The oxidative modifications in the macromolecules of sunflower seed during germination

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The oxidative modifications in the macromolecules of sunflower seed during germination

Les modifications oxydatives des macromolécules dans la graine de tournesol au cours de la germination

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List of abbreviations

ABA	Abscisic Acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACN	Acetonitrile
Ado-Met	S-adenosyl methionine
BSA	Bovine serum albumin
CBS	Cystathionine β-synthase
CLD	Cyclophilin-like domain
CNBr	Cyanogen bromide
COFRADIC	Combined fractional diagonal chromatography
DCPIP	2,6-Dichlorophenolindophenol
DHA	Dehydroascorbate
DTNB	Ellman's Reagent, 5,5'-Dithiobis-(2-Nitrobenzoic Acid)
DTT	Dithiothreitol
ET	Ethylene
FA	Formic acid
FW	Fresh weight
GSH	Glutathione SH
GSSG	Glutathione disulfide
G6PDH	Glucose-6-phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
HCN	Hydrogen cyanide
HG	Homogalacturonan
His	Histidine
•OH	Hydroxyl radicals

HOCl	Hypochlorous acid
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
Met	Methionine
Met-SO	Methionine sulfoxide
MPD	Morphophysiological dormancy
mRNA	Messenger ribonucleic acid
MSR	Methionine sulfoxide reductases
MV	Methylviologen
PAC	Paclobutrazol
PBS	Phosphate buffered-saline
PCA	Principal Component Analysis
PD	Physiological dormancy
PM	Plasma membrane
PME	Pectin methyl-esterase
PMEI	Pectin methyl-esterase inhibitor proteins
PPP	Pentose phosphate pathway
RP-HPLC	Reversed phase- high performance liquid chromatography
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TCA	Tricaboxylic acid
XEH	Xyloglucan endohydrolase
XET	Xyloglucan endotransglucosylase
XGA	Xylogalacturonan
XTH	Xyloglucan endotransglcosylase/hydrolase

Chapter 1 Introduction

Chapter 1 Introduction

In higher plants, the new individuals are generated by sexual reproduction. The physiological and biochemical features of the seed containing the embryo as the new plant in miniature, determine the subsequent plant survival. Thus, the seeds act a critical role in the plant life cycle by enabling the dispersal and survival of the species. In nature, most organisms live in unpredictable environments and typically experience conditions that are suboptimal for growth and reproduction. A very common response for some organisms to environmental stresses is to enter a reversible state of reduced metabolic activity, quiescence or dormancy. By doing so, these organisms can drastically lower their energetic expenditures and evade unfavourable conditions that would otherwise reduce the fitness of the population. Moreover, even plants have evolved many ways to disperse their offspring by dispersing their seeds, unlike animals, they are limited in their ability to seek out favourable conditions for survival and growth. Seed dormancy is an adaptive trait that enables seeds to overcome periods that are unfavourable for seedling establishments. It is defined as the inability of seeds to germinate under apparently favourable conditions so that to ensure that seed can germinate at the most appropriate moment (Finch-Savage and Leubner-Metzger, 2006; Bentsink and Koornneef, 2008). Indeed, the lack of dormancy at harvest in many cultivars of wheat results in the adverse effects of pre-harvest-sprouted wheat grains on end-product quality and led to serious worldwide economic losses (Bewley and Black 1994; Gubler et al., 2005; Wang et al., 2015). Therefore, seed dormancy is important for plant ecology and agriculture. Dormancy is induced during seed development and is maintained during the late maturation phase in most of orthodox seeds, which prevents pre-harvest sprouting. The transition from seed dormancy to germination is a complex process, it is influenced by some internal factors and multiple environmental factors such as light, temperature and humidity (Finkelstein et al., 2008). Seed dormancy release is a very fascinating process that consists in a dramatic change in seed physiology. Many authors have underlined the role of Reactive Oxygen Species (ROS) in this process (Bailly, 2004a; El-Maarouf-Bouteau et al., 2007, 2015; Su et al., 2016). Hence, dormancy alleviation involves some oxidative changes underlined by the action of ROS which are produced from embryogenesis to germination. ROS accumulation can be beneficial for seed germination and seedling growth by regulating cellular growth, ensuring a protection against pathogens, controlling the cell redox status, cell wall loosening, testa and endosperm weakening, as well as protein oxidation thus playing a signaling role in seed dormancy alleviation (El-Maarouf-Bouteau et al., 2007; Su et al., 2016).

I. Seed Development and Composition

Seed development in higher plants begins with a double fertilization process that occurs within the ovule and ends with a maturated seed primed to become the next plant generation (Goldberg et al., 1994). The major events that occur during seed development are shown in figure 1. Embryo development can be divided up into two phases, the first or “embryogenesis” involves cell divisions associated with morphogenetic events which form the basic cellular pattern for the development of the shoot-root organs and the primary tissue layers, and it also programs the regions of meristematic tissue formation. Following the cell division arrest at the end of the embryo growth phase, the seed enters the second phase, which is called “maturation phase”, this process involves cell growth and the storage of reserves, such as proteins, starch and oils, required as 'food and energy supply' during germination and seedling growth (Goldberg et al., 1994; Holdsworth et al., 2008). The seeds enter then, into a metabolically quiescent state related to dehydration after dry maturation, which represents the normal terminal event in the development process of orthodox seeds. Genetic studies in Arabidopsis have identified genes that provide new insight into molecular event underlying plant development (Le et al., 2010). Dormancy process is initiated during seed maturation and reached a maximum in harvest-ripe seeds (Karssen et al., 1983; Ooms et al., 1993; Raz et al., 2001). Seeds may remain in this dry dormant state from several days to many years and still retain their viability.

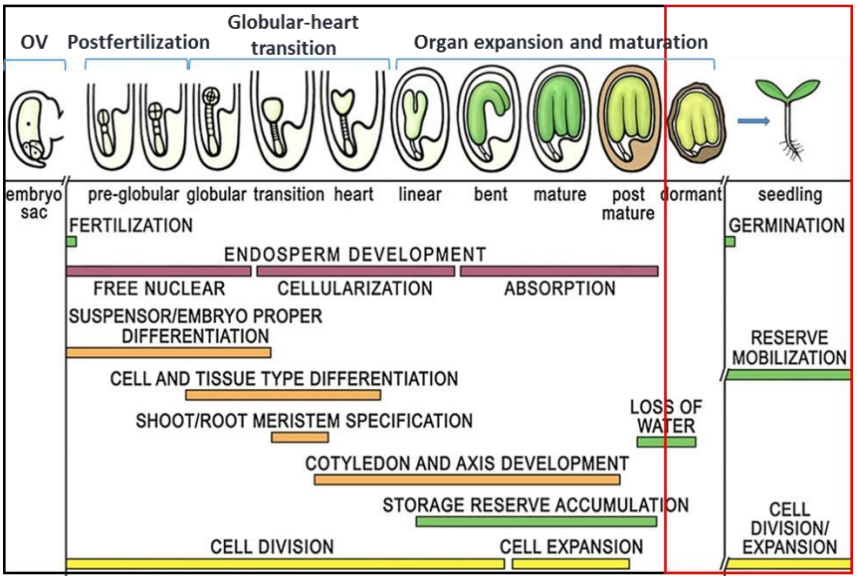

Fig. 1. A generalized overview of seed development and stages of the life cycle. OV, unfertilized ovule; Postfertilization, also called proembryo, this stage involved in terminal and basal cell differentiation, formation of suspensor and embryo proper; Globular-heart transition, differentiation of major tissue-type primordia at this stage,

including establishment of radial (tissue-type) axis, visible appearance of shoot-root, hypocotyl-radicle development and differentiation of root meristem; Organ expansion and maturation, enlargement of cotyledons and axis by cell division and expansion before seed maturation, and following seed maturation storage reserves such as RNA, proteins and lipids are accumulated, and then after maturation involve in loss of water (dehydration) and dormancy status formed to inhibit precocious germination. Diagram adapted from Le et al. (2010), developmental events were modified from Goldberg et al. (1994).

II. Dormancy and Control of Germination

Baskin and Baskin (2004) have summarized the hierarchical system of classification for seed dormancy. The system has divided dormancy into five classes: (1) is physiological dormancy (PD), which contains three depth levels: deep, intermediate and non-deep. Depending on the PD depth level, dormancy can be released by different stratification treatments (imbibition at low or warm temperatures) or GA treatment; (2) morphological dormancy (MD), which caused by a delay of embryo development; (3) morphophysiological dormancy (MPD), it is a combinational dormancy (PD + MD); (4) physical dormancy (PY) is due to the existing of water-impermeable layers of palisade cells in the seed coat and it also can be released by mechanical or chemical scarification; and (5) combinational dormancy (PY + PD). However, inside all of these classifications, PD (non-deep level) is the most common kind of dormancy because it occurs in part of gymnosperms and in all major clades of angiosperms, and depending on the species, the dormancy alleviation correlated with stratification, a period of dry storage (after-ripening) or gibberellins (GAs) treatment. In the last decade, the works by Finch-Savage and Leubner-Metzger (2006) and Cadman et al. (2006) have provided insight into the molecular mechanisms of non-deep PD. Two terms of physiological seed dormancy are distinguished; the intrinsic molecular mechanisms determined by seed components, namely embryo and coat dormancy. The latter corresponds to the case when the intact seed is dormant but the isolated embryo can germinate normally, therefore, the seed coat enclosing the embryo exerts a constraint that the embryo cannot overcome. By contrast, embryo dormancy is characterized by the inability of the embryo itself to germinate normally after removal of the seed coat. Sunflower seeds are characterized by an embryo covered by seed-coats and a pericarp easily removable, this can help to deeply investigate the mechanism of embryo dormancy and eliminate the influence of coat dormancy.

Control of seed germination and growth is crucial to the survival of the next generation, there are several critical determinants for the transitions from dormancy to germination and from germination to growth (Fig. 2). At dispersal, the mature seed, when it encounters favourable environmental conditions, which can include light of a given wavelength, sufficient water availability, optimal temperatures, and adequate oxygen, releases dormancy and commences germination. Visible evidence of the completion of germination is usually radicle protrusion and elongation. Some seeds must be exposed to environmental cues, such as periods of warmdry conditions (after-ripening), moist chilling, or even smoke, to release dormancy (Stephen et al., 1986; Egerton-Warburton, 1998) (Fig. 2). The dormancy status reduces during afterripening, consequently, seeds are able to complete germination in wide range of environmental conditions. After-ripening largely depends on environmental conditions during seed storage and germination conditions (Donohue et al., 2005), while seed covering structures, moisture and temperature are the main factors to decide the speed of after-ripening (Manz et al., 2005). However, these primary dormant seeds may enter into a second stage of dormancy, called secondary dormancy, during imbibition if the environmental conditions are unfavourable for germination (Fig. 2, Bewley and Black, 1994; Kermode, 2005). Secondary dormancy is a safety mechanism that is implemented from the seed being exposed to adverse conditions once it falls from plant and function in imbibed seeds, it can be induced by anachronistic environmental conditions such as too low or high temperature, hypoxia and prolonged exposure to darkness or light (Khan and Karssen, 1980; Leymarie et al., 2008; Hoang et al., 2013 a, b, 2014).

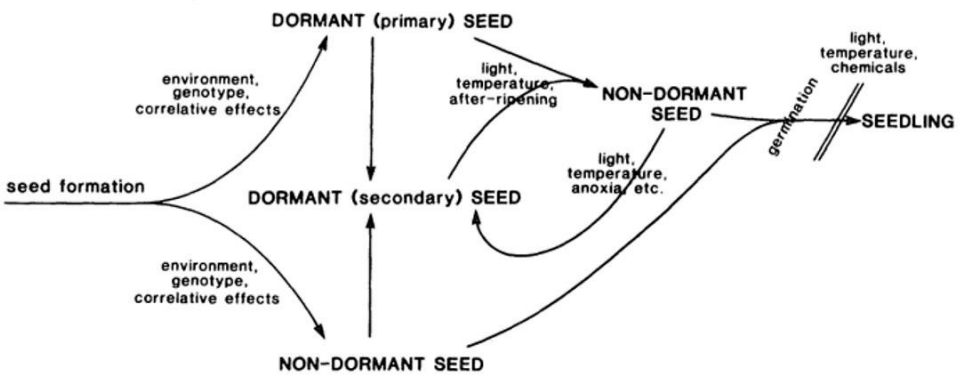

Fig. 2. Relationship between dormancy and germination. The control of germination exists at the state of seed dormancy and the operation of environmental factors such as light, temperature and air humidity effect both dormancy and germination. The diagram is from Bewley and Black (1985).

