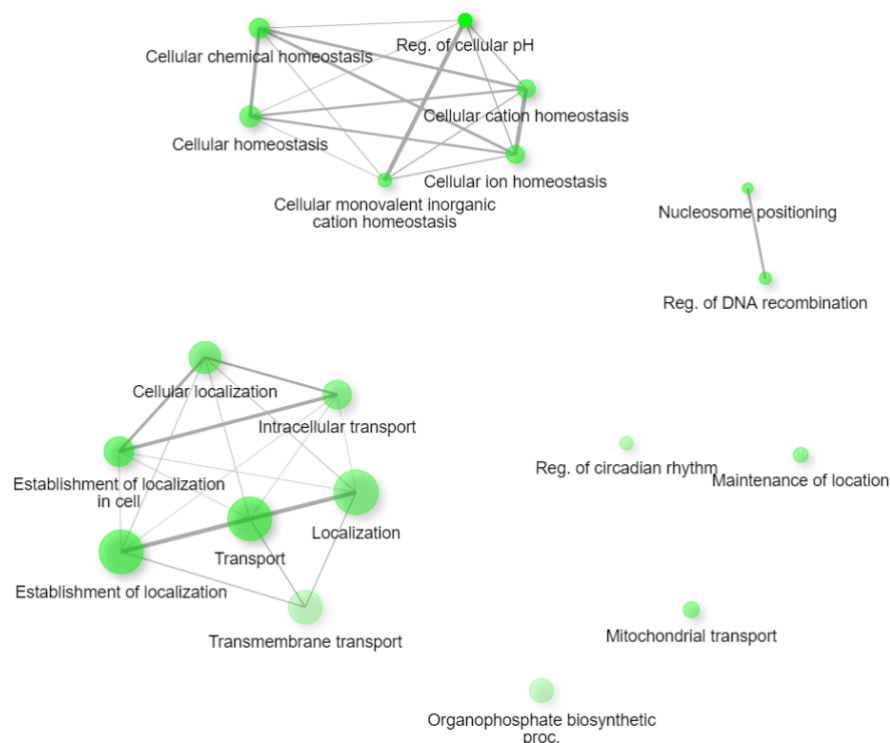
Similarly, we also performed a GO analysis with the 588 down regulated genes by plasma at 24 h (Figure 13 and 14). The down regulated genes were mostly found in the categories "Cellular homeostasis" and "transport pathway". The GO analysis of down regulated genes also evidenced categories related to gene expression regulation (nucleosome positioning).

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**Figure 13** - Chart Gene Ontology classification of Biological Processes of 24 hours of imbibition down regulated interaction DEGs (507 genes) selected by False Discovery Rate (FDR cutoff of 0.05) and sorted by Fold Enrichment.

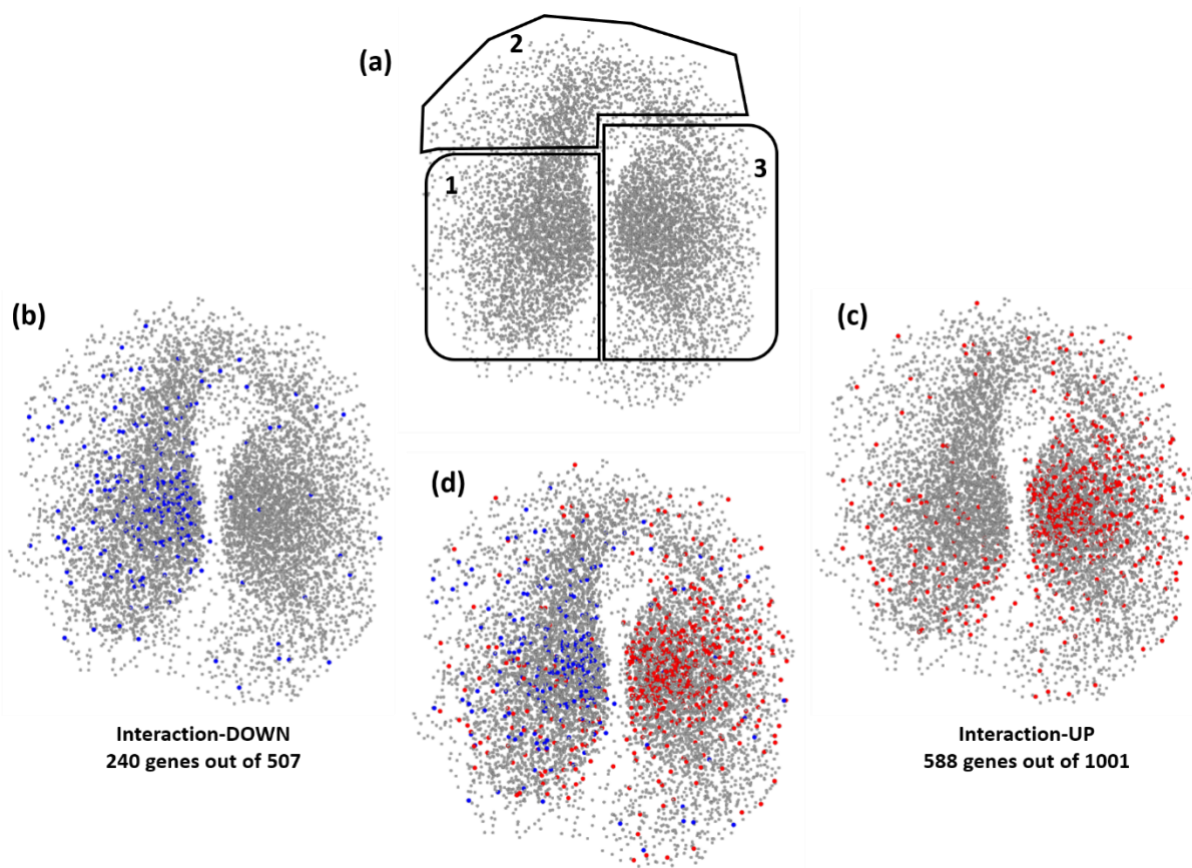
Chapter 4: Deciphering the biological mechanisms and morphological changes induced by ambient air cold plasmas to release the dormancy of Arabidopsis seeds



**Figure 14** - Network Gene Ontology classification of Biological Processes of 24 hours of imbibition down regulated interaction DEGs (507 genes) selected by False Discovery Rate (FDR cutoff of 0.05) and sorted by Fold Enrichment.

Finally, we also localized the transcripts whose abundance was modified by C2APs treatment on the SeedNet network. SeedNet network is a referential transcriptomics network model describing interactions in dormant and germinating Arabidopsis seeds. In this network, there are 3 main clusters associated with dormancy and germination (Figure 15(a)). The first region corresponds to co-expressed genes related to dormancy and the third region corresponds to germination and the second one to the transition between dormancy and germination. Using the Cytoscape software, up and down regulated genes by C2APs treatment were highlighted in the SeedNet network. On the 507 down regulated genes, 240 were found in the network and on the 1001 up regulated genes, 588 were found in the network. In Figures 15(b), (c) and (d), these genes are represented in the SeedNet network. Interestingly, most down-regulated genes (in blue dots) are located in the dormancy region whereas most of the up-regulated genes (in red dots) are located in the germination region.





**Figure 15** - Study of interaction DEGs into the SeedNet Network (Bassel et al., 2011). SeedNet gene co-expression network (a) with genes associated with seed dormancy in region 1, genes associated with dormancy-germination transition in region 2 and genes associated with germination in region 3. Interaction down DEGs into the SeedNet network (240 genes out of 507) in blue dots (b), Interaction up DEGs into the SeedNet network (588 genes out of 1001) in blue dots (c), Interaction DEGs into the SeedNet network: merge of down and up (blue and red dots) regulated DEGs (d).

