Investigating cold atmospheric plasmas as oxidizing agents of anhydrobiotic systems Application to seed biology

This Doctoral project is proposed by C. Bailly from Seed biology group (UMR 7622 SU CNRS, IBPS) (Team 1) and T. Dufour from Laboratory of Plasma Physics (Team 2). Lena Tarras, M2 student and currently in internship in the two laboratories will be supported as a candidate to the IPV program.

Scientific context and objectives of the study

Cold atmospheric plasmas (CAP) are weakly ionized gases composed of active species including electrons, ions, metastables, reactive oxygen and nitrogen species (ROS, RNS) as well as photons from UV and visible spectral ranges. CAP can be considered as gaseous media out of thermodynamic equilibrium where electrical, chemical, radiative, thermal and fluid-mechanical properties are combined. They can be used to modify the surface and in-depth properties of materials through etching, roughening or chemical functionalization. More recently, they have been investigated to respond to issues in Life Sciences, especially in an emerging research field called "plasma agriculture" where seed biology occupies a central position. Behind the "plasma seed" interaction lie fundamental issues of heterogeneous chemistry, diffusion of chemical reactive species.

Team 1 and Team 2 have been investigating the effects of CAP on seed biology for 6 years up-to-date (Judée et al., 2018; Dufour et al., 2021) . Together they have shown that CAP exposure can stimulate the germination of many seed species but also participate in the elimination of seed borne pathogens. This research has led to the innovation of a device that enables to treat the dry seeds in a plasma of ambient air. A patent has been deposited both on this device and on the related treatment method which combines plasma physical parameters with seed biological properties.

Dry seeds are anhydrobiotes, i.e. they can withstand severe desiccation that occurs at the end of their development on the mother plant. They can then survive into a state of suspended life in which their metabolism comes to a standstill. During dry storage, the low moisture content of seeds is associated with high cytoplasmic viscosity (i.e. glassy state) that prevents molecular mobility and normal metabolism. However, dramatic physiological changes occur at the same time including dormancy alleviation and loss of viability on a longer term. The works carried out in Team 1 have demonstrated that the key determinants of the physiological changes occurring upon anhydrobiosis are oxygen diffusion and subsequent oxidation mechanisms (Oracz et al., 2007; Bazin et al., 2011, Bailly, 2019). It has been shown that dry seed tissues progressively evolve towards a more oxidized status, suggesting that non-enzymatic reactions continuously generate ROS. However, the kinetics of these events is very slow, ranging from months for seed dormancy alleviation to several decades for seed ageing. These time constants dramatically limit the feasibility of experimental approaches, making the biology of anhydrobiotic systems far from being understood. J. August (3rd year PhD student in Teams 1 & 2) is currently investigating the molecular bases of CAP on seeds. He has demonstrated that CAP generates ROS within seed tissues which suggests that CAP can be used as an oxidizing agent of dry biological tissues. This preliminary work demonstrates that, at a fundamental level, CAP could be used as a cutting-edge technology to understand the mechanisms of oxidative signaling in anhydrobiotic biological systems, on a short time scale.

In addition, bacterial and fungi naturally live within seed tissues and can also survive in a dry resting state. Some of them can be beneficial for plant fitness (eg. fungi endophytes producing biostimulant compounds) while others (mostly bacteria) can cause diseases in seedlings and significantly reduce crop yields. CAP have been successfully applied to reduce seed borne pathogens; their biocidal effect relying on the plasma-generated reactive species (Adhikari et al., 2020). However these disinfection mechanisms remain largely un-elucidated, especially how the oxidative stress generated by CAP in the seed/pathogen microenvironments can destroy microbial cells rather than seed cells. It has also been shown that all the micro-organisms of a same microbiote do not show the same biological response after their exposure to CAP. This biological selectivity deserves to be bridged

with chemical selectivity of a plasma source and its ability to deliver a single type of reactive species, at a desired concentration and without generating other reactive side-products.
The objectives of the project are:

- **To innovate a chemically selective plasma source, producing targeted radicals or targeted reactive species.** Hence, the anhydrobiotic seeds will be exposed to different types of oxidizing atmospheres (ie. plasma exposures), each one being ruled by its own chemical pathways which relies on physical parameters (temperature, electrical charge per streamer). The PhD student will investigate the homogeneous chemistry reactions occurring in the plasma phase as well as the heterogeneous chemistry mechanisms taking place in the seeds and at the plasma/seed interface
- **To understand how an oxidative intracellular environment (generated by CAP) can modify seed biology in the dry state, and to identify the molecular targets of ROS.** The ability of cold plasma in enhancing the oxidation rate in the presence of oxygen opens novel fields of investigation for deciphering the cellular and molecular bases of the events occurring during storage of dry seeds, especially the events leading to dormancy alleviation and seed ageing. Here the PhD student will take advantage of CAP for controlling the time lapse necessary for dormancy release and loss of viability of dry seeds, leading to unique findings in the field of biology of dry systems.
- **To decipher how plasma-generated ROS can alter microbial diversity within seeds.** The doctoral student will investigate the nature of the oxidative mechanisms underlying microbial inactivation and will establish whether CAP has similar effects whatever the microbial population. At a practical level, the interaction of plasma-generated ROS with seeds and microbial cells and their microenvironment should be established to allow the development of this technology at a larger scale.