A number of high-throughput, large scale-omics studies have been investigated to gain insight into molecular networks of seed dormancy and germination. Bentsink and Koornneef (2008) have presented the first high-throughput, large scale-omics studies investigating seed dormancy and germination, including transcriptomics, proteomics and metabolomics. Several transcriptomic analyses have provided gene expression profiles underlying dormancy-related differential gene expression (Gao et al., 2012; Dekkers et al., 2016). These new approaches and derived data sets provide an unprecedented level of detail concerning genome expression associated with germination potential, which suggest that RNA translation or post-translation are the major levels of control for germination completion, by contrast, transcriptome changes might reflect alteration in dormancy status or enhancement of germination vigor and effects on post-germination functions that relate to seedling growth. Indeed, Layat et al. (2014) and Basbouss-Serhal et al. (2015) have shown the importance of translational regulation in the control of this process. The absence of correlation between transcriptome and translatome reported suggests that translation regulation and consequently proteome change should be considered more than transcriptional regulation. Thus, proteomics is an increasingly important tool for the study of several plant functions because it allows the investigation of the underlying molecular processes in plant physiology (Trindade et al., 2018). Proteomic change investigated in dormant and non-dormant seeds pointed out the importance of metabolism, energy production, protein metabolism, cell growth and defense classes (Pawlowski and Staszak, 2016; Xia et al., 2018a). Post-translational modifications (PTMs) represent another level of regulation in seeds especially phosphorylation, carbonylation and thiol oxidation (see below). Metabolomics represent the last branch of high throughput functional genomics that allowed measuring biochemical activity directly by monitoring the substrates and products converted by cell metabolism (Saito and Matsuda, 2010). Metabolomics is not yet actively investigated in seeds. During Arabidopsis seed stratification, Fait et al. (2006) have shown the importance of the groups of storage proteins, stress response and detoxification in dormant state and energy, amino acid metabolism, folding and mRNA and protein metabolism in non-dormant state. The comparison between two wheat cultivars with contrasting dormancy status revealed several raffinose family oligosaccharides as key markers (Das et al., 2017). Moreover, in sunflower seeds, Xia et al. (2018b) showed that among more than 100 metabolites quantified, only sugars present significant change between dormant and non-dormant seeds. These results highlight the importance of energy and metabolism regulation in non-dormant state. Beyond the importance

of the level of regulation, the elements brought specially by the proteome and metabolome underlined the importance of central metabolism. Targeted enzyme activity can provide integrated information about gene expression and PTMs. Recently, Xia et al. (2018a) have highlighted PTMs regulation of enzymes involved in central metabolism in dormancy alleviation in sunflower by combining proteomic analysis and enzymatic profiling. In sum, multiple tevel of regulation has been shown in dormancy alleviation and germination processes.

III. Cellular Events During Germination

The dehydrated state of mature seed helps to withstand drought and extreme temperatures. Germination begins with water uptake by the dry seed during imbibition and ends with the embryonic axis or radicle elongation (Finch-Savage and Leubner-Metzger, 2006). During this process, a sequence of cellular events is initiated following seed water uptake which ultimately leads to emergence of the radicle and complete germination successfully. Metabolism commences in the seeds as soon as their cells are hydrated. Respiration and protein synthesis have been recorded within minutes of imbibition, using components conserved in the dry seed (Black et al., 2006; Galland et al., 2014). This is followed by synthesis of RNA, and DNA repair and synthesis. Numerous enzymes are either activated or *de novo* synthesized during germination, including lipases, proteinases, phosphatases, hydrolases, calmodulin, carboxypeptidases and others that appear to be particularly associated with this process (Mayer and Poljakoff-Mayber, 1982; Bewley and Black, 1985; Cocucci and Negrini, 1991; Washio and Ishikawa, 1994).

III.1. Seed Water Uptake

Water uptake by a mature dry seed has been defined as triphasic (Fig. 3). Phase I is a rapid initial uptake, imbibition is probably very fast into the peripheral cells of the seed and small tissues such as radicle. Metabolism can be activated from this phase within minutes of imbibition. The phase I is followed by a plateau (phase II), also called germination *sensu stricto*, both of dormant and non-dormant seeds are metabolically active during this phase, and for nondormant seeds, the major metabolic events that take place at this time is the preparation for radicle emergence. A further increase in water uptake in the phase III occurs only after radicle elongation. The dormant seeds are blocked in entering this phase because they cannot complete

the germination process (Bewley, 1997a). The duration of each of these phases depends on several factors, including inherent properties of the seed such as seed size, seed coat permeability and genotypes, and also some environment factors such as temperature, and the moisture content and composition of medium (Bewley and Black, 1985).

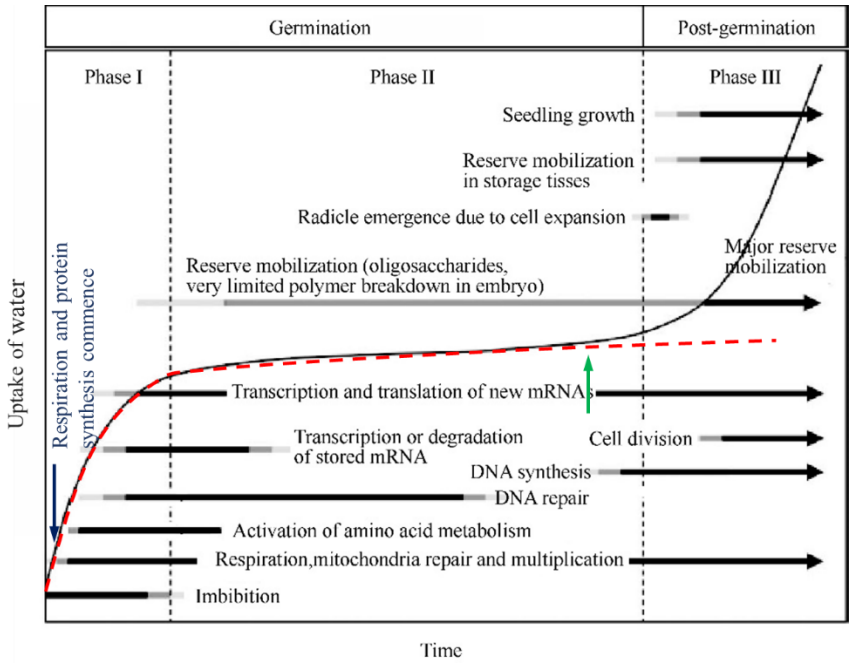

Fig. 3. Triphasic pattern of water uptake and time course of major events involved in germination and subsequent post germination. The time for events to be completed varies from several hours to many weeks, depending on the plant species and germination conditions. Red dot line represents imbibition curve of seed that cannot complete germination and did not enter into Phase III. Adapted from Bewley (1997a) and Wang et al. (2015).

Before imbibition, dry seeds have a low moisture content, 5 to 12%, depending on the species. This low moisture content contributes to a remarkably low rate of metabolism. Marked changes in metabolism occur as soon as the seed imbibes (Copeland and McDonald, 2001). As the dry seed starts to take up water during phase I, influx of water into the cells lead to temporary structural perturbations, especially on membranes accompanied by a massive leakage of cellular solutes and low molecular weight metabolites including ions, sugars and amino acid into the surrounding imbibition solution (Powell and Matthews, 1978). This is a transition symptomatic of the membrane phospholipid components from the gel phase achieved by drying during seed maturation, to the normal and hydrated liquid-crystalline state (Crowe and Crowe, 1992). However, in order to deal with the damage imposed during dehydration, storage and rehydration, seeds activate a series of repair mechanisms during imbibition (Fig. 3), which

include membrane repair and therefore the membranes return to more stable configuration and solute leakage is curtailed within a short time of rehydration. Dry seeds contain mRNAs stored during maturation, also called long-lived transcripts to indicate that they survived desiccation (Rajjou et al., 2004). Over 10 000 different stored mRNAs have been identified in transcriptome analyses of Arabidopsis (Nakabayashi et al., 2005; Kimura and Nambara, 2010; Okamoto et al., 2010). Similar numbers were found in barley and rice (Howell et al., 2009; Sreenivasulu et al., 2008). More transcripts (about 32 000) have been shown in sunflower (Meimoun et al., 2014). The dry seed transcriptome mirrors the process of seed maturation as well as prepares the seed for the following germination (Weitbrecht et al., 2011). As showed by Kimura and Nambara. (2010), the major portions of the dry seed transcriptomes are very similar between seeds of non-dormant Col and dormant Cvi Arabidopsis accession, and the majority of stored mRNAs are of the LEA (late embryogenesis abundant) group or transcripts of storage proteins. Meimoun et al. (2014) brought the evidence that there is no significant difference between D and ND at dry state in sunflower seeds. Hence, mature seeds contain ready to use mRNA for cell functioning upon imbibition, the difference between D and ND might be due to change mRNA and or protein quality and/or environment such as redox regulation that fine-tuned gene expression or protein activity.

III.2. Respiration

Upon imbibition, the rise in water content induces a parallel rise in the rate of metabolism including respiration and O_2 consumption. One of the first changes upon imbibition is the resumption of respiratory activity, which can be detected within minutes. Three respiration pathways operate in a seed during germination: the glycolysis, the pentose phosphate pathway (PPP) and the tricarboxylic acid cycle (TCA cycle or Krebs's cycle). They produce key intermediates in metabolism and energy in the form of adenosine triphosphate (ATP), and reducing power in the form of reduced pyridine nucleotides, the nicotine adenine dinucleotides (NADH and NADPH) (Côme and Corbineau, 1989; Black et al., 2006). Both of the glycolytic and PPP are restored during the phase I. Enzymes of the TCA cycle and the terminal oxidases are usually present in the dry seed and become activated or are resynthesized when oxygen is high enough in internal structures (Nicolas and Aldasoro, 1979; Salon et al., 1988). Germinating seeds frequently produce ethanol in many species (Morohashi and Shimokoriyama, 1972). This is often the result of an internal deficiency in oxygen that is caused

by restrictions to gaseous diffusion by the structures that surround the seed and by the dense internal structure of most seeds. This oxygen deficiency may result in more pyruvate production than used for activities of the TCA cycle and electron transport chain. Application of inhibitors of respiration can break dormancy in several kinds of seeds including sunflower, lettuce, rice and barley (Côme and Corbineau, 1989; Bewley and Black, 1994). It has been demonstrated that cyanide, which can inhibit terminal oxidation and the TCA cycle in the mitochondria, the glycolysis inhibitor, fluoride, and electron acceptors such as nitrate, nitrite and methylene blue can break seed dormancy of sunflower and Arabidopsis (Oracz et al., 2007; Arc et al., 2013). Consequently, the concept of the pentose phosphate pathway playing a unique role in dormancy breaking arises largely from these studies. Thus, the system is established as dormant seeds are deficient in an alternative oxygen-requiring process essential for germination, which is depleted of oxygen because of its lower affinity for this gas than the cytochrome pathway of respiration, so inhibitors of glycolysis, TCA cycle and terminal oxidation reactions of the mitochondrial electron transport chain can broke dormancy by activating pentose phosphate pathway, suggested as the alternative oxygen-requiring process essential for germination (Fig. 4).

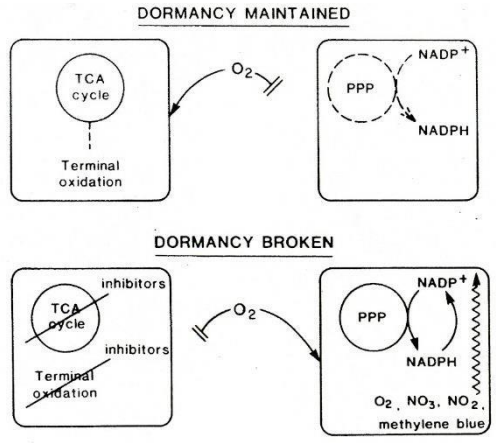

Fig. 4. The pentose phosphate pathway and dormancy breakage. The consumption of oxygen by conventional respiration is blocked by the inhibitors, and oxygen then become available for other processes, meanwhile, the pentose phosphate pathway requires oxygen for the oxidation of reduced NADP (NADPH). From Bewley and Black (1985).