## 4. Discussion and conclusion

### 4.1. Cold plasma treatment modifies dry seed infrastructure

A major interest of C2APs treatment is that they can modify seed physiology, i.e. the availability to germinate, after being applied in dry resting seeds. Surface properties are known to be affected by plasma treatment (Stolárik et al., 2015), but in addition we demonstrate here that C2APs treatments can also have an effect on seed infrastructure. Non-invasive tomographic techniques have already been used to describe the 3D structure of mature Arabidopsis seeds (Cloetens et al 2006). Here, in accordance with the results of these authors, it has been shown that air channels, i.e. void intercellular spaces, are present

within all seed tissues, from radicle tip to both cotyledons (Figure 2). Such spaces constitute a network that allows air diffusion within the dry tissues. This is interesting with regards to the actual hypothesis of seed dormancy release in anhydrobiosis, which postulates that it is induced by oxidative processes (Bailly, 2019). Our observations suggest that gaseous oxygen can easily diffuse within dry seeds which reinforce this hypothesis. In addition, tomography also indicate that plasmas can be produced within the void spaces of the tissues when seeds were exposed to C2APs treatment, and we therefore postulate that C2APs do not only modify seed surface properties but that they propagate in the dry tissue and can in turn also physically affect internal structures of the seed. This was verified when measuring the characteristics of the pore network (Figure 4; tables 2, 3 and 4). C2APs treatments from 15 min to 1 h indeed increased porosity and pore density and they dramatically increased volume and surface of pores, demonstrating their direct effect on internal seed physical properties. Additional analyses of other parameters (ie tortuosity, edges and vertex characteristics) brought the same conclusions. Similar results were found when the seed water content was reduced at 3 %<sub>DW</sub>. Nevertheless, no difference was demonstrated with heating treatment applied to the seed, surely indicating a more important interaction of structural change of the pore network with heating than with the plasma treatment.

#### 4.2. Effects of cold plasma treatment on anhydrobiotic oxidative processes

The increase of tissue porosity can be linked with oxygen diffusivity, as it has already been proposed with tomato fruit tissues (Xiao et al., 2021). We thus assume that changes in porosity and tortuosity of the seed pore network induced by C2APs treatments might also be associated with increased oxygen diffusion in the dry state. Since estimating diffusion of gaseous oxygen within dry Arabidopsis seeds is unfeasible, we assessed the potential effect of internal plasmas and/or higher oxygen diffusion by estimating seed H<sub>2</sub>O<sub>2</sub> content and lipid peroxidation, a well-known marker of oxidative damages in plant tissues. However, we did not highlight any change in oxidative components in dry Arabidopsis seeds treated by plasmas (Figure 5). However biochemical methods used here were probably not sensitive enough for revealing subtle changes in ROS homeostasis and we therefore assessed ROS production in Arabidopsis seeds using cell imaging techniques (Figure 6). It must be noted

that in this case seeds were not strictly dried since a short imbibition period (2 h) for required for being able to dissect the seeds. Nevertheless, DCFH-DA fluorescence revealed that C2APs treated seeds contained a much higher level of ROS (Figure 6). Air plasma contain reactive oxygen and nitrogen species such as excited nitrogen and oxygen molecules ( $N_2$ ,  $O_2$ ) and hydroxyl radical (OH), reactive species of short lifespan: oxygen (O), nitric oxide (NO) and nitrogen dioxide ( $NO_2$ ) and long lifespan: ozone ( $O_3$ ) which could interact with seed tissues and formed superoxide ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical (OH) or singlet oxygen ( $^1O_2$ ) (Waskow et al., 2021). It is therefore not surprising ton evidence that ROS content of Arabidopsis increases after C2APs treatment, although this has not been clearly evidenced up to date (Yan et al., 2022). Interestingly, in food, it has been proposed that ROS from plasma could interact with lipids and initiate oxidative processes, including at low water content (Gavahian et al., 2018). In dry rice grains, it was demonstrated that cold plasma slightly increased lipid peroxidation and that this process depended upon the processing time (Lee et al., 2018), and it is possible that the lipid peroxidation assessment was carried out after too small duration of treatment (15 min, Figure 5). Here, interestingly, high level of lipid peroxidation was observed at high water content after plasma treatment (Figure 5) demonstrating that C2APs treatment can indeed generate lipid oxidation, and that this oxidation is favoured when seed water content increased from 7 to 30 %<sub>DW</sub> (Figure 5). This was associated with a higher dormancy release at high water content (Chapter 2). We therefore propose that the beneficial of C2APs treatment on seed dormancy is associated with ROS accumulation and related oxidative processes, in agreement with the concept of oxidative window (Bailly et al., 2008).

### 4.3. Cold plasmas treatments modify physiology of imbibed seeds

To get a more comprehensive perception of plasmas effect on seed dormancy release we also assessed their long-term effect, i.e. during seed imbibition. In agreement with the observation that plasma can trigger lipid oxidation we evidenced an increase in electrolyte leakage during imbibition of C2AP treated seeds, thus revealing changes in lipid membrane properties (Figure 7). Measurement of seed conductivity usually reveals loss of seed germinability and it is often used as an indicator of loss of seed viability. However, it has long been established that membranes would also play a role in seed dormancy ((Hilhorst, 1998); (Hallett and Bewley, 2002)) and a range of compounds that affect cell membrane

properties and electrolyte leakage also release dormancy through an anaesthetic effect (Hallett and Bewley 2002). This would suggest that membrane lipid might be a major target of C2APs treatment in seeds but the direct link between germinability and modification of plasma membranes properties has to be further explored. The effect of cold plasma on cell membranes has already been demonstrated in other biological systems such as Chondrosarcoma cells (Haralambiev et al., 2020) or *Escherichia coli* and *Saccharomyces cerevisiae*, for example (Gan et al., 2021). Oxygen consumption of C2APs treated seeds was also higher indicating a rapid activation of mitochondrial respiration by the treatment (Figure 8). This is likely to be associated with increased energy production and reducing power but also ROS production (Bailly 2019). In addition, plasma treated seeds became able to better germinate in hypoxia, thus demonstrating that the treatment indeed released seed dormancy (Cueff et al., 2022).

#### 4.4. Cold plasmas treatments modify seed transcriptome

Lastly, we also demonstrate that plasma treatments have an indirect effect on seed transcriptome during subsequent seed germination. Oracz et al. (2007) and Bazin et al. (2011) previously showed that oxidative processes associated with seed after-ripening triggered changes in transcript abundance during seed germination, thus allowing seed germination. Similar effects were observed here, but we propose that C2APs treatments dramatically accelerated this effect which occurred after 15 min of treatment whereas dry after-ripening in *Arabidopsis* seeds generally requires weeks (Leymarie et al., 2012). The transcriptome of plasma treated seeds indeed progressively diverged from the one of the control seeds during seed imbibition (Figure S5) which directed the seed developmental program towards germination. Our study is the first transcriptomic analysis of the effect of plasma treatment on seed germination and dormancy breaking. Hayashi et al. (2016) had studied the gene expression profile of *Arabidopsis* seeds just after oxygen plasma exposure using DNA microarray but without comparison to dry control. They showed that C2APs treatments were associated with increase in energy production of plants via photosynthesis and carbon fixation and auxins productions. Another study on cold plasma effect that focused on transcriptomic analysis on *Arabidopsis* seedlings (Cui et al., 2021) showed that most of the DEGs are associated with defence, stimuli or stress-responsive genes and encoded proteins with oxidoreductase activity. Here, as expected, we first identified up-

regulated genes associated with some classical pathways of seed dormancy release and regulation of germination such as response to hydrogen peroxide and response to hormones (Figures 11 and 12), that were included in the SeedNet network (Figure 15). In addition, we also identified transcriptomics footprints that could be more specific to C2APs treatments. For example, we have found that the plasma treatment triggered pathways in response to karrikin. Karrikin refers to a group of plant regulators that can be produced during forest fires and that stimulate the germination of many seeds, including Arabidopsis seeds (Waters et al., 2014). Several studies have shown that these compounds might be associated with regulators involved in hormonal signalling and tolerance to abiotic stress leading to a stimulation of germination and a decrease in seed dormancy ((Meng et al., 2017), (Wang et al., 2018), (Sami et al., 2021)). We also found that indole glucosinolate metabolism processes were induced by the plasma treatment. Indole glucosinolates are plant secondary metabolites that can be related to auxin biosynthesis (Liu et al., 2019). This is supported by the presence of secondary metabolic processes that emerge from the analysis showing the action of cold plasma treatment on the activation of seed metabolism through secondary metabolism. Interestingly, many processes related to oxygen response have been found: cellular response to hypoxia, cellular response to decreased oxygen levels, response to hypoxia, response to oxygen levels, response to oxidative stress, response to oxygen-containing compounds. Gene regulatory pathways founded and related to these biological processes are consistent with the results of the experiences presented in this study. This may explain the increase in germination rate of dormant seeds and the enhancement of germination under hypoxia observed after the plasma treatment. Further works could focus on the determination of transcription factors associated with the activation and formation of proteins regulating germination.