Workplan and Methodologies

1. Innovating a versatile and chemically-selective plasma source

The solicited PhD student will innovate a plasma process that can selectively generate reactive species or radicals. Hence, the process will allow the production of either ozone, or atomic oxygen, or NO radicals, or OH radicals without generating other active species considered here as by-products. For this, the doctoral student will have to modify the gases and vapors feeding the cold plasma source (helium with/without O_2,, N_2, CO_2 and H_2O) but also study the influence of physical parameters that rule the plasma chemical pathways, especially the features of the applied voltage (magnitude, sinusoidal/pulse profile, frequency), electrical charge of the streamers, plasma temperature and plasma energy. The biological effects of each of the plasma processes will be evaluated as detailed in sections (2), (3) and (4).

Electrical, spectral and chemical properties of plasma will be investigated. The active species will be identified by combining optical emission spectroscopy (OES) with mass spectrometry (MS). Using MS, spatial profiles of the main reactive species, positive/negative ions will be determined, while the chemical and kinetic mechanisms will be identified and quantified, comparing two mass spectrometry operating modes: conventional ionization (70eV) and soft ionization (electron attachment). Using OES, physical parameters of the plasma will be measured, in particular the electronic temperature, the gas temperature, the electron density (broadening of the H_β line) and the electric field (deconvolution of He line at 492.8 nm). The values of these physical parameters will be used to calculate the rate constants of the chemical reactions providing the reactive species.

2. Plasma-induced heterogeneous chemistry at the surface and core of seeds

To bridge chemical reactivity of cold plasma with oxidative signaling mechanisms, seeds will be exposed to a cold plasma of helium mixed with an isotopic gas ($^{18}O_2$ at M=36 g/mol or $^{15}N_2$ at M=30 g/mol). The resulting isotopic reactive species will be monitored within the plasma phase and the seeds, whether in surface (seed coat) or in volume (cotyledons, embryonic axis). For this, the isotopic reactive species will be measured combining mass spectrometry (gaseous phase) and secondary ion

mass spectrometry (applied on seed coat and embryo separated). Hence, it will be possible to verify whether the plasma-generated isotopic ROS are the same as those functionalizing the outer coating of the seeds and if they have also diffused within the seeds core. This will allow the PhD student to verify whether the ROS involved in oxidative signaling and decontamination have a direct "plasma" origin and/or can be transformed into other stabilized species functionalizing the seed intercellular spaces.

3. Oxidative signaling in anhydrobiotic systems

By controlling the chemo-selectivity of the plasma source as well as its physical parameters (temperature, energy, density of streamers or ionization waves) (see 1), the doctoral student will treat dormant *Arabidopsis thaliana* seeds and will characterize the plasma effects on both dormancy alleviation (short treatment) and longevity (longer treatment). ROS content within dry seeds and during their germination will be determined, using biochemical and cell imaging approaches. In particular, the PhD student will develop microfluidic hydroponic chips for microscopic study of *Arabidopsis thaliana* seeds which will allow monitoring of real-time ROS production with the use of genetically modified seed lines expressing fluorescent ROS nanosensitive probes.

The oxidative modifications generated by CAP will also be studied, especially the oxidative modifications of mRNAs since Team 1 has previously shown the involvement of this mechanism in seed after-ripening (Bazin et al., 2011). Here, Nanopore technology will be developed in collaboration with ArtBio Platform (IBPS) in order to design novel tools that will allow direct identification (eg. guanine oxidation to 8-oxoguanine) of oxidized mRNAs extracted from seeds. In addition, we will determine whether ROS can directly alter chromatin status in the dry state and thus modify gene expression during subsequent imbibition. Indeed, we have recently demonstrated that ROS could modify chromatin compaction through an unknown mechanism in Arabidopsis seeds (Jurdak et al., 2022). Cytogenetic studies, RNAseq approaches and targeted studies of oxidative modifications of histones will address this point.

4. CAP effects on seed microbiote

First, the effect of CAP on seed microbiote will be assessed using a "without a priori" approach on various ecotypes of Arabidopsis seeds (Col, Ler and Cvi). DNA metabarcoding will be performed to describe the microbiote of each ecotype after a CAP treatment relying on the selective production of O_3, O, NO or OH species (designed in 1). Second, the PhD student will carry out a targeted study through the use of several pathosystems such as Arabidopsis/Alternaria, Tomato/Xanthomonas and Sunflower/Fusarium. Effect of plasma on seed microbiote – especially DNA damage – will be assessed by qRT-PCR, using known molecular markers of the pathogens as well as conventional methods. Plant pathogenic microbiotes (bacteria and fungi, originated from international collections of micro-organisms, eg. https://www6.inrae.fr/cirm/) will be also directly treated by CAP. Then, they will be viewed by confocal microscopy in order to assess the integrity of the cellular membranes. Thanks to the ability of the plasma source to selectively produce O_3, O, NO or OH species at different concentrations (see 1), microbial tolerance/resistance mechanisms will be characterized.

References

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