In a recent enzymatic study, Xia et al. (2018a) have provided the evidence that temperature can also promote dormancy break by decreasing glycolysis and TCA cycle activities and increasing sucrose metabolism in sunflower seeds. Indeed, the activity of the respiratory enzymes related to TCA cycle (Aconitase, MDH) and glycolysis (Aldolase, enolase,

PGK, GAPDH) decreased in phase I of germination *sensu stricto* and subsequently an increase in the enzyme related to sucrose metabolism (UGPase) was found. Consequently, one can hypothetize that such enzymes are the target of dormancy release regulatory events probably related to PTMs processes which remain however to be characterized in this process.

III.3. RNA and Protein Synthesis

Enzyme activation begins during Phases I and II of imbibition. Phase II which can vary widely in duration, is characterized by a stabilized water and oxygen uptake. The seed undergoes many processes essential for germination following Phase II of water uptake, including a sequential translation of mRNA related with antioxidant mechanisms, cell detoxification, protein fate, energy, and amino acids metabolism occurs (Copeland and McDonald, 2001; Galland and Rajjou, 2015). Proteomic approaches unveiled the main importance of protein synthesis during seed imbibition in order to meet the increasing demand for proteins for seedling growth (Rajjou et al., 2004; Kimura and Nambara, 2010, Galland et al., 2014). Dry mature seeds contain a large number of mRNA species, supposed to be ready for protein synthesis upon imbibition. Transcription inhibitor did not prevent seed germination suggesting that transcription is not strictly requested for seed germination (Rajjou et al., 2004). Previous studies have demonstrated that sunflower seed dormancy release by after-ripening is associated with oxidation of specific subsets of stored mRNAs and differential accumulation of polysome-associated mRNAs but not related to transcriptomic changes, the translatome differs between germinating and nongerminating sunflower embryos (Bazin et al., 2011; Meimoun et al., 2014; Layat et al., 2014). In agreement with this, Basbouss-Serhal et al. (2015) have shown that there is no correlation between transcriptome and translatome in Arabidopsis, and that germination regulation is translational implying a selective and dynamic recruitment of messenger RNAs to polysomes in both dormant and non-dormant seeds. Hence, translation machinery components are highly represented in transcriptomic and proteomic studies. By contrast, seed proteome appears quite unique and diverse when compared to other plant developmental stages. Seed proteins encompass several functional classes from primary and secondary metabolism to structural and antimicrobial defence. In a proteomic study on *Arabidopsis thaliana* seeds, Galland et al. (2014) have underlined the importance of neosynthesis of some specific proteins during seed germination *sensu stricto* by selective mRNA translation as a major regulatory mechanism for the sake of radicle protrusion and

germination completion. They have shown that during phase I, stored mRNAs are transalted and a restart of the late seed developmental program as exemplified by the synthesis of seed storage proteins is in action (i.e. cruciferin and late embryogenesis abundant proteins). The transition from phase I to phase II was seen to be characterized by the action of important remodeling/repair (i.e. rotamase cyclophilin), antioxidant (i.e. monodehydroascorbate reductase), and detoxification mechanisms (i.e. mercaptopyruvate sulfurtransferase 1). As for phase II, the combined action of the proteasome (20S proteasome subunit) and peptidases (peptidase S8/S53, tripeptidyl peptidase II) is active for the degradation of seed storage and other proteins in order to fuel amino-acid-incorporating metabolism (glutamine synthetase 1.3). Entering to phase III of water uptake, proteins involved in seedling establishment are neosynthetized in preparation for the seedling growth (i.e. actin and tubulin). Therefore, it can be assumed that mRNA translation and protein post translational modifications constitute the main levels of control for germination completion. These processes are highly regulated in plants and represent rapid and efficient way to cope with environmental variations (Galland and Rajjou, 2015).

III.4. Cell Wall Modification and Cell Elongation

Cell growth which is regarded as an irreversible increase in cell volume can occur through two processes: by expansion (increase in cell size in two or three dimensions) or by elongation (expansion which is constrained preferentially to one dimension). During elongation or expansion, existing cell wall architecture must be modified to permit incorporation of new material, thus increasing the surface area of the cell and inducing water uptake by the protoplast (McCann and Roberts, 1994). Many conditions have to take part like the turgor pressure in the primary cell wall which is a prerequisite to drive cell expansion (Cosgrove, 1993), in addition to the extensibility of the cell architecture done by mechanisms operating for discrete biochemical loosening of the matrix to permit microfibril separation and insertion of newly synthesized polymers (McCann and Roberts, 1994; Hepler et al., 2013). The constraint on expansion needed for elongation rely in part on the presence of cellulose/xyloglucan network where possible rearrangements of microfibrils within and between layers require enzymes that act on the xyloglucans that cross-link them into the network. Small proteins, termed expansins, may displace xyloglucans from cellulose microfibrils by disrupting the hydrogen-bonding

between the xyloglucan backbone and the microfibril (Cosgrove, 1993, 2005). Moreover, McCann and Roberts (1994) have explained that cellulose microfibrils undertake changes in orientation during cell growth. Hence, unidirectional microfibrils are inserted into the wall by apposition at the plasma membrane, then previously deposited randomly-oriented microfibrils will gradually move to the outer layers of the wall and become progressively 'diluted' by oriented microfibrils. Thus, either the old layers of microfibrils will be integrated into newer layers or the microfibrils within the old layers will become separated so far that they do not contribute significantly to wall thickness. Pectin takes as well a big part in this process. Elongation is correlated with increased esterification in pectin and the cessation of elongation with de-esterification in many species like carrots (McCann and Roberts, 1994) maize (Kim and Carpita, 1992), tobacco (McCann et al., 1994) and Arabidopsis (Derbyshire et al., 2007; Peaucelle et al., 2015). Indeed, Derbyshire et al. (2007) have shown that a minimum level of about 60% of degree of esterification is required for normal cell elongation in Arabidopsis hypocotyls.

Moreover, the pectic network in the wall is replaced by newly-synthesized highly-esterified pectins, and older un- or de-esterified pectins are contributing to the increase in surface area of the middle lamella region. Hepler et al. (2013) suggested that newly secreted pectin becomes inserted into the wall matrix where it loosens the bonds between the existing pectate linkages causing a reduction in bulk viscosity. Thus, the newly secreted pectin would compete away Ca^{2+} from the existing matrix, further weakening the wall and allowing turgor-driven expansion. From the above mentioned processes, one can conclude that one part of the cell wall extensibility might be the synthesis of highly esterified pectins that may alter the rheology of the pectic network. Another part might be the coordinated secretion or activation of xyloglucan endotransglycosylase (XET) and expansins during elongation to permit rearrangement of the cellulose/xyloglucan network as new microfibrils and xyloglucans are inserted. Furthermore, Peaucelle et al. (2011) have explained that increased cell wall hydration may in turn facilitate the sliding of wall polymers or the mobility of wall-modifying agents (expansins, Xyloglucan endotransglcosylase/hydrolase, XTH) thus increase extensibility. Besides, Barnes and Anderson et al. (2018) have shown that many enzymes are involved in the elongation process. For example, elongating Arabidopsis stems exhibit relatively high expression of polygalacturonases (PG), pectate-lyases (PL), XTH, ß-1,3-xylosidase, ß-D-xylosidase, α-L arabinofuranosidase, ß-D-glucuronidase, and ß-D-mannosidase genes (Minic

et al., 2009). Little is known about the occurrence of these processes in seeds, especially during germination *sensu stricto*, but they may operate to prepare radicle protrusion. In fact, in seeds of some species, decreasing the endosperm mechanical resistance to such a level that radicle can protrude through the weakened tissues is a condition in proceeding for germination. Table 1 represents a general overview of cell wall related genes and proteins characterized in many seed species associated to seed dormancy and germination. Most cell wall modifying enzymes are represented suggesting their involvement in seed dormancy and germination. Thus, An and Lin (2011) have demonstrated that cell wall synthesis and modification pathway genes are preferentially up-regulated within the early germination phase, which may function to loosen cell walls for subsequent cell expansion and division. Hence, it has been demonstrated that cell wall and protein degradation pathways are in direct relation with plant hormones and ROS in the germination process (Müller et al., 2013; Miransari and Smith, 2014; Xiong et al., 2015).

For germination process, cell wall weakening is thought to be a prerequisite through the induction of cell wall hydrolases and the decrease in the force required for radicle protrusion.

Dynamic changes in the cytoskeleton and cell wall would assist cell expansion and reserve nutrition deposition which are essential for the germination process.

Table 1. Cell wall genes and proteins in relation with seed dormancy and germination in various plant species.

Protein Name	Specie	Dormancy/ Germination	Reference
Cellulose synthase catalytic subunit	Barley	Germination	(An and Lin, 2011)
Cellulose Synthase	Barley	Germination	(An and Lin, 2011)
	Red rice	Dormancy	(Gianinetti et al., 2018)
Pectin methylesterase	Cedar	Dormancy / germination	(Ren and Kermode, 2000)
	Red rice	Germination	(Gianinetti et al., 2018)
	Lepidium sativum	Germination	(Scheler et al., 2015)
	Arabidopsis	Germination	(Möller et al., 2013)
Putative pectin methylesterase	Barley	Germination	(An and Lin, 2011)
	Arabidopsis	Dormancy	(Holdsworth et al., 2008)
Pectin-acetylesterase	Red rice	Germination	(Gianinetti et al., 2018)
Invertase/pectinmethyl- Esteraseinhibitors			
Pectin methylesterase inhibitors	Arabidopsis	Germination	(Möller et al., 2013)

Hemicelluloses synthases	Red rice	Germination	(Gianinetti et al., 2018)
Xyloglucan endo-1,4-beta-D-glucanase	Barley	Germination	(An and Lin, 2011)
Putative xyloglucan endotransglycosylase	Barley	Germination	(An and Lin, 2011)
Xyloglucan endotransglucosylase/Hydrolase (XTH)	*Lepidium sativum*	Germination	(Morris et al., 2011)
	Arabidopsis	Germination	(Linkies et al., 2009)
	Sunflower	Dormancy	(Xia et al., 2018b)
	Barley	Germination	(An and Lin, 2011)
Xyloglucan endotransglycosylase (XET)	Barley	Germination	(Barrero et al., 2009)
	Tomato	Germination	(Chen et al., 2002)
	Arabidopsis	Dormancy	(Holdsworth et al., 2008)

Beta-D- xylosidase	Sunflower	Dormancy	(Xia et al., 2018b)
Putative arabinogalactan-like protein Arabinogalactans	Barley	Germination	(An and Lin, 2011)
Putative polygalacturonase isoenzyme 1 beta subunit Polygalacturonase-inhibiting protein	Barley	Germination	(An and Lin, 2011)
Polygalacturonase	Tomato Red rice Sunflower	Germination Germination Dormancy	(Sitrit et al., 1999) (Gianinetti et al., 2018) (Xia et al., 2018b)
Putative extensin-like protein	Barley	Germination	(An and Lin, 2011)

Putative mannan	Barley	Germination	(An and Lin, 2011)
Endo-1,4-beta-mannosidase	Barley	Germination	(Bewley, 1997b)
Endo-ß-mannanase	*Picea glauca*	Germination	(Downie et al., 1997)
	Tomato	Germination	(Nonogaki et al., 2000)
Endo- ß -1,4-mannanase	*Lepidium sativum*	Germination	(Morris et al., 2011)
Expansin	Barley	Germination	(An and Lin, 2011)
	Barley	Dormancy breaking	(Barrero et al., 2009)
	Tomato	Germination	(Chen and Bradfond, 2000)
α –expansin	*Lepidium sativum*	Germination	(Morris et al., 2011)
	Arabidopsis	Germination	(Linkies et al., 2009); (Weitbrecht et al., 2011)
ß-expansin	Barley	Germination	(An and Lin, 2011)
Glucan endo-1,3- β –glucosidases	Barley	Dormancy breaking	(Barrero et al., 2009)
β –glucosidases	Arabidopsis	Germination	(Gallardo et al., 2002)
β-1,3-glucanases	Tobacco	Dormancy breaking and	(Leubner-Metzger, 2005)
Endo-1,4-β-glucanase	Red rice	Germination	(Gianinetti et al., 2018)
		Germination	
Xylosidase	*Lepidium sativum*	Germination	(Morris et al., 2011)

α- and β-xylosidases	Red rice	Germination	(Gianinetti et al., 2018)
Glycosyl hydrolases	Red rice	Dormancy	(Gianinetti et al., 2018)
Cell wall peroxidase	Maize	Germination	(Lizkay et al., 2003)
β -galactosidases	Chickpea	Germination	(Hernández-Nistal et al., 2014)

IV. Hormonal Regulation of Seed Dormancy and Germination

The plant hormones are crucial signaling molecules that coordinate the regulation of seed dormancy and germination. Hormonal regulation may be a highly conserved mechanism of seed dormancy among seed plants (Nonogaki, 2014). ABA is a positive regulator of dormancy induction and maintenance, while GAs release dormancy and promote the completion of germination by counteracting the effects of ABA (Bewley and Black, 1994). The balance of ABA and GAs levels and sensitivity is a major regulator of dormancy status; environmental signals regulate this balance by modifying the expression of biosynthetic and catabolic enzymes to determine seed dormancy or germination outcomes (Fig. 5). Mediators of environmental and hormonal response include both positive and negative regulators. The net result is a slightly heterogeneous response, thereby providing more temporal options for successful germination (Finkelstein et al., 2008).