## 4.5. Conclusion

In conclusion, this study allowed revealing a sequence of events that may participated to the beneficial effect of C2APs treatments on Arabidopsis seed germination. Using a variety of techniques, we have shown that these treatments, when performed in the dry state, alter physical and biological seeds component that are probably closely related. Our results validate the hypothesis of seed dormancy release by oxidative processes in anhydrobiosis and they show that a rapid increase of seed oxidative status is sufficient for realising seed dormancy in the dry state. They also identify a set of biological events, including changes in membrane properties, activation of respiration and change in gene expression that are

#### Chapter 4: Deciphering the biological mechanisms and morphological changes induced by ambient air cold plasmas to release the dormancy of *Arabidopsis* seeds

triggered by the treatment of dry seeds by C2APs, but expressed during seed imbibition. Altogether these results should help to better understand the cellular basis of C2APs treatments on seed germination and will help to design novel protocols of treatment adapted to other seed species.



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# **General Conclusion & Perspectives**

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This work brings new discoveries in the application of cold plasmas for agriculture and in particular for seed treatment through the release of Arabidopsis seeds dormancy. The innovative approach of this multidisciplinary study allowed to highlight multiple mechanisms playing on the interaction between plasma and seed physiology.

First, this work has evidenced a range of cellular events that are associated with the effects of plasmas in dry seed tissues, before these ones get imbibed. The results show that seed water content is a particularly important factor for plasma treatment. It has been shown that plasma treatment could alter water sorption properties in seed tissues; these changes being possibly related to the modification of water binding radicals. Moreover, considering the seeds as dielectric materials, it was shown that seed water content both increased their dielectric properties and played on the electrical properties of the plasma. The study of the effect of plasma treatment on the release of Arabidopsis seed dormancy also demonstrated the importance of seed water content in modulating this effect. It was shown that seed dormancy release was significantly improved when the cytoplasm of the seeds changed from a glassy state to a rubbery state, either after increasing water content or by increasing the temperature. The cytoplasmic rubbery state is characterised by a decrease in viscosity and an increase in metabolic activity that might play a role in plasma treatment efficiency. It can be hypothesised that increased molecular mobility may favour diffusion of reactive oxygen species generated and kinetics of oxidative reactions associated with plasma effect.

The changes in seed water binding properties might be explained by the effect of plasma on seed infrastructure. Indeed, plasma treatment has been shown to increase seed porosity, demonstrating that plasma effects are not only restricted to seed surface, but that they can modify inner parts of the seed. This can have a direct effect on the water binding properties because in the strong-binding sites, which are modified by plasmas, water is associated with macromolecular surfaces and biological interfaces by ionic bonding (Hay et al. 2022). In addition, the increase of seed internal volumes caused by plasmas could be associated with a higher diffusion of gases from the atmosphere, including oxygen, within seed tissues. This could be associated with seed dormancy release, since it is known that in the dry state this process is triggered by oxidative reactions. Although the occurrence of oxidative processes and ROS generation is not possible to assess experimentally, this work brings a set of indirect evidence suggesting that short plasma treatment can generate an oxidative environment in dry seeds that allows dormancy alleviation.

Secondly this work has also highlighted germination-related biological processes in imbibed seeds that were induced by plasma treatments in the dry seeds. As shown in other biological systems, plasmas appeared to have an effect on cell membranes, pointing out again the putative role of membrane fluidity and properties in the control of seed germination. The results have shown that plasma can alter cell metabolism, as evaluated by respiratory activity. This work is also the very first to investigate the effect of plasma on seed transcriptome during imbibition, and it was demonstrated that plasmas induced reprogramming of gene expression towards germination. New regulatory pathways emerged from the analysis of the results, such as response to karrikin or pathways linked to the metabolism of indole glucosinolates. Interestingly the RNAseq analysis also revealed that processes linked to the response to oxygen, hypoxia and oxidative phenomena were induced by plasmas. Taken together, these results lead to a better understanding of the interaction between seeds and plasma by showing the influence of the seed cytoplasm physical state on the effect of the treatment and they highlight cellular processes and morphological changes associated with the plasma treatment on seed germination. This will help to design new treatment protocols suitable for other seed species.

These results open up new perspectives on the study of the interaction between seeds and plasma and on the mechanisms associated with the effects of plasma treatments on seed physiology from the dry state. If seed water content is a crucial parameter affecting the interaction between plasma and seeds, one can wonder if temperature and relative humidity in the reactor during the treatment can have an impact on this one (Patil et al., 2014). These two parameters could influence the properties of the plasma discharge by modulating the quantities of reactive species generated but also the interaction with the seeds (Zhou et al., 2021). Biological processes triggered by the plasma treatment highlighted in this study have shown the role of oxidative processes and the generation of ROS in seed tissues. These oxidative phenomena triggered by plasma deserve to be better understood in order to detect the different mechanisms operating in dry seeds. Further studies could deepen the knowledge of these processes by using different gaseous products (Misra et al., 2014). Applying the discharge in different mixtures of gases could modulate the generation of reactive species in the tissues. In particular, it could be envisaged to treat seeds in atmospheres in which the oxygen level varies during the discharge, in order to determine whether the processes triggered by plasma could be dependent on the plasma oxygen level. The study of oxygen diffusion in the tissues would help to understand the influence of the treatment on the physiological state of the seeds.

One could also target more deeply the ROS generated in the tissues that were observed by confocal microscopy. For example, by electron paramagnetic resonance (EPR) or electron spin resonance (ESR) spectroscopy, one could detect and quantify the production of superoxide ( $O_2^{\cdot-}$ ) or hydroxyl radical ( $OH^{\cdot}$ ). It would also be relevant and interesting to evaluate the evolution of metabolic activity and the change in physiology of the seeds when stored in an atmosphere where oxygen availability is limited.

The analysis of the tomography data could be further developed by determining, for example, the location of morphological changes. By segmenting these changes over different parts of the seeds (cotyledons and embryonic axis), the construction of models of oxygen diffusivity through the tissues could provide a more in-depth understanding of the effect on the intercellular spaces formed by the pore network. Cell segmentation could also be achieved and their reorganisation could be revealed. Another question is whether plasma treatment can have an effect on cell membranes and cell walls. Electron microscopy experiments of fixed tissues could show a potential effect on the ultrastructure of the seeds after treatment.

From the transcriptomic data obtained in this study, further analysis and data mining of the genes up-regulated by the plasma treatment would allow the identification of transcription factors related to the germination enhancement, even though it could remain difficult to determine whether these transcription factors and target genes would be related to the germination process and/or would be more specific to the plasma treatment. Transcriptomic studies outside the context of dormancy, where the treatment accelerates germination (germinative vigour) or where the treatment removes pathogens from infected seeds (decontamination), could also be considered.

The experiments in this study were conducted on *Arabidopsis* seeds of the Columbia Col-0 ecotype grown and harvested dormant. Comparison of the same plasma treatments on seeds of the same species, but from different ecotypes, would be relevant to understand the effects of the treatment. For *Arabidopsis* seeds, one could consider experiments on several ecotypes with different physiological traits (Fletcher, 2022) and evaluate their responses to the plasma treatment. In addition, since our hypothesis is that plasma treatment induces an oxidative environment in dry tissues which leads to oxidative signalling during imbibition and then releases seed dormancy, it would be very interesting to extend this study to other species with different reserve natures. Indeed, *Arabidopsis* seeds are rich in lipids that favour auto-oxidation reactions through the tissues. It is

legitimate to ask whether these plasma treatments would work in the same way for other seed species with less lipid but more protein (protein seeds) or more carbohydrate (starch seeds).

Moreover, on the basis of analysis to elucidate the effect of cold plasma, investigations in the field are continuously necessary to construct the optimal treatment conditions. When is the ideal moment to treat seeds with plasma; right after harvest, before storage or before sowing (how many hours/days before sowing) are all questions that remain to be answered. Studies must also focus also on the durability of the treatment effect. For example, it was shown that the plasma treatment can have a 4 months retention time effect on cotton seed germination (de Groot et al., 2018). However, the effect of the retention time on physiological response of seeds to plasma treatment must be studied deeper.

Altogether the results obtained during this Thesis bring novel sets of data that allow to better understand the biological mechanisms involved in plasma effects on seed germination. This should participate in the development and diffusion of this technology towards the stakeholders in a context of both global change and sustainable agriculture.