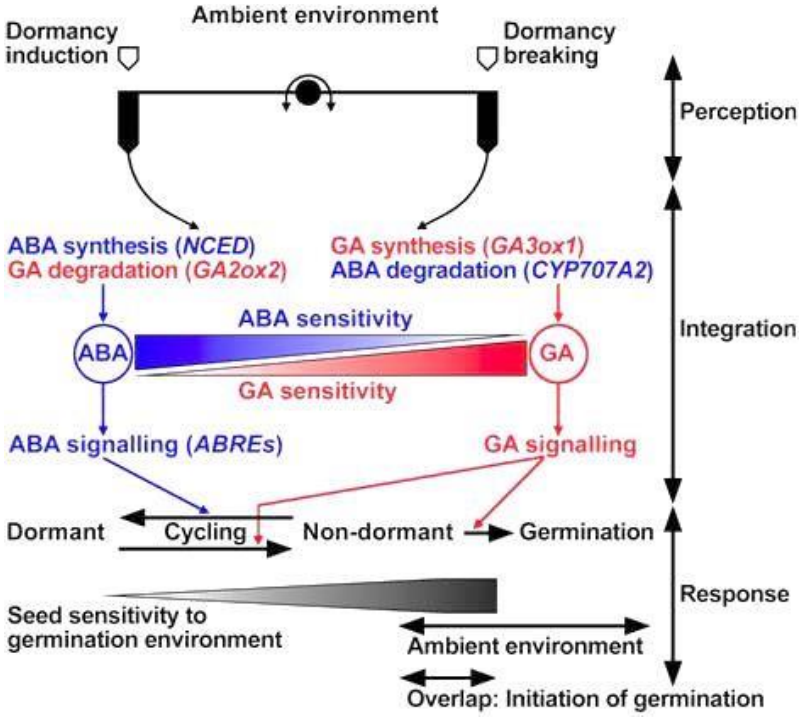

Fig. 5. Model for the regulation of dormancy and germination by abscisic acid (ABA) and gibberellins (GA) in response to the environment. ABA synthesis and signaling (GA catabolism) dominate the dormant maintains, whereas GA synthesis and signaling (ABA catabolism) dominate the transition to germination. The complex interplay among hormone synthesis, degradation and sensitivities in response to ambient environmental conditions can result in dormancy cycling. NCED: Cloning of 9-cis-epoxycarotenoid dioxygenase, GA2ox2: gibberellin 2 beta-dioxygenase 2, GA3ox1: gibberellin 3-beta-dioxygenase 1, CYP707A2: abscisic acid 8'-hydroxylases 2). From Finch-Savage and Leubner-Metzger (2006).

ABA plays a prominent role in seed dormancy maintaining (Hilhorst, 1995; Kermode, 2005). Since the discovery of ABA in the early 1960s, many efforts have been devoted to understanding of ABA regulation. During seed development, ABA is known to initiate the embryo maturation, synthesis of storage reserves and late embryogenesis-abundant (LEA) proteins, and initiation of seed dormancy (Phillips et al., 1997). The level of ABA is determined by the rate of biosynthesis and catabolism. Since ABA is well known to be responsible for dormancy induction and maintenance, the capacity of seed to germinate is normally associated with a low amount of ABA. As demonstrated by previous studies in several species, the endogenous ABA content rapidly declines upon imbibition in ND and D seeds during the early phase of germination, this decrease continues in ND seeds while subsequent *de novo* ABA synthesis in the imbibed D seeds occurs leading to the higher ABA content required for dormancy maintenance (For review, Bentsink and Koormneef, 2008).

GAs are important during the early and the late phase of germination and counteract the ABA inhibition by regulating both of ABA metabolism and signaling (Ogawa et al., 2003). The GA metabolism pathway in plants has been studied for a long time, and a number of genes encoding the enzymes involved in this pathway have been identified (Frigerio et al., 2006). Bioactive GAs control diverse aspects of plant growth and development, including seed germination, stem elongation, leaf expansion, and flower and seed development. Though a hundred GAs identified from plants, only few such as GA1 and GA4 among them, are thought to function as bioactive hormones (Yamaguchi, 2008). The concentration of bioactive GAs in a given plant tissue are determined by the rates of their synthesis and deactivation.

Ethylene (C_2H_4) is the other major hormone acting as a positive regulator of seed germination (for review, Corbineau et al., 2014). Ethylene and its immediate precursor (1aminocyclopropane-1-carboxylic acid, ACC) completely break seed dormancy and improve seed germination. Previous studies have shown that sunflower seed is highly sensitive to ethylene during dormancy alleviation and germination (Corbineau et al., 1990; Oracz et al., 2008). Indeed, Xia et al. (2018b) transcriptomic analysis showed a high similarity between the non-dormant (ND) and dormant (D) treated with ethylene suggesting ethylene involvement in dormancy alleviation of sunflower seed. EL-Maarouf-Bouteau et al. (2015) have shown that the effect of ethylene on germination involves ROS production. In addition, the antagonistic interaction between ABA and C_2H_4 during germination has been shown in various species and was reviewed by Arc et al. (2013). Hence, Linkies et al. (2009) have presented that during the late phase of germination, ethylene plays an important role in counteracting the ABA-induced

inhibition of endosperm cap weakening and rupture by interfering with ABA signaling. Moreover, it has been shown that in sunflower seeds, exogenous GAs has a limited effect on the stimulation of germination of dormant seeds, contrary to ethylene (Corbineau et al., 1990; Oracz et al., 2008).

V. Reactive Oxygen Species Action in Seed Biology

Reactive oxidative species (ROS), are partially reduced or activated derivatives of oxygen. The reduction of oxygen gives rise to superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and singlet oxygen ($^1O2$). The various ROS react with different substrates: for example, $HO^{\bullet}$ reacts rapidly with all types of cellular components, whereas $O_2^{\bullet-}$ reacts primarily with protein Fe-S centers and $^1O2$ is particularly reactive with conjugated double bonds as found in polyunsaturated fatty acids (PUFAs) and aromatic amino acids (Requena and Stadtman, 1999; Requena et al., 2001). ROS have been widely considered as detrimental to plants and seeds due to their highly reactivity and toxic effect leading to cell death by causing damage to proteins, lipids, carbohydrates, and DNA (Mittler et al., 2004, El-Maarouf-bouteau et al., 2011). However, in the last decade, advances in plant physiology signaling pathways have led to reconsider the concept of 'oxidative signalling' instead of 'oxidative stress' (Foyer and Noctor, 2005). Hence, ROS play a key role in various events of seed life. In orthodox seeds, ROS are produced from embryogenesis to germination, i.e., in metabolically active cells, but also in quiescent dry tissues during after ripening and storage, owing various mechanisms depending on the seed moisture content. Yet, upon prolonged storage or storage under inappropriate conditions (temperature, relative humidity) the ROS content could reach a critical level, being harmful for the seed and inducing an oxidative stress and related damage (Bailly, 2004a; Bailly et al., 2008). Thus, under normal conditions, ROS accumulation can be beneficial for seed germination and seedling growth by regulating cellular growth, ensuring a protection against pathogens, controlling the cell redox status, cell wall loosening, testa and endosperm weakening, as well as protein oxidation thus playing a signaling role in seed dormancy alleviation (El-Maarouf-Bouteau et al., 2007; Su et al., 2016). In addition, they interact in the transduction pathway of hormones related to germination, as abscisic acid and gibberellins and are likely to control numerous transcription factors and properties of specific protein through their carbonylation (Oracz et al., 2007; 2009).

V.1. ROS Production and Scavenging System

In plants, and more especially in seeds, ROS may derive from the mitochondrial respiratory chain or be produced through the action of enzymes such as NADPH-oxidase which is a plasma membrane-bound enzyme that catalyzes the production of $O_2^{\bullet-}$, cell wall-bound peroxidases that catalyze the one-electron oxidation of several substrates to release either hydroxyl radical or superoxide or hydrogen peroxide, oxalate oxidases which catalyze the oxidation of oxalate to CO_2 and H_2O_2, or from the apoplastic amine oxidases that produce H_2O_2 from amine oxidation (Bailly, 2004a). ROS production can be enhanced by many abiotic stresses, such as drought stress, salt stress, heat shock, low temperature, nutrient deprivation, and high light (Malan et al., 1990; Prasad et al., 1994; Tsugane et al., 1999; Desikan et al., 2001; Mittler, 2002) as well as some biotic stresses, such as pathogen infection and wounding, also trigger a ROS burst. However, at shedding in dry seeds such as in mature orthodox seeds (seed moisture content is generally below 0.10 g H_2O/g dry weight (DW)), respiration is prevented and enzymes are presumably not active, which suggests the existence of other mechanisms for ROS production which is thought to be initiated at dry state after harvest in sunflower seed (Oracz et al., 2007). Henceforth, non-enzymatic reactions are known to occur, such as the Amadori and Maillard reactions (Priestley, 1986; Sun and Leopold, 1995) and lipid peroxidation (McDonald, 1999; Bailly, 2004a). Bailly et al. (2008) have suggested that ROS could accumulate during dry storage but would become actors of cell regulatory mechanisms only after seeds get imbibed. It has indeed already been demonstrated that hydration of seeds causes a release of free radicals from the trapped state (Priestley et al., 1985). Conversely, the absence of free water and an elevated cytoplasm viscosity, would allow ROS to travel within the cell. Therefore, short-lived ROS, such as $HO^{\bullet}$, would react with sensors closed to their production site, whereas long-lived ROS, such as H_2O_2, could reach targets far from their production site (Møller et al., 2007). Moreover, H_2O_2 are thought to be more selective in the targets that they damage, with modifications occurring to specific molecules at particular sites. In general, the most reactive radicals tend to be the least selective such as hydroxyl radical (Davies, 2005). However, H_2O_2 is a simple molecule and it does not have the required specificity to selectively trigger complex cellular processes, as those that are involved in the control of seed germination. This implies that H_2O_2 is likely to act as a primary messenger by oxidizing compounds that will in turn act as second messengers (Møller and Sweetlove, 2010).

In contrast, seed imbibition is associated with a tight regulation of ROS homeostasis, which involves both ROS producing enzymes discussed earlier and ROS detoxifying enzymes

(catalase, superoxide dismutase,...) (El-Maarouf-Bouteau et al., 2013). Thus, seeds must be endowed with a ROS removing system that tightly regulates their concentration. Scavenging strategies use numerous scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), aldehyde dehydrogenase (ADH), methionine sulfoxide reductases (MSRs) and catalase (CAT) or use non-enzymatic antioxidants such as ascorbate, glutathione, flavonoids, alkaloids and tocopherol to detoxify the ROS excess (Noctor and Foyer, 1998; Apel and Hirt, 2004; Job et al., 2005; Wang et al., 2012). Indeed, Oracz et al. (2009) have proposed that the stimulating embryo elongation would be the result of the inhibition of ROS-scavenging enzymes, such as CAT and SOD, in conjunction with the stimulation of ROS-generating enzymes. Thus, the germination process could be regulated by a narrow balance between ROS production and scavenging.

V.2. ROS in the Regulation of Dormancy Alleviation and Germination

In order to overcome dormancy, seeds undergo stratification or dry storage (after-ripening). Studies have stated that alleviation of seed dormancy, including in the dry state, was mainly related to a change in the seed transcriptome. Although Oracz et al. (2007) have proposed ROS production and proteome oxidation as a key regulatory mechanism in the breaking of dormancy. Hence, the regulation of this phenomenon is multifactorial and requires a coordinated activation of various molecular mechanisms. Many reports have shown that the transition from a quiescent seed to a metabolically active organism is associated with ROS generation. Production of hydrogen peroxide has been demonstrated at the early imbibition period of seeds of soybean, (Puntarulo et al., 1988, 1991; Gidrol et al., 1994), radish (Schopfer et al., 2001), maize (Hite et al., 1999), sunflower (Bailly et al., 2002), wheat (Caliskan et al., 1998), pea (Wojtyla et al., 2006) and tomato (Morohashi, 2002) seeds. Nitric oxide (NO) (Caro et al., 1999; Sarath et al., 2007), hydroxyl radicals (Schopfer et al., 2001) and superoxide radicals (Puntarulo et al., 1991; Schopfer et al., 2001) also accumulate during the germination of seeds of various species. Moreover, the crosstalk between ROS, hormones and enzyme regulation in the germination process (Fig. 6) have been proposed (Liu et al., 2010; Bahin et al., 2011; Bailly et al., 2008; El-Maarouf-Bouteau et al., 2015). It has been shown that ROS could have an effect on transcriptional activity in germinating seeds (Oracz et al., 2009; Leymarie et al., 2012). Thus, El-Maarouf-Bouteau et al. (2015) have revealed that ROS interact at the transcriptional level with ABA signalling pathway and this is mainly done by decreasing the amount of key targeted transcripts. Liu et al. (2010) have shown that H_2O_2 mediates the up-

regulation of ABA catabolism, probably through a NO signal, and also promotes GAs biosynthesis. However, Bahin et al. (2011) have shown that in barley, H2O2 activate GA signalling and synthesis rather than repression of ABA signaling to achieve dormancy alleviation. It has been shown in many species (soybean, Arabidopsis and sunflower seed) that ROS promote ethylene signalling and inhibit ABA action (Ahlfors et al., 2004; Oracz et al., 2008; Jaspers et al., 2009; El-Maarouf-Bouteau et al., 2015). Moreover, El-Maarouf-Bouteau et al. (2007) have shown that, during dry after-ripening, sunflower seeds become able to fully germinate since they accumulate high amount of hydrogen peroxide and exhibit a low detoxifying ability through catalase by the decrease in CATA1 transcript, one of the four genes coding for catalase in sunflower (Bailly et al., 2004b), during the early phase of imbibition. Moreover, it has been proposed that ROS could oxidize specific proteins during seed imbibition (Job et al., 2005; Oracz et al., 2007; Barba-Espín et al., 2011), thus driving the germination process, or oxidize mRNAs, thus preventing their translation (Bazin et al. 2011). Nevertheless, Bailly et al. (2008) suggested that seed germination occurs when the seed ROS content is enclosed within an oxidative window that allows ROS signaling but not ROS damage.

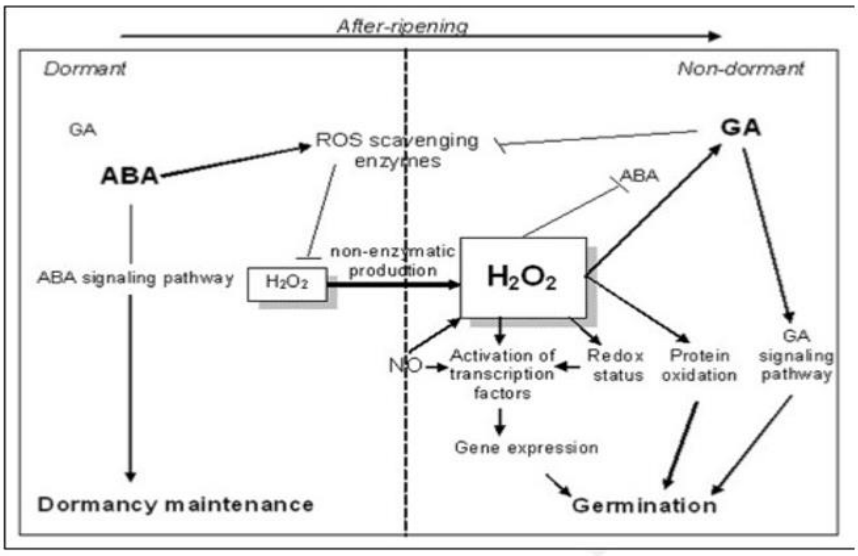

Fig. 6. Hypothetical model proposing a central role of ROS in seed dormancy release and germination. In dormant state, high amount of ABA induces an active signaling pathway involved in dormancy maintenance. Lower ABA concentration in nondormant seeds would be associated with a higher level of ROS, presumably H_2O_2, which could in turn interfere with ABA and GA signaling pathways, modify redox status and induce protein carbonylation. NO may interfere with H_2O_2 and activate GA signaling. The size of the letters is proportional to the relative amount of the compounds (ABA, GA, H_2O_2). From El-Maarouf-Bouteau and Bailly (2008).

Methylviologen, a ROS generating compound, is known to alleviated dormancy in green tissues (Slooten et al., 1995) and seeds such as sunflower and pea seeds (Oracz et al.

2007, 2009; El Maarouf-Bouteau et al., 2015; Wang et al., 2012). This water- and lipid-soluble compound is reduced in the light by the photosynthetic electron transport chain, but it is also (although less rapidly) reduced in the dark by other as from reductants PSI in chloroplasts. When reduced, MV reacts with oxygen thus yielding superoxide (Slooten et al., 1995; Xiong et al., 2007; Oracz et al., 2007; Whitaker et al., 2010). Many studies have presented the suppression of the inhibitory effect of ABA by MV treatment (Oracz et al., 2009; El-Maarouf-Bouteau et al., 2015). Moreover, Oracz et al. (2009) have presented that MV treatment entailed the same specific pattern of protein oxidation as seen with hydrogen cyanide.

All these results show that ROS are involved in dormancy alleviation and germination probably by acting at different levels of cellular processes including central metabolism and hormone signalling.

V.3. ROS and Protein Oxidation

Proteins are, with nucleic acids and lipids, the most sensitive molecules to *in vivo* oxidation (El-Maarouf-Bouteau et al., 2013). Oxidation of proteins, which makes up 68% of the oxidized molecules in the cell (Rinalducci et al., 2008), can occur through a number of different mechanisms, such as the formation of disulfide cross-links and glycoxidation adducts, oxidation of methionine (Met), nitration of tyrosine (Tyr) residues, and carbonylation of specific amino acid residues (Davies, 2005). Indeed, protein oxidation can be caused directly by ROS or by co-products of oxidatively modified lipids, amino-acids or sugars (Shacter, 2000). Proteomic investigations have highlighted that seed proteins are subjected to a large number of PTMs (see later). Hence, ROS can affect proteins by the oxidation of their amino acids in a posttranslational manner which may affect protein functions including localization, complex formation, stability and activity (Arc et al., 2011). Depending on the oxidized amino acid, ROS damage of proteins can be either irreversible (i.e., carbonylation) thus triggering the degradation of the oxidized proteins by the cytosolic 20S proteasome or can be reversed through the action of thioredoxins, peroxiredoxins, or glutaredoxins (cysteine oxidation) or by methionine sulfoxide reductase (methionine oxidation) (El-Maarouf-Bouteau et al., 2013). The relationship between carbonylation, Met sulfoxidation and phosphorylation have been unraveled recently showing that sulfoxidation alters protein carbonylation (Moskovitz and Oien, 2010; Oien et al., 2011) and phosphorylation (Hardin et al., 2009). Such interactions are a potentially direct interface between redox sensing and signaling, and cellular protein kinase/phosphatase-based signalling (see below).

Figure 7 shows the direct link of some PTMs with seed germination. Protein post-translational modifications are among the fastest and earliest of plant responses to changes in the environment, making the mechanisms and dynamics of PTMs an important area in the plant science. Indeed, they greatly expand proteome diversity, increase functionality, and allow for rapid responses, all at relatively low costs for the cell (Friso and Van Wijk 2015). Plant PTMs have been implicated in the regulation of a number of regulatory and metabolic processes (Arsova et al., 2018). Hence, PTMs play key roles in plants through their impact on signaling, gene expression, protein stability and interactions, and enzyme kinetics. Worth-mentioning that PTMs can occur spontaneously (non-enzymatically) due to the physico-chemical properties of reactive amino acids and the cellular environment (i.e. pH, oxygen, ROS and metabolites) or through the action of specific modifying enzymes (i.e. carboxylases, acetyltransferases…) and can be reversible or irreversible on specific amino acid residues (for review check: Ryšlavá et al., 2013). Most occurred PTMs in plants are: phosphorylation, S-nitrosylation and nitration, acetylation, deamidation, lipidation, N-linked glycosylation, ubiquination, sumoylation, sarbonylation, methylation, glutathionylation, oxidation, S-guanylation, and formylation. PTMs can be intensified by co-factors like NAD or glutathione intermediates which regulate proteins involved in gene transcription, protein synthesis and degradation via the proteasome, hormone metabolism, where comes the interlink of PTM with post-transcription modifications (Skelly et al., 2016). Several PTMs, which has been characterized in seed dormancy such as oxidation, carbonylation and phosphorylation, will be discussed below.

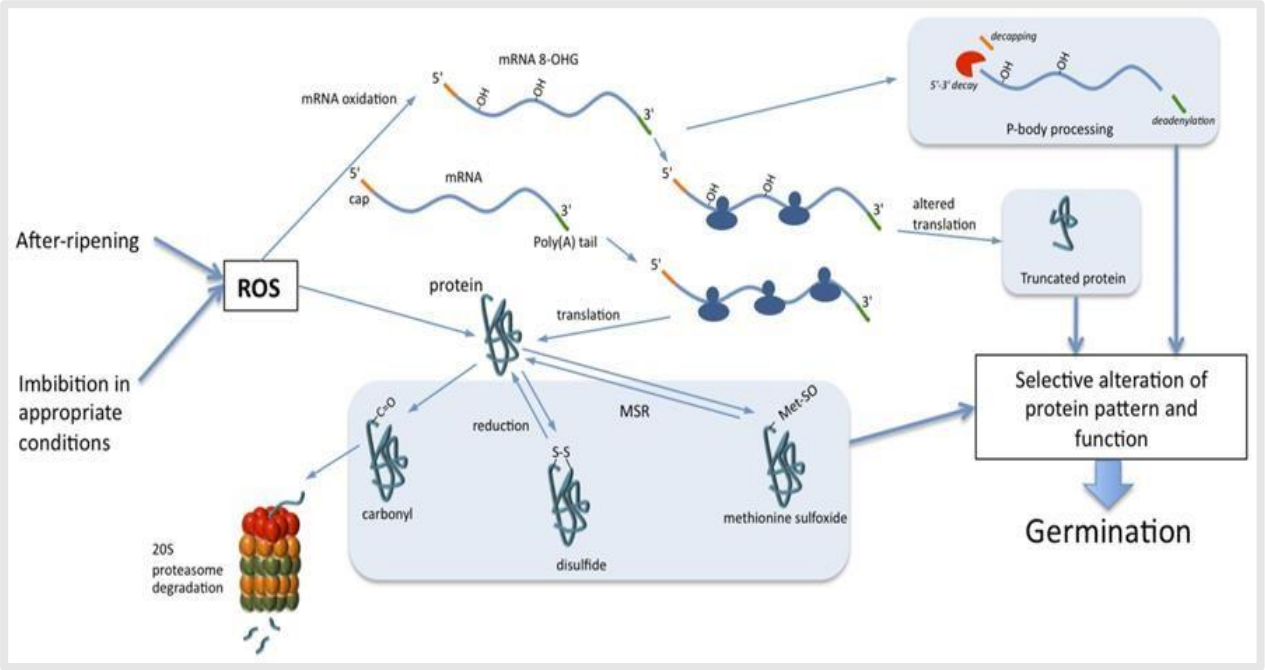

Fig. 7. Role of mRNA and protein oxidation in seed germination. During non-dormant seed imbibition, ROS, accumulated during dry after-ripening or produced during imbibition, trigger selective oxidation of mRNA and proteins. Oxidation of mRNAs leads to their decay through the action of P-bodies and/or to the alteration of translation. Proteins can be irreversibly oxidized by carbonylation, which leads to their cytosolic degradation through the 20S proteasome. Oxidation of cysteine and methionine induces formation of disulfide bonds and methionine sulfoxide, respectively. Reduction of disulfide bonds occurs through the action of thioredoxins, peroxiredoxins, or glutaredoxins when methionine sulfoxides are reduced to methionine by methionine sulfoxide reductase (MSR). The selective oxidation of a subset of mRNAs and proteins guides cell signaling pathways toward the completion of seed germination. From El-Maarouf-Bouteau et al. (2013).

V.3.1. Carbonylation

Protein carbonylation is a widely used marker of protein oxidation (Ballesteros et al., 2001; Das et al., 2001; Mostertz and Hecker, 2003; Johansson et al., 2004). It occurs by direct oxidative attack on Lys, Arg, Pro, or Thr residues of proteins, thus inhibiting or altering their activities and increasing their susceptibility toward proteolytic cleavage (Job et al., 2005). Carbonylation seems to be irreversible and unrepairable modification (Dalle-Donne et al., 2003). To avoid their toxic accumulation, carbonylated proteins are degraded through the action of the 20S proteasome in the cytosol (Nyström, 2005). However, this type of protein oxidation is not shown to be always deleterious. Hence, a number of proteins (soluble and storage) were shown to be carbonylated during germination of Arabidopsis and sunflower seeds (Job et al., 2005; Oracz et al., 2007; El-Maarouf-Bouteau et al., 2007). The latter authors suggested that,

in the sunflower seed system, protein carbonylation may result from an accumulation of ROS themselves and from accumulated lipid peroxidation products such as MDA. They have found that the use of hydrogen cyanide or methylviologen has led to carbonated proteins that were specifically associated with breaking of dormancy in the imbibed state of sunflower seed axes. Job et al. (2005) have suggested that protein carbonylation during germination is used as a means to adapt embryo metabolism to the oxidative conditions encountered during germination. Thus, protein carbonylation may play a key role in dormancy alleviation by buffering ROS to protect seed cell after active metabolism resumption, by facilitating reserve mobilization and probably by regulating enzymes that can be involved in metabolism or hormone signalling.

V.3.2. Sulfur-centers oxidation

The ease of oxidation of sulfur centers makes Cys, cystine and Met residues major sites of oxidation within proteins (Davies, 2005). The most likely way in which ROS can interact with proteins is through thiol modification of Cys residues, which can be oxidized to varying degrees to cause changes in protein conformation and activity (Hancock et al., 2006). Reversible disulfide bond formation from thiol groups of cysteine is probably the post-translational oxidative modification that has been the most extensively studied in seed physiology (Buchanan and Balmer, 2005; Arc et al., 2011). The thiol of cysteine can be oxidized to a disulphide (PSSP), sulfenic acid (PSOH), sulfinic acid (PSO_2H) or sulfonic acid (PSO_3H). In general, cysteine oxidation can be induced by hydrogen peroxide, superoxide, or nitric oxide and its derivatives, as well as more highly reactive species such as hydroxyl radicals (Rinalducci et al., 2008). It is thought that the formation of protein disulphides and glutathionylation can protect the protein from further, potentially more damaging, oxidation thus irreversible oxidation of proteins and their subsequent degradation (Dalle-Donne et al., 2007; Spadaro et al., 2010). Hence, formation of disulfide bridges between cysteine residues occurs during seed maturation and has been proposed to provoke compaction of proteins, rendering them less sensitive to protease action or inactive (Buchanan and Balmer, 2005). Upon rehydration, deglutathionylation can be catalyzed by glutaredoxins (GRXs) and protein disulphide bonds can be reduced through NADPH-dependent thioredoxins (TRXs) (Alkhalfioui et al., 2007). Indeed, formation of disulfide bonds occuring during maturation drying is a process that would be associated with the down regulation of metabolic activities in mature seed (Buchanan and Balmer, 2005). Thus, the reversibility of this process during germination, likely through the action of thioredoxins (Trxs), participates allowing reactivation of metabolism and mobilization

of reserves after imbibition to sustain germination and seedling establishment (Colville and Kranner, 2010; Châtelain et al., 2013). It has also been proposed that the mobilization of protein reserves during germination would require disulfide bond reduction since it increases solubility and facilitates proteolytic attacks (Alkhalfioui et al., 2007). This suggests that germination might be associated with a subtle balance between oxidation and reduction of cysteines on targeted proteins.

Met oxidation is a hallmark of oxidative conditions and aging in all organisms (Stadtman, 2006). Oxidation of methionine usually yields methionine sulphoxide (R-SOCH$_3$, abbreviated as MetSO), a relatively commonly detected oxidative modification in biological systems that can be reversed by chemical reduction or by the action of methionine sulphoxide reductases (MSRs), although a more severe attack can result in the formation of the sulphone (R-SO$_2$CH$_3$) which is thought to be irreversible and damaging to protein. MetSO can be reversed through the specific action of methionine sulfoxide reductase A and B on S-and R-diastereomers, respectively (Davies, 2005). The role of oxidation of methionine MetO and its repair by MSRs has not yet been widely documented in seeds, although several lines of evidence support the possible involvement of MSRs in maturation and germination processes. Met residues on proteins can act as sacrificial antioxidants, and that the interconversion of Met to Met sulfoxide may act as a reversible redox switch to control the activity and function of proteins (Davies, 2005; Rinalducci et al., 2008; Châtelain et al., 2013). In addition, seed longevity may be linked to the abundance of both MSR types. Such a general role is supported by the negative relationship between the level of MSR activity and the levels of proteins oxidized by carbonylation in numerous organisms (Moskovitz and Oien, 2010). Thus, the involvement of MSRs in ROS scavenging could occur in seeds to limit the extent of damage resulting from the oxidative conditions reigning during maturation and imbibition (Châtelain et al., 2013).

Moreover, as a matter of cross talk between these PTMs, sulfoxidation may be involved in protein carbonylation and phosphorylation (Hardin et al., 2009; Moskovitz and Oien, 2010; Oien et al., 2011). Moskovitz and Oien (2010) have presented the fact that methionine sulfoxide modifications to proteins may precede the formation of protein-carbonyl adducts because of consequent structural changes that increase the vulnerability of amino acid residues to carbonylation. Ghesquière et al. (2014) have suggested that MSR B1 had a higher contribution in protecting the brain cerebellum from protein-carbonyl accumulation. In addition, it was shown that the lack of or compromised MSR activities (mainly of MSR A) may cause faster

accumulation of protein-carbonyls in yeast by ageing (Moskovitz and Oien, 2010). Thus, it is suggested that in the lack of MSRs, enhanced protein–carbonyl accumulation may be due to an increased oxidation prompted by Met-SO which may cause conformational changes to proteins, leading to further irreversible oxidation. Moreover, Hardin et al. (2009) have presented the fact that Met-SO can couple oxidative signals to changes in protein phosphorylation. Hence, oxidation of surface-exposed Met residues in kinase substrate proteins, can inhibit the phosphorylation of nearby sites and thereby couple oxidative signals to changes in protein phosphorylation. Contrariwise, overexpression of MSR reduces Met-SO near the phosphorylation site, so increasing the amount of phosphorylated proteins (Hardin et al., 2009; Tarrago et al., 2009). Thus, Met oxidation regulates the ability of kinases to bind the target protein, thereby directly linking oxidative signals to changes in protein phosphorylation. On the other hand, it seems that phosphorylation-dephosphorylation may also play an important role in seeds. Indeed, it has been shown that enzymes involved in central metabolism are targeted of phosphorylation among several other PTMs. Consequently, it can influence the activity of several enzymes such as the UDP-glucose pyrophosphorylase, phosphoglucomutase, enloase, malate dehydrogenase, fructose-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase during dormancy alleviation in sunflower seeds (Xia et al., 2018a).

V.4. ROS in Cell Wall Loosening

There is a wealth of evidence suggesting that ROS would play a key role in cell wall loosening in growing tissues (Fry, 1998; Liszkay et al., 2004). Hydrogen peroxide (H_2O_2) has been shown to promote cell expansion through modification of some cell wall component (pectin) and contribute to cell wall loosening through hydrolases action in plant roots, suggesting that ROS and enzymatic cell wall loosening might act synergistically *in planta* (Xiong et al., 2015). It has been shown that hydroxyl radical (•OH), cleaves cell wall polysaccharides in a site-specific non-enzymatic reaction by an oxidative scission of backbone bonds (Fry et al., 2001; Schweikert et al., 2002; Liszkay et al., 2003, 2004; Renew et al., 2005) and can, in this way, cause wall loosening shown in fruits (Chen et al., 2016), seeds (Müller et al., 2009) and plants (Schopfer, 2001). Penfield et al. (2006) have presented the effect of ABA treatment or paclobutrazol (PAC, an inhibitor of GA synthesis) by down-regulation cell wall loosening enzymes and other (xyloglucan endotransglycosylase-related proteins, pectin methylesterase and expansins). Hence, Morohashi (2002) and Müller et al. (2007) showed that

H_2O_2 reversed the inhibitory effect of ABA on endosperm rupture, underlining the cross talk between these two compounds.

VI. Objectives of the Thesis on Sunflower Seed Dormancy and Germination

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops in the world. It represents a major renewable resource for food (oil), feed (meal), and green energy. France is a major sunflower producer (1.4Mt/year, 1st European producer) and sunflower production contributes significantly to the French economy. French breeders are ranked first in terms of sunflower seed production. Rapid and homogenous emergence and stand establishment are critical factors for sunflower production. Sunflower seeds provide an excellent system for studying dormancy because they are deeply dormant at harvest, and this dormancy is progressively lost during dry storage (Corbineau et al., 1990). Otherwise, sunflower seed has a small and simple forms, with one fruit containing a single seed and the seed envelops consists of the ovary wall (pericarp, Fig. 8a) and testa. Sunflower embryo is composed of two primary organ systems: the axis and cotyledon (Fig. 8b). Both of these two organs have distinct developmental fates; the axis corresponds to the hypocotyl-radicle region of the embryo. It contains the shoot and root meristems, which will grow into the mature plant after seed germination. The cotyledon, as a terminally differentiated organ, is involved in reserves supply for subsequent seed germination and seedling growth before photosynthetically active phase.

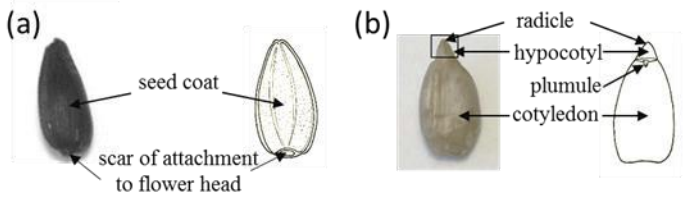

Fig. 8. Structure of sunflower seed. (a) Sunflower fruit, which is composed of an embryo surrounded by covering seed coat derived from the walls of the ovule; (b) Sunflower seed after removal of the fruit pericarp. the plumule made up of two embryonic leaves and a terminal (apical) bud, the two embryonic leaves will become the first true leaves of the seedling, and the terminal bud contains the meristem at which later growth of the stem takes place; two cotyledons involved in food supplying for the germinating seedling; hypocotyl and radicle, represent the axis which will grow into the part of the stem below the first node and primary root respectively. Adapted from Oracz et al. (2009).

In order to fill the shortage of the study of seed dormancy/germination in sunflower, our seed biology group (IBPS, Sorbonne University) has contributed for a large part. The physiological dormancy concerns both of seed coat- and embryo in Sunflower. As described in

Corbineau et al. (1990), the inability of freshly sunflower seed to germinate at high temperatures (25 to 40°C) resulted principally from a seed coat-imposed dormancy, whereas poor germination at temperatures below 25 °C was mainly due to an embryo dormancy. However, this embryoimposed dormancy can be overcome during after-ripening or by applying exogenous ethylene or some chemicals (Methylviologen). Unlike in Arabidopsis, exogenous GA has a limited effect on the stimulation of germination of dormant sunflower seeds, while the antagonistic interaction between ABA and ethylene play a critical role (Corbineau et al., 1990; Oracz et al., 2008; Arc et al., 2013; El-Maarouf-Bouteau et al., 2015; Xia et al., 2018b).