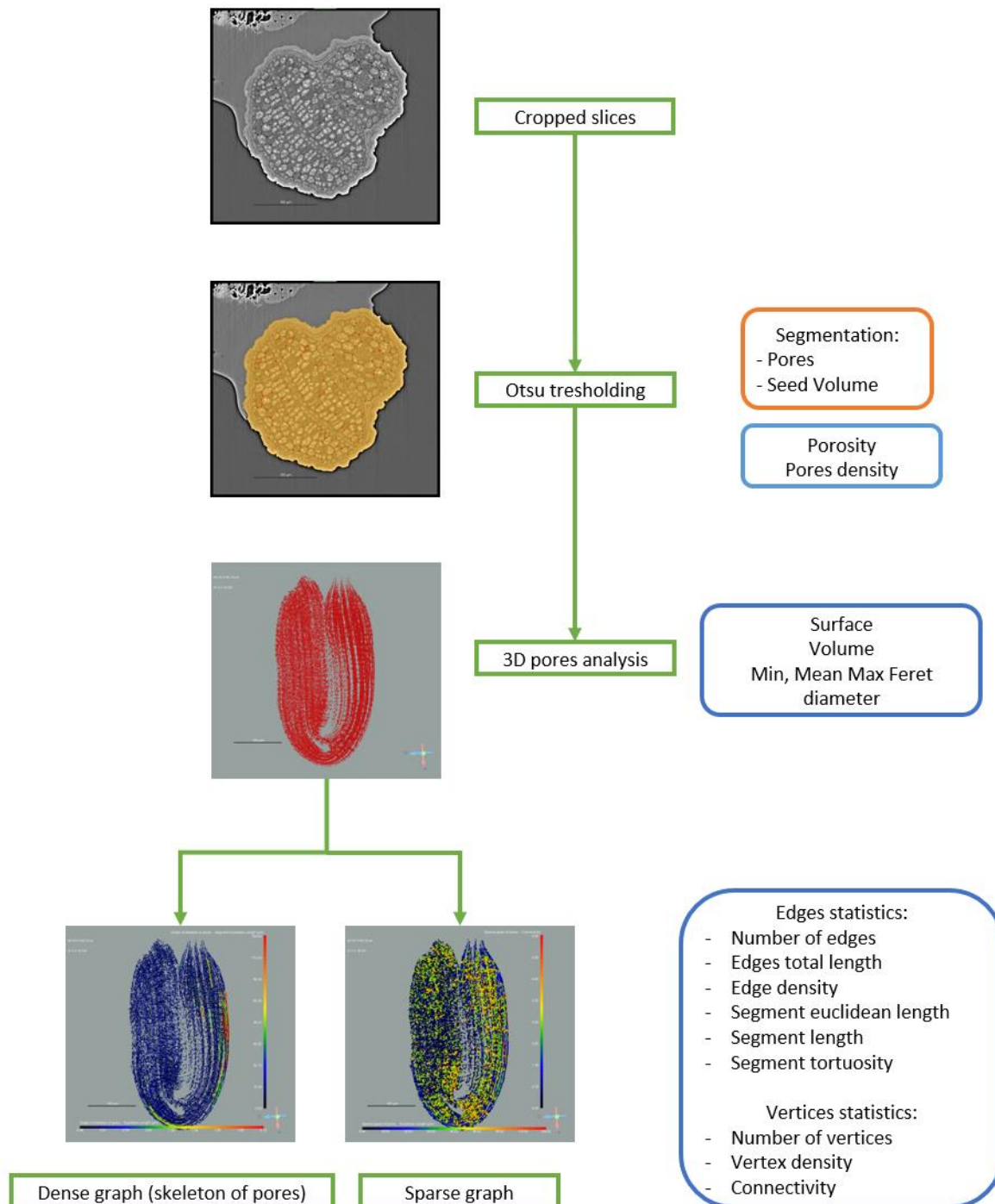


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# Annex

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## Annex Chapter 4



**Figure S1** - Protocol analysis of microtomography images: step (green boxes), segmentation (orange box), statistical calculations (blue boxes).

**Table S1** - Segmentation parameters of pores and seed volume of all condition replicates.

	<b>Pore Volume (<math>\times 10^4 \mu\text{m}^3</math>)</b>	<b>Pores (<math>\times 10^5</math> voxels)</b>	<b>Seed volume (<math>\times 10^6 \mu\text{m}^3</math>)</b>	<b>Seed volume (<math>\times 10^7</math> voxels)</b>	<b>Pore density (<math>\times 10^{-2} \mu\text{m}^{-3}</math>)</b>	<b>Porosity (%)</b>
<b>CTRL1</b>	20.7	64.9	13.3	41.5	48.8	1.56
<b>CTRL2</b>	5.9	18.4	11.5	35.9	16	0.51
<b>CTRL3</b>	30.7	96.1	15.5	48.5	62	1.98
<b>CTRL4</b>	25.4	79.5	15.8	49.5	50.3	1.61
<b>CTRL5</b>	29.3	91.8	18.7	58.3	49.2	1.57
<b>CTRL6</b>	30.8	96.4	16.1	50.4	59.8	1.91
<b>CTRL7</b>	19.6	61.4	19.3	60.5	31.8	1.02
<b>CTRL8</b>	25.3	79.1	15.1	47.3	52.3	1.67
<b>CTRL9</b>	22.3	69.6	14.6	45.6	47.7	1.53
<b>CTRL10</b>	31.7	99	18.1	56.5	54.8	1.75
<b>P15'-1</b>	30.3	94.8	13.5	42.3	70.1	2.24
<b>P15'-2</b>	48.3	150.9	19.6	61.4	76.9	2.46
<b>P15'-3</b>	21.5	67.1	11.7	36.6	57.3	1.83
<b>P15'-4</b>	41.6	130.1	16.3	50.9	80	2.56
<b>P15'-5</b>	38.8	121.2	15.5	48.4	78.3	2.5
<b>P15'-6</b>	39	122.1	18.4	57.4	66.5	2.13
<b>P15'-7</b>	33.5	104.8	15.4	48.3	67.9	2.17
<b>P15'-8</b>	47.4	148.2	14.4	45.1	102.9	3.29
<b>P15'-9</b>	58.5	182.9	16	50.1	114.1	3.65
<b>P30'-1</b>	30.2	94.4	11.8	36.9	80	2.56
<b>P30'-2</b>	25.3	79	13.5	42.1	58.6	1.87
<b>P30'-3</b>	44.8	140.2	15.7	49.1	89.3	2.86
<b>P30'-4</b>	36.9	115.3	13.3	41.5	86.9	2.78
<b>P30'-5</b>	32	100.2	13.1	41	76.4	2.44
<b>P30'-6</b>	39.4	123.2	16	50.1	76.9	2.46
<b>P30'-7</b>	62.7	196	15.3	47.8	128.4	4.1
<b>P30'-8</b>	30.5	95.5	14.6	45.7	65.4	2.09
<b>P30'-9</b>	30.7	95.9	12.7	39.8	75.4	2.41
<b>P30'-10</b>	42.5	133	16.1	50.4	82.5	2.64
<b>P1h-1</b>	37.2	116.5	13.7	42.8	85	2.72
<b>P1h-2</b>	47.4	148.1	16.6	52.4	88.4	2.83