Despite the huge progress that has been made the last decade owing to the emergence of the omics approaches, the molecular mechanisms regulating seed germination and dormancy are far from being resolved. For example, how a dormant seed acquires the ability to germinate during dry after-ripening or imbibition in optimal temperature is unknown. ROS have been proposed to be key players in sunflower seed dormancy and germination (Oracz et al., 2007; Bailly et al., 2008; El-Maarouf-Bouteau et al., 2015). In fact, ROSgenerating compound, methylviologen, and respiration inhibitor hydrogen cyanide (HCN) can alleviate sunflower embryo dormancy during imbibition. Moreover, sunflower seed dry afterripening is associated with ROS accumulation, which induces selective protein oxidation in anhydrobiosis (Oracz et al., 2007, 2008). Bazin et al. (2011) also reported that mRNA oxidation occurs during sunflower seed dry after-ripening, which highlights a potential mechanism of seed dormancy alleviation in which targeted mRNA oxidation can fine-tune the cell signaling pathway that controls germination by targeting mRNA decay and by regulating selective translation. Both of these studies clearly document that non-enzymatic processes leading to selective translation and selective protein degradation are probably key events occurring during after-ripening (El-Maarouf-Bouteau et al., 2013). Moreover, multiscale omics analyses, ie, transcriptomics, proteomics, metabolomics (Xia et al., 2018 a and b) and translatome analyses (Layat et al., 2014) have underlined the importance of central metabolism related- and cell wall modifying enzymes in sunflower seed dormancy alleviation. Thus, to explore the regulation of such enzymes and the involvement of cell wall change in dormancy alleviation and germination, this work aimed to characterize the changes occurring in sunflower seed cell wall during germination *sensu stricto* process, in particular tackling the action of pectin methyl-esterase (PMEs) and the contribution of other factors (ROS, ethylene) on cell-wall loosening. Moreover, targeted protein oxidation (Met-sulfoxydation) involved in the germination process has been

studied by a proteomic analysis to integrate a supplemental level of regulation, such as posttranslational modifications.

The results obtained will allow us to characterize the processes that take place from the beginning of the imbibition to the radicle protrusion, the last step of germination *sensu stricto*.

Chapter 2 Cell wall loosening during dormancy alleviation in sunflower seeds

Abstract

Germination involves biomechanical properties modifications that influence cell-wall loosening towards radicle protrusion. The involvement of different seed compartments was well studied in endospermic seeds. It was shown that endosperm softening play an active role in the regulation of seed germination. The aim of this work was to investigate cell wall modifications in non-endospermic seeds, through dormancy alleviation process during the germination *sensu stricto*. Germination was induced on non-germinating Dormant (D) sunflower (*Helianthus annuus* L.) seeds by after-ripening, methylviologen, a Reactive Oxygen Species (ROS) generating compound, or ethylene treatments (Non-Dormant (ND), D/MV and D/ET, respectively). Cell wall sugar contents have been quantified by gas chromatography and the degree of pectin methylesterification has been assessed by pectin methyl-esterase (PME) activity and *in situ* localization of low- and high- esterified pectin. No significant monosaccharide content change has been recorded between D and all the other treatments upon the imbibition times tested (3, 15 and 24h). Greater immunofluorescence signal of methylesterified epitopes was observed in D as compared to the other treatments, especially ND. Moreover, AFM analyses confirm the softer texture of ND, D/MV and D/ET comparing to D. Interestingly, this softness concerns only the meristematic zone. The possible independent pathway of ROS acting non-enzymatically on the mechanical expansion and cell wall permeability along with the role of ethylene on other cell wall hydrolases allowing cell wall loosening, specifically meristematic cells softening, are discussed.

Keywords: after-ripening, cell-wall loosening, dormancy-alleviation, ethylene, methylviologen, pectin methyl-esterase, reactive oxygen species, sunflower seed.

I. Introduction

Seed dormancy is characterized by the inability of the seed to germinate after harvest even in favorable environmental conditions. One can distinguish embryo dormancy when the embryo is unable to germinate, or seed-coat imposed dormancy corresponding to the inability of nondormant embryo to overcome the surrounding layers of seed coat. Germination *sensu stricto* starts with water uptake and ends with radicle protrusion corresponding to cell elongation (Bewley, 1997). Thus, cell wall weakening is thought to be a prerequisite through the induction of cell wall remodeling enzymes and the decrease in the force required for radicle protrusion during germination. Accordingly, dormancy mechanism may rely on some mechanical restraint to keep the steady stage of the seed. Plant cell growth is determined by the interplay between cell wall extensibility and water content (Peaucelle et al., 2012). During seed germination, the force exerted by water is one of the most important in plant's life cycle as upon imbibition, seeds pick up more than 50 % of their dry matter of water in cells filled with reserves. Furthermore, morphological and physiological characteristics of seeds are of high importance. Most seeds of angiosperm species contain embryo enclosed by one or more seed-covering layers. Such layers serve as the mechanical barrier that prevents embryo elongation in coatimposed dormancy. Consequently, it's important to study mechanical property of seed cell wall in the context of the given type of dormancy and seed morphology.

Elongation is correlated with increased esterification in pectin and the cessation of elongation with de-esterification in many species like maize (Kim and Carpita, 1992), tobacco (McCann et al., 1994) and Arabidopsis (Derbyshire et al., 2007; Peaucelle et al., 2015). Pectin methylesterases (PMEs; E.C. 3.1.1.11), a family of enzymes which is specific for the pectin matrix, catalyze the specific demethylesterification of homogalacturonan (HG) within plant cell walls, releasing methanol and protons creating negatively charged carboxyl groups and pectin with lower degree of methylesterification (Pelloux et al., 2007). The demethylesterified HG can either form Ca^{2+} bonds (demethylesterified in blockwise fashion by the action of plant PME), which promote the formation of the 'egg-box' model structure resulting in gel formation so intercellular adhesion, or become a target for pectin-degrading enzymes, such as polygalacturonases and pectate lyases, affecting the texture and rigidity of the cell wall. Indeed, PMEs play a key role in pectin remodeling *in muro* (Pelloux et al., 2007). PMEs constitute a family of 66 members in Arabidopsis. Wang et al. (2013) have shown that all PMEs have a

conserved pectin esterase domain (Pfam01095), but only group 2 has in addition a PME inhibitory domain (Pfam04043) which in turn can be regulated by endogenous PME inhibitor proteins (PMEI) through a non-covalent reversible reaction (Jolie et al., 2010). PMEIs form an equally large gene family of 69 members in Arabidopsis (Peaucelle et al., 2008; Wolf et al., 2009).

A wealth of studies have been done to highlight the role of PMEs in cell-wall loosening in plants and fruits but scarce studies have been done on seeds. Nevertheless, in seeds, primary plant cell wall compartments are also the target of many enzymes involved in different cell wall polysaccharides as cellulose and hemicellulose matrix and linkage, which are shown sometimes to be correlated together in cell-wall loosening (Cosgrove, 2005). For example, in seeds of white spruce, weakening of the micropylar end of the megagametophyte is associated with endo-β-mannanase activity, the corresponding gene (*LeMAN2*) being expressed as well in the micropylar endosperm cap of tomato seeds (Downie et al., 1997; Nonogaki et al., 2000). Tobacco endosperm cell walls may be weakened by a β-1,3-glucanase since its activity increases within the micropylar endosperm before radicle emergence (Leubner-Metzger et al., 1995). Other candidate is xyloglucan endotransglcosylase/hydrolase (XTH) enzymes that catalyzes either the cleavage or molecular grafting of the β-(1,4)-xyloglucan backbone through endotransglucosylase (XET) activity or its irreversible shortening through the endohydrolase (XEH) activity (Shi et al., 2015). The activity of XET, which is capable of reversibly cleaving xyloglucan molecules, increases in the apical region of maize seedling roots during their elongation (Wu et al., 1994) and are expressed in the endosperm cap of tomato (*Solanum lycopersicum*) seeds during germination (Chen et al., 2002). In sunflower seeds, transcriptomic analysis has underlined the alteration of gene expression of cell wall modifying enzymes, such as XTH, Beta-D- xylosidase, or polygalacturonase in sunflower seed dormancy alleviation (Xia et al., 2018).

Primary cell wall is subject to enzymatic and non-enzymatic reactions contributing to its loosening (Fry, 1998; Cosgrove, 2005; Xiong et al., 2015). Non-enzymatic reactions include the action of Reactive Oxygen Species (ROS), some non-enzymatic proteins as expansins and hormones as ethylene and auxin being involved in cell-wall loosening. Auxin can play a role in cell-wall loosening by the control of apoplast pH which is in part mediated through the auxinmediated phosphorylation of H+-ATPase (Hocq et al., 2017), promoting the action of ROS and/or by inducing the action of some cell wall enzymes and expansins (Fry et al., 2001; Schopfer, 2001; Durachko and Cosgrove, 2009; Xiong et al., 2015). Expansins are a group of

non-enzymatic wall proteins that induce wall stress relaxation and extension. They mediate acid induced growth, also referred as creep, by disrupting the non-covalent linkages that hold microfibrils in place in the cell wall (Fry, 1995; Cosgrove, 1999; Cosgrove, 2000; Cosgrove, 2005) and possess the ability to disrupt hydrogen bonds between glucan chains (cellulose and/or XG) (McQueen-Mason and Cosgrove, 1994) thus creating a "lubricating" effect (Peaucelle et al., 2012). Furthermore, *in vitro* and *in vivo* studies have highlighted the role of ROS, specifically hydroxyl radicals (•OH), in cleaving cell wall polysaccharides (xylan, xyloglucan, pectin, galacturonan, arabinogalactan and cellulose) in a site-specific reaction causing immediate chain scission (Miller, 1986; Fry, 1998; Schopfer, 2001; Müller et al., 2009).

Many reports have shown that the transition from a quiescent seed to a metabolically active organism is associated with ROS generation. Production of hydrogen peroxide has been demonstrated at the early imbibition period of seeds of soybean (Puntarulo et al., 1988, 1991; Gidrol et al., 1994) radish (Schopfer et al., 2001), maize (Hite et al., 1999), sunflower (Bailly et al., 2002), wheat (Caliskan and Cuming., 1998), pea (Wojtyla et al., 2006) and tomato (Morohashi, 2002) seeds. Moreover, the crosstalk between ROS, hormones and enzyme regulation in the germination process have been proposed (Bahin et al., 2011; Bailly et al, 2008; El-Maarouf-Bouteau et al., 2015). In sunflower seeds, it was shown that ROS interact with ethylene and ABA signaling in inducing dormancy alleviation (El-Maarouf-Bouteau et al., 2015).

To explore the occurrence of cell wall extensibility in such hydrated organ subjected to ROS and hormones during dormancy release and germination, this work aimed to characterize the changes occurring in sunflower seed cell wall during germination *sensu stricto* process by measuring its softness through atomic force microscopy in several parts of the axis (the elongated part of the embryo) and by characterizing PMEs contribution comparing afterripening, ROS and ethylene treatments.

II. Materials and Methods

II.1. Plant Material and Treatment

Seeds of sunflower (*Helianthus annuus*, "LG5665"), harvested in 2015 near Montlémar (Drôme, France) and purchased from Limagrain (www.limagrain.com), were used in this study. At harvest, dormant seeds (D) were stored at -20ºC until use in order to maintain their dormancy. After-ripening (ND) was performed by placing seeds at 25ºC and 70% relative humidity for at least 3 months to break their dormancy. Treatment with 1-Aminocyclopropane1-Carboxylic Acid (ACC) and methylviologen (MV) (Sigma-Aldrich) was carried out by placing hulled seeds in darkness at 10ºC on a cotton wool moistened with ACC solution of 1mM and MV solution of 0.1 mM. After 3 hours, D/MV embryos were carefully rinsed three times with distilled water and put on a moistened cotton wool for the rest of the treatment. Treatment with ethylene (ET) was carried out by placing hulled seeds in darkness at 10ºC on a cotton wool moistened in a tightly closed glass jar continuously in the presence of 100 ppm gaseous ethylene.

II.2. Germination Tests

Germination tests were performed with seeds without pericarp (embryos) in darkness, in 9-cm petri dishes (25 seeds per dish, three replicates) on a layer of cotton wool moistened with distilled water. Petri dishes were placed at 10ºC, a suboptimal temperature for dormant sunflower seed germination (Corbineau et al., 1990). An embryo was considered as germinated when the radicle had elongated to 2–3 mm. Germination counts were made daily for 7 days and germination percentage means are the results of six replicates ± SD.

II.3. Atomic Force Microscopy (AFM)

Data were collected following the protocol described in Braybrook and Peaucelle (2013) using a standalone JPK NanoWizard (Germany). Axis samples were cut as cross sections by a blade using an optical microscope and immobilized on glass slide surrounded by stiff agarose. In order to avoid turgor pressure, all samples were plasmolyzed in hypertonic solution (0.55 M, 10% Mannitol) 30 min prior to measurement. Six axes (5 µm bead) were analyzed for each

treatment used (D, ND, D/MV and D/ACC) at 24 hours of imbibition. The maximum relative set point was 3V with an "extend speed" of 40 µm/sec and the "z length" of 5 µm with a sample rate = 3000 Hz. The sample zone was a 40*40 µm square area. For young modulus measurement using force spectroscopy, with a tip speed of 150nm/s, a retraction length of 1.5µm, were used and a constant maximum force was imposed; this value was determined for each experiment to obtain a maximum deformation at all points of the sample of about 200 nm. Images were obtained using JPK Data Processing Software (JPK Instruments AG, Germany).

II.4. Cell Wall Monosaccharide Composition

The monosaccharide composition of sunflower axis cell wall fractions of the different treatments (D, ND, D/MV and D/ET) at different imbibition times (0, 3, 6, 15 and 24 h) were analyzed by Gas Chromatography coupled to a Flame Ionization Detector (GC-FID, USA) in the laboratory of Glycobiology and Extracellular Plant Matrix, at Rouen University. Alcohol Insoluble Residues (AIR) fractions were extracted by grinding 15 axes (around 60 mg) of sunflower seeds, washed two times with 2 mL of 70% (v/v) ethanol, 2 mL of chloroform:methanol (1:1) overnight, and then soaked in 2 ml acetone for 2 hours before drying. Hydrolysis of each fraction (1mg) was performed using the method described in Moore *et al*. (2006). Chromatographic data were integrated with GC Star Workstation software (Varian). A temperature program was optimized for the separation of the most common cell wall monosaccharides such as arabinose, rhamnose, fucose, xylose, glucuronic acid, mannose, galactose, and galacturonic acid. The results correspond to the means of three measurements ±SD.

II.5. Pectin Methyl-Esterase (PME) Activity

PME activity was measured for each of the different treatments used (D, ND, D/MV and D/ET) at 0, 24h and 48 hours of imbibition. Soluble protein were extracted from 20 sunflower axes (around 100 mg). Protein were assayed using Bradford reagent (Bio-Rad) with Bovine Serum Albumin (BSA) as a standard. The supernatants were freshly used since the PME activity can be affected by freezing. A coupled enzymatic assay was performed as described by Grsic-Rausch and Rausch (2004) using a spectrophotometric plate reader (Tecan Infinite F500, Switzerland). Reaction rates were recorded at 340 nm. The reaction was performed at room

temperature for 15 min. The change in absorption per unit time over the linear part of the reaction is calculated for each well, and used to calculate the increase in concentration of PME. One nKat PME activity was defined as 1 nmol NADH formed per sec. Enzyme activities were measured for three independent replicates ±SD.

II.6. Immunofluorescence Studies and Histological Examination

Analyses have been performed for the different treatments used (D, ND, D/MV and D/ET) at 24 hours of imbibition based on the method prescribed in Möller *et al.* (2013). 30 µm axes sections (vibratome LEICA VT 1000S) were incubated overnight in a blocking solution of paraformaldehyde (PFA) 4% (w/v). After rinsing five times in phosphate buffered-saline (PBS 1x and 0.1% Tween 20), samples were blocked by goat serum (1/30) which was added for 20 min in dark then the sections were rinsed again 5 times to be incubated in the primary antibodies LM20 and 2F4 (1/10 and 1/100 respectively; Plant probes; www.plantprobes.net) overnight at 4ºC. Following incubation (2h) of the secondary antibody (FITC-anti-mouse IgG1FITC for 2F4 and FITC-Anti-Rat IgG IgA IgM for LM20, Sigma-Aldrich) used at a dilution of 1:100 at room temperature in the darkness, the sections were rinsed again 5 times and examined via fluorescence microscopy (ZEISS, imager z1 (https://www.zeiss.com)). Images were recorded by AxioVision 4.8 Microscopy software.

II.7. Statistical Analysis

Two way ANOVA was used as a statistical analysis for significance among treatments and imbibition times. Significant means ($\alpha < 0.05$) were induced by using Tukey's and Games Howell's honestly significant difference test. These analyses were performed using the SPSS16.0 statistical software**.**

III. Results

III.1. Germination Assay

Figure 1 shows the germination behavior of D, ND and D treated with MV (D/MV), ethylene (D/ET) and ACC (D/ACC; ACC being the direct precursor of ethylene) upon imbibition on water in darkness at 10ºC. ND seeds germinated fully by 72 hours, whereas D reached a maximum of 20% after 7 days. Ethylene treatment on dormant seeds have succeeded to alleviate dormancy by around 90% at day 3. D/ACC and D/MV have improved germination of D as germination rates reached 40 and 63% respectively after 7 days of imbibition**.**

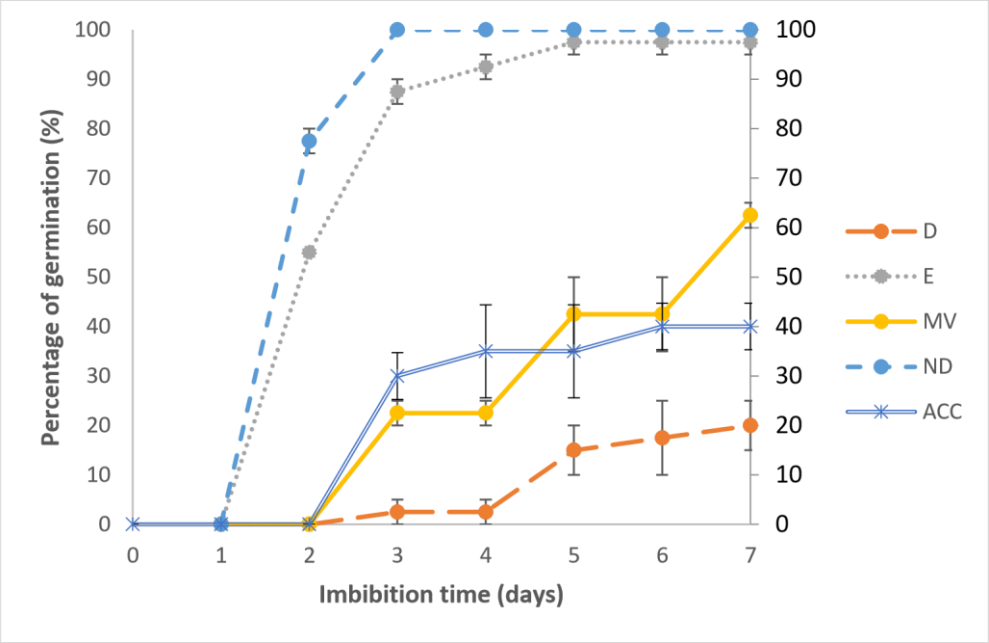

Fig. 1. Germination of D, ND, D/ET, D/ACC and D/MV sunflower embryos (i.e. naked seeds without pericarp) upon imbibition time (days). Small dashes (ND), big dashes (D), perfect line (D/MV), dots (D/ET) and double lines (D/ACC). Dormant embryos were treated with 0.1 mM methylviologen and with 1mM ACC. MV treatment was carried out for 3 hours before transferring the embryos to wet cotton wool at 10˚C. Values are means of six replicates ± SD.

III.2. Wall Stiffness Assessment

AFM technique permits to indent a material and record the force and the deformation. From this data it is possible to extract the stiffness of a solid material through its apparent Young's modulus (EA, the coefficient of elasticity). The higher the EA, the less elastic (or

stiffer) the tissue (Peaucelle et al., 2011). In this work AFM was used to study cell-wall mechanical changes related to the degree of stiffness in each treatment used (D, ND, D/MV and D/ACC) at 24 hours of imbibition. Measurement has been performed at several area of the seed axis to map this change (Fig. 2 A). Figure 2 (B and C) shows the difference between D and ND moving from a rigid to a soft state, respectively, especially at the meristematic zone (corresponding to the middle column). D/ACC and D/MV have presented a softer tendency than D but at a lesser manner than ND (Fig. 2 B, C, D and E). Statistical analyses of the average of Young's moduli confirm that all treatments corresponding to germinating seeds are less stiff comparing to non-germinating seeds, dormant ones (Fig. 2 F).

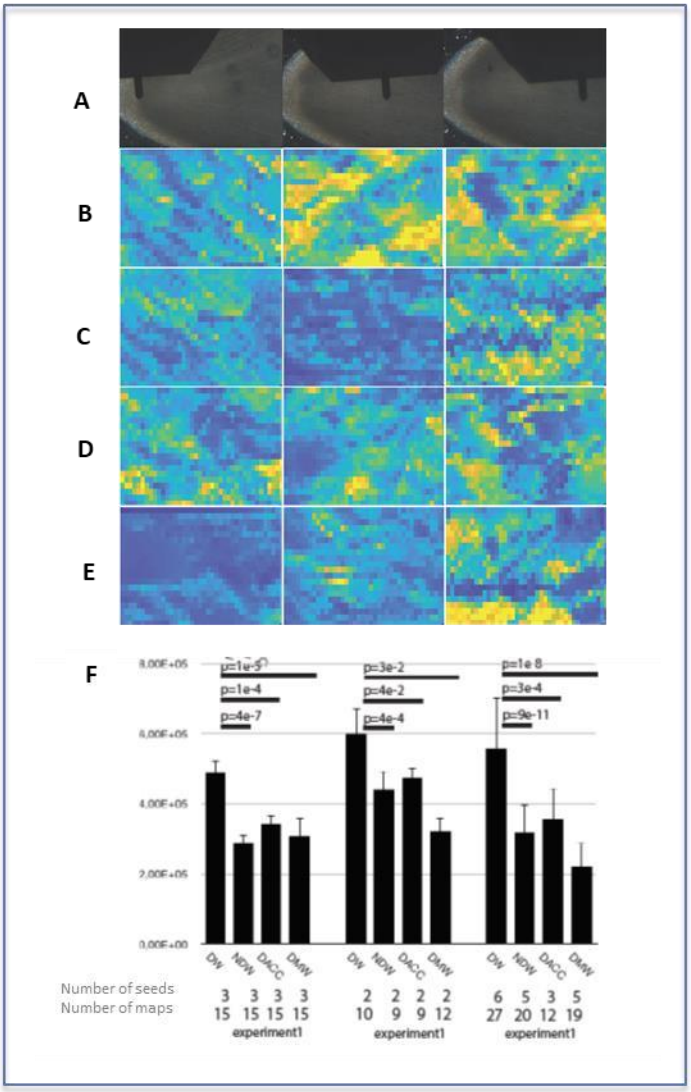

Fig. 2. Wall Stiffness in sunflower seed axis. (A) sunflower axis measured along the axis, from the tip to the base (from the left to the right, the tip, meristem and the base). (B, C, D and E) stiffness maps of cells in the three areas presented in (A), corresponding to dormant (D), non- dormant (ND), D treated with ACC (D/ACC) and methylviologen (D/MV), respectively. (F) Average Young's moduli measured on cell walls of the meristematic cells.

III.3. Characterization of Cell Wall Monosaccharide Composition

In order to assess the cell wall composition change between germinating (ND, D/ET and D/MV) and non-germinating seeds (D), quantification of cell wall monosaccharide have been done using gas chromatography. It was shown that sunflower seed cell walls contain mainly arabinose (43,5 %) with the presence of xylose, galacturonic acid, galactose, rhamnose, mannose, fucose and glucuronic acid (15, 11, 10, 8, 7, 1 and 0.7% respectively) (Table 1), suggesting that pectin groups are present such as homogalacturonan which is a polymer of