## Annex

<b>P1h-3</b>	41.8	130.8	20	62.5	65.5	2.09
<b>P1h-4</b>	66.6	208.4	15.2	47.5	137.2	4.39
<b>P1h-5</b>	30.5	95.4	14.3	44.6	66.9	2.14
<b>P1h-6</b>	38.6	120.8	14.2	44.5	84.9	2.71
<b>P1h-7</b>	41.	129.6	16.6	51.9	78.2	2.5
<b>CH1</b>	24.7	77.3	13.8	43.2	55.9	1.79
<b>CH2</b>	42.2	132.1	16.4	51.4	80.4	2.57
<b>CH3</b>	28.6	89.5	14.2	44.3	63.2	2.02
<b>CH4</b>	23.6	73.8	15.4	48.2	47.9	1.53
<b>CH5</b>	24.3	76	13	40.6	58.6	1.87
<b>CH6</b>	31.2	97.6	13.1	40.9	74.7	2.39
<b>CH7</b>	31.8	99.3	16.6	52	59.7	1.91
<b>CH8</b>	28.4	89	13.6	42.6	65.4	2.09
<b>PH1</b>	31.5	98.7	15.1	47.1	65.5	2.09
<b>PH2</b>	24.5	76.6	12.7	39.7	60.4	1.93
<b>PH3</b>	32.6	101.9	15.1	47.6	67.6	2.16
<b>PH4</b>	17.6	55	11.5	35.9	47.9	1.53
<b>PH5</b>	26.2	82	13.7	42.7	60.2	1.92
<b>PH6</b>	26.2	81.9	15.3	47.7	53.7	1.72
<b>PH7</b>	39.7	124.1	18.7	58.5	66.3	2.12
<b>PH8</b>	39.1	122.4	16.9	52.7	72.6	2.32
<b>PH9</b>	27.2	85	11.5	36.1	73.6	2.35
<b>CLW01</b>	23.5	73.5	14.2	44.6	51.6	1.65
<b>CLW02</b>	23.7	74.1	16	50	46.3	1.48
<b>CLW03</b>	23.9	74.8	14	43.8	53.4	1.71
<b>CLW04</b>	26	81.2	19.2	60.1	42.3	1.35
<b>CLW05</b>	22.6	70.8	15.7	48.9	45.2	1.45
<b>CLW06</b>	24.9	77.8	14.1	44.2	55	1.76
<b>CLW07</b>	25.2	78.9	15.4	48.2	51.2	1.63
<b>CLW08</b>	25.2	78.7	13.7	42.9	57.3	1.83
<b>CLW09</b>	20.6	64.5	13.5	42.1	47.9	1.53
<b>CLW10</b>	29.6	92.4	16.2	50.6	57.1	1.82
<b>PLW01</b>	27.9	87.4	17.3	54	50.6	1.62
<b>PLW02</b>	26.1	81.7	14.8	46.1	55.3	1.77
<b>PLW03</b>	36.6	114.5	19.4	60.6	59.1	1.89
<b>PLW04</b>	44.7	139.8	15.1	47.2	92.7	2.96
<b>PLW05</b>	58.4	182.6	23	71.8	79.6	2.54

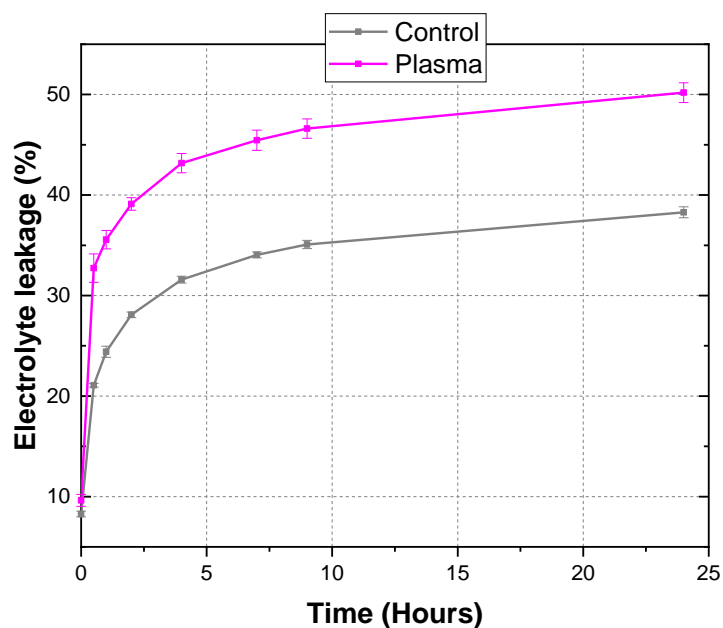
<b>PLW06</b>	27.8	86.9	15.6	48.7	55.9	1.79
<b>PLW07</b>	25.4	79.6	13.7	42.8	58.2	1.86
<b>PLW08</b>	34.3	107.4	17.2	53.8	62.4	1.99
<b>PLW9</b>	22.2	69.4	17.7	55.4	39.2	1.25

**Table S2** - Sparse graph parameters (number of vertices, number of edges, edges total length, vertex density, edge density) of all groups: control dormant seeds (CTRL), seeds treated during 15 min (P-15'), seeds treated during 30 min (P-30'), seeds treated during 1h (P-1h), seeds heated at 60 °C during 15 minutes (C-H), seeds heated at 60 °C and treated during 15 minutes (P-H), seeds with low water content at 3 %<sub>DW</sub>(LW) and seeds with low water content at 3 %<sub>DW</sub> and treated during 15 min (P-LW). Means +/- SD.

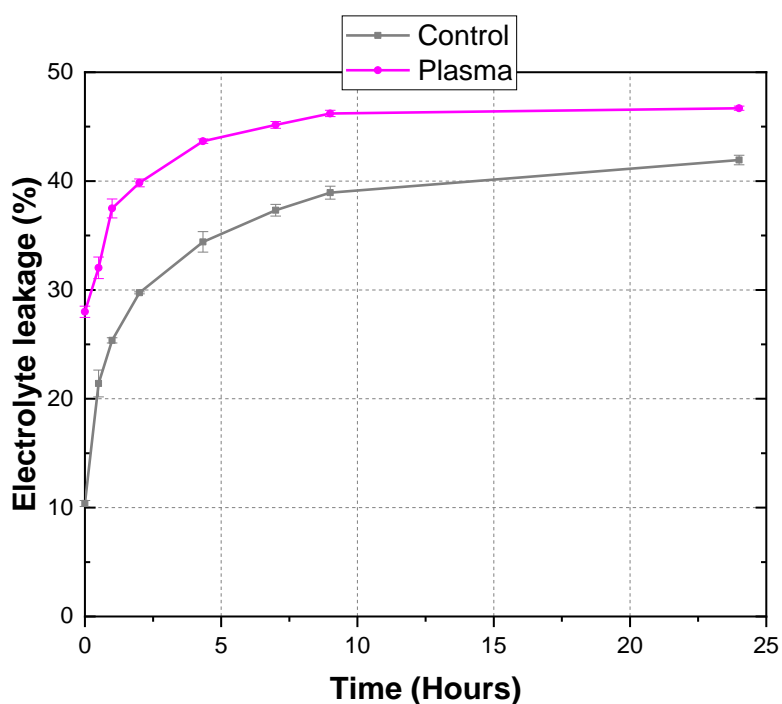
	CTRL	P-15'	P-30'	P-1h	C-H	P-H	C-LW	P-LW
<b>Number of vertices (x10<sup>4</sup>)</b>	6.7	12.4	10	12.4	11.1	9.4	7	10.5
	±	±	±	±	±	±	±	±
	1.2	1.7	0.9	1.3	0.7	1.1	0.4	1.2
<b>Number of edges (x10<sup>4</sup>)</b>	3.2	5.9	4.9	6	4.9	4.5	3.2	5
	±	±	±	±	±	±	±	±
	0.6	0.8	0.4	0.5	0.4	0.5	0.1	0.8
<b>Edges total length (mm)</b>	137.8	211.1	181.1	217.9	173.3	169.4	143	192.5
	±	±	±	±	±	±	±	±
	17.1	19.7	10.5	17.8	11.9	16.7	4.6	21.7
<b>Vertex density (x10<sup>5</sup> mm<sup>-3</sup>)</b>	42	78	71	79	76	63	46	63
	±	±	±	±	±	±	±	±
	8	9	6	8	4	6	3	8
<b>Edge density (x10<sup>5</sup> mm<sup>-3</sup>)</b>	20	37	35	38	34	31	21	30
	±	±	±	±	±	±	±	±
	3	4	3	3	2	3	1	5

**Table S3** - Edge and Vertex statistical properties of sparse graphs for all groups: control dormant seeds (CTRL), seeds treated during 15 min (P-15'), seeds treated during 30 min (P-30'), seeds treated during 1h (P-1h), seeds heated at 60 °C during 15 minutes (C-H), seeds heated at 60 °C and treated during 15 minutes (P-H), seeds with low water content at 3 %<sub>DW</sub> (LW) and seeds with low water content at 3 %<sub>DW</sub> and treated during 15 min (P-LW). Means +/- SD.

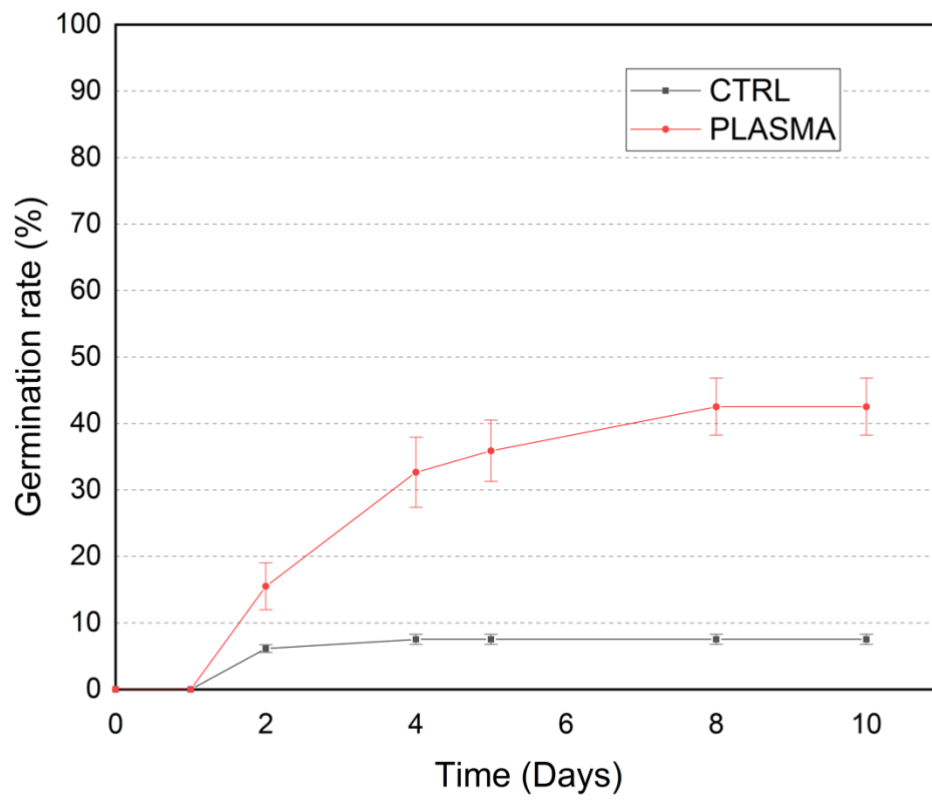
		CTRL	P-15'	P-30'	P-1h	C-H	P-H	C-LW	P-LW	
Edge	Euclidean length (μm)		112.4	96.9	112.9	97.1	79.6	112	110.7	96.5
		Max	±	±	±	±	±	±	±	±
			6	9.8	7.6	13.7	5.8	12.5	6.7	5.2
			4.56	3.75	3.78	3.75	3.53	3.85	4.5	4.07
		Mean	±	±	±	±	±	±	±	±
			0.24	0.2	0.18	0.13	0.13	0.18	0.15	0.25
Vertex	Connectivity		6.2	7.8	8.7	7.3	7.3	6.7	6.7	7.1
		Max	±	±	±	±	±	±	±	±
			0.3	0.5	1.1	0.4	0.3	0.2	0.3	0.4
			0.94	0.95	0.99	0.98	0.89	0.99	0.93	0.93
		Mean	±	±	±	±	±	±	±	±
			0.06	0.02	0.03	0.04	0.03	0.03	0.03	0.04



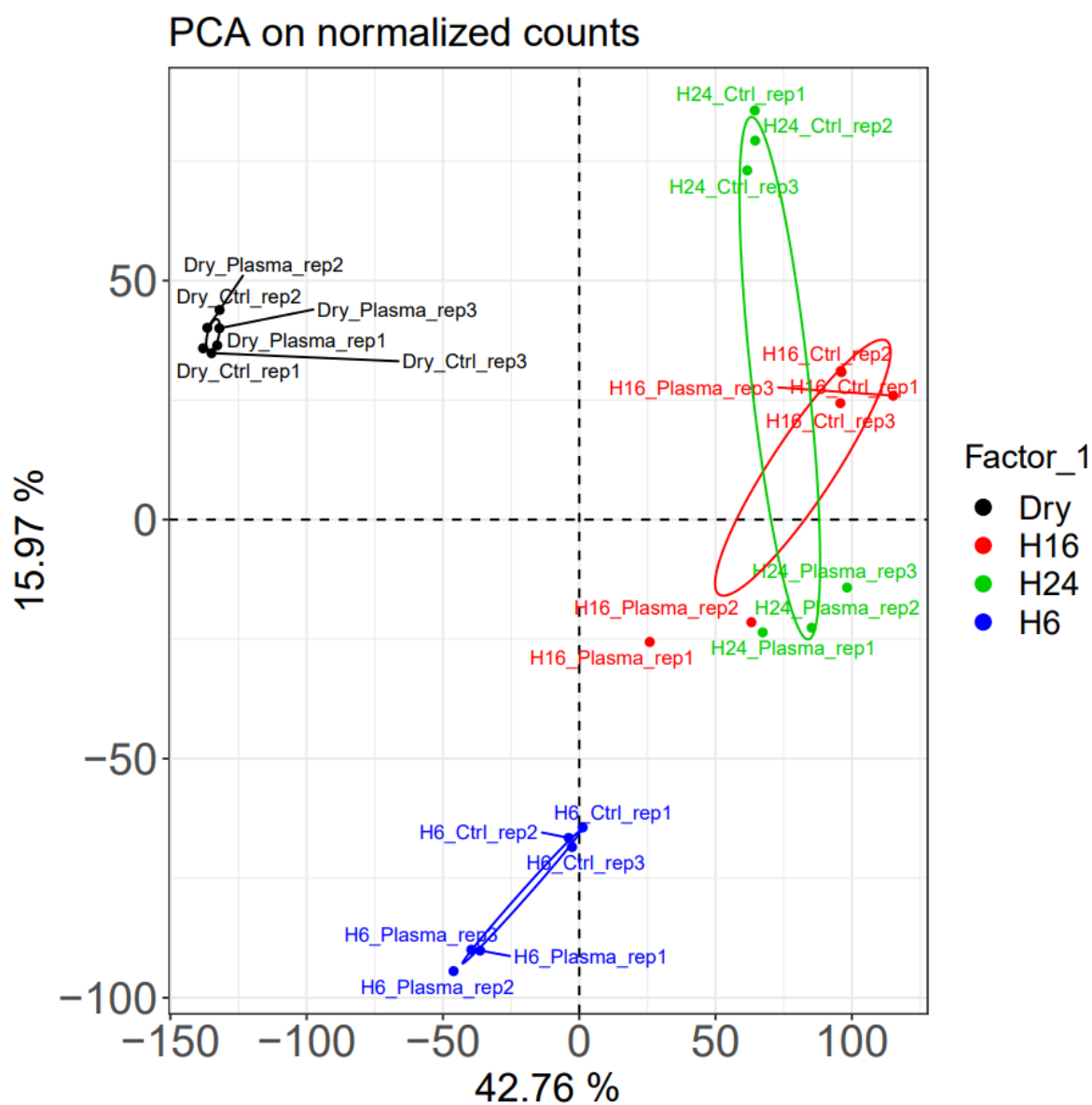
**Figure S2** - Kinetics of electrolyte leakage (%) for 30 mg of control dormant *Arabidopsis* seeds (Control) and plasma treated seeds (Plasma) over 24 hours. Means of 3 replicates of 30 mg of *Arabidopsis* seeds for each group with SD.



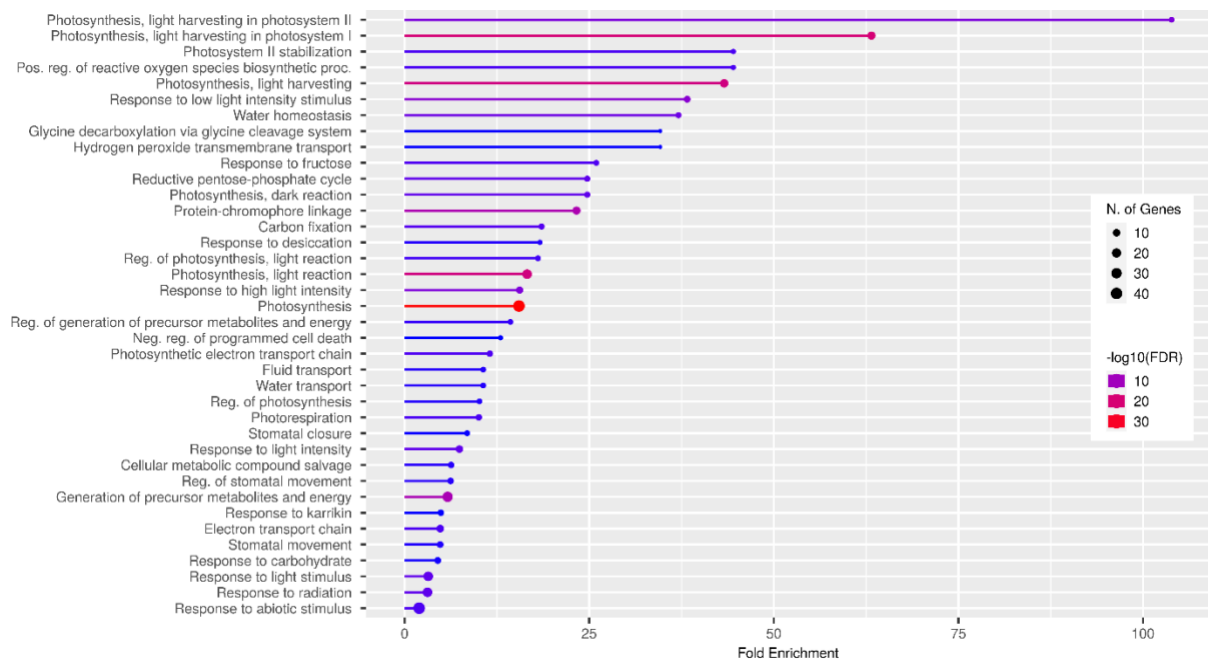
**Figure S3** - Kinetics of electrolyte leakage (%) for 30 mg of control dormant *Arabidopsis* seeds (Control) and plasma treated seeds (Plasma) over 24 hours. Means of 3 replicates of 30 mg of *Arabidopsis* seeds for each group with SD.



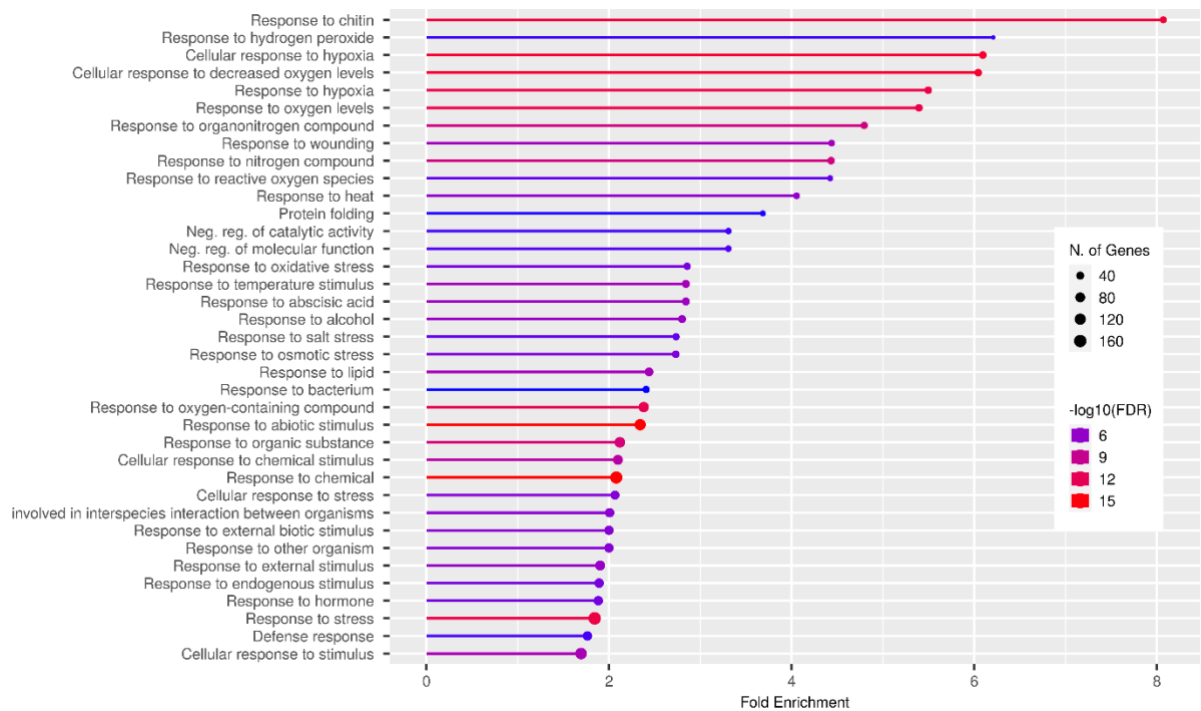
**Figure S4** - Germination curves of control dormant seeds (CTRL) and plasma-treated seeds (PLASMA).



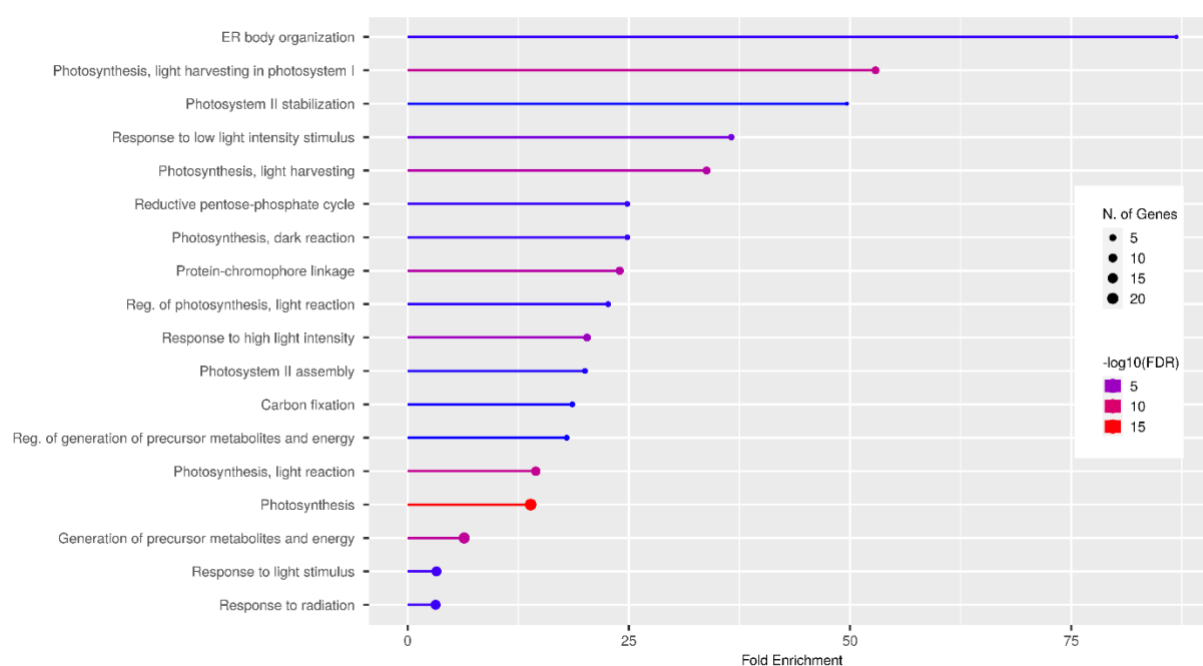
**Figure S5** - PCA diagram of the control and plasma conditions over the imbibition time points.



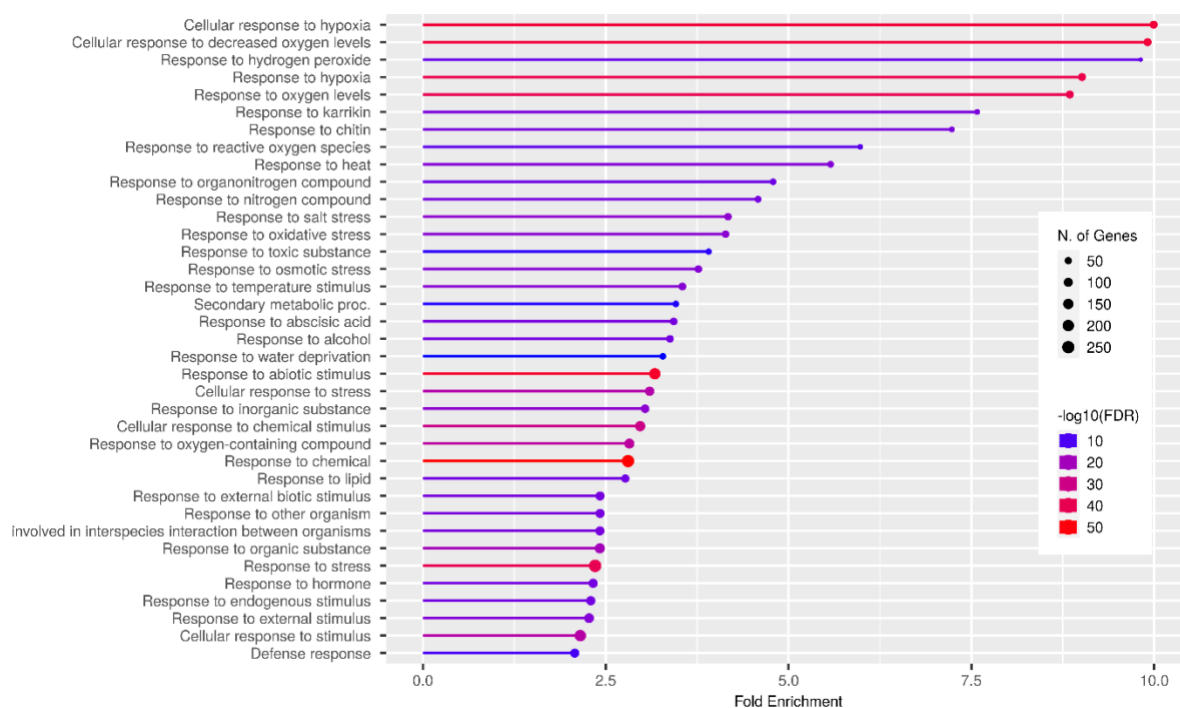
**Figure S6** - Chart Gene Ontology classification of Biological Processes of 6 hours of imbibition down regulated interaction DEGs selected by False Discovery Rate (FDR cutoff of 0.05) and sort by Fold Enrichment.



**Figure S7** - Chart Gene Ontology classification of Biological Processes of 6 hours of imbibition up regulated interaction DEGs selected by False Discovery Rate (FDR cutoff of 0.05) and sort by Fold Enrichment.



**Figure S8** - Chart Gene Ontology classification of Biological Processes of 16 hours of imbibition down regulated interaction DEGs selected by False Discovery Rate (FDR cutoff of 0.05) and sort by Fold Enrichment.



**Figure S9** - Chart Gene Ontology classification of Biological Processes of 16 hours of imbibition up regulated interaction DEGs selected by False Discovery Rate (FDR cutoff of 0.05) and sort by Fold Enrichment.





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# Scientific production

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## Scientific production

### Publications

Release of Arabidopsis seed dormancy by cold ambient air plasma relies on cytoplasmic glass transition, J. August, T. Dufour, C. Bailly, *Plants*, 2022 (Submitted).

Interactions between Arabidopsis seeds and cold ambient air plasma: deciphering the role of seed water content, sorption sites and bioelectrical parameters, J. August, T. Dufour, C. Bailly, 2022 (To be submitted).

Deciphering the biological mechanisms and morphological changes induced by cold ambient air plasma to release Arabidopsis seed dormancy, J. August, N. Chaumont, J. Perrin, T. Weitkamp, C. Paysant-Le-Roux, M.L. Martin, J. Caïus, C. Bailly, T. Dufour, 2022 (To be submitted).

### Conferences

9<sup>th</sup> Central European Symposium on Plasma Chemistry (CESPC9) + COST Action CA19110 Plasma Applications for Smart and Sustainable Agriculture (PIAgri), September 4-9, 2022, Vysoké Tatry, Slovakia. From anhydrobiosis to germination processes: effect of a cold ambient air plasma treatment on Arabidopsis seed dormancy. J. August, T. Dufour, C. Bailly - Oral - **Young scientist prize for the best presentation.**

## Scientific Production

Advances in Seed Science Research at Department of Seed Science and Technology, University of Agricultural Sciences, Bangalore, India on 9<sup>th</sup> August, 2022. Cold plasma Production and Applications in Seed Science. J. August – Invited Speaker.

Séminaire BIS – Laboratoire de Biologie du Développement – Institut de Biologie Paris-Seine. 5 avril 2022. Investigating the action mechanisms of cold atmospheric plasmas on seeds anhydrobiosis. J. August, C. Bailly, T. Dufour.

Congrès Graines 2021 – Colloque Graines du Réseau Français de Biologie des Graines. Visioconference, France, October 25th-27th, 2021. Rôle de la transition vitreuse dans l'efficacité des traitements au plasma froid sur la levée de dormance des graines d'Arabidopsis. J. August, C. Bailly, T. Dufour – Oral et poster – **Prix poster** attribué par le conseil scientifique du réseau français de biologie des graines.

International Workshop on Plasma Agriculture IWOPA, March 1-3, 2021, Greenwald, Germany. Release of Arabidopsis thaliana seed dormancy by air dielectric barrier discharges. J. August, T. Dufour, C. Bailly - Poster.

HAPPYBIO 2020 – Workshop in plasmas for biology, Edition 2. Orsay, France, November 12th-13th, 2020 Levée de dormance d'Arabidopsis Thaliana par décharge à barrière diélectrique dans l'air à pression atmosphérique. J. August, C. Bailly, T. Dufour – Oral.

## Patent

T. Dufour, J. August, C. Bailly. France Brevet numéro FR2109995



## **Étude des effets d'un plasma froid d'air ambiant sur la physiologie des graines d'*Arabidopsis***

Les plasmas froids d'air ambiant (PFA2) sont des gaz faiblement ionisés composés d'espèces réactives de l'oxygène et de l'azote, d'électrons libres énergétiques, d'espèces métastables et de photons dans les domaines spectraux UV et visible. En raison de leurs propriétés électriques, thermiques, radiatives et chimiques, ces procédés apparaissent pertinents pour améliorer les propriétés germinatives des semences. De plus, les plasmas froids sont une solution technologique prometteuse pour remplacer les produits agrochimiques et faire face aux problèmes de l'agriculture moderne. Les études menées pendant la thèse ont permis de comprendre les mécanismes d'actions des PFA2 sur la physiologie des semences d'une espèce modèle en biologie : *Arabidopsis thaliana*. Une première étude a été menée pour comprendre comment la teneur en eau peut modifier les propriétés électriques des graines et leur interaction avec les propriétés du plasma froid. Puis, l'observation et l'analyse des modifications physiologiques des semences traitées par PFA2 ont permis (i) d'identifier les conditions biologiques et physiques optimales à la levée de dormance des semences et (ii) de lier ce phénomène à la transition vitreuse cytoplasmique des tissus de la graine. Enfin, pour comprendre les effets du plasma sur les propriétés germinatives des semences, les processus biologiques déclenchés après exposition au PFA2 ont été analysés de l'état sec à la phase d'imbibition des graines menant à la germination. Le rôle des réactions oxydatives, des changements anatomiques (évalués par tomographie), et de la régulation de l'expression des gènes ont été mis en évidence. Les résultats obtenus ont été corrélés et discutés afin d'explicitier les mécanismes régissant la levée de dormance et le changement d'état physiologique des graines d'*Arabidopsis* par le traitement au PFA2.

**Mots-clés : plasma froid, semences, Arabidopsis, physiologie, dormance, transition vitreuse, réactions oxydatives, tomographie, transcriptomique.**

### **Investigating the effects of cold ambient air plasma on *Arabidopsis* seed physiology**

Cold ambient air plasmas (C2APs) are weakly ionised gases composed of reactive oxygen and nitrogen species, energetic free electrons, metastable species and photons in the UV and visible spectral ranges. Due to their electrical, thermal, radiative and chemical properties, these processes appear to be relevant for improving the germination properties of seeds. Furthermore, cold plasmas are a promising technological solution that can be an alternative to agrochemicals to face modern agriculture issues. The studies carried out during the thesis consisted in understanding the mechanisms of action of C2APs on the seed physiology of a model species in biology: *Arabidopsis thaliana*. A first study was carried out to understand how water content can modify the electrical properties of seeds and their interaction with the properties of cold plasma. The observation and analysis of the physiological modifications of C2APs-treated seeds allowed (i) to identify the optimal biological and physical conditions of seed dormancy release and (ii) to link this phenomenon to the cytoplasmic glass transition within seed tissues. Finally, to understand the effects of plasma on the germinative properties of seeds, the biological processes triggered after exposure to C2APs were analysed from the dry state to the seed imbibition phase leading to germination. The role of oxidative reactions, anatomical changes (studied by tomography), and regulation of gene expression were demonstrated. The results obtained were correlated and discussed in order to highlight the mechanisms governing the dormancy breaking and the change in physiological status of *Arabidopsis* seeds induced by C2APs treatment.

**Keywords: cold plasma, seeds, Arabidopsis, physiology, dormancy, glass transition, oxidative processes, tomography, transcriptomic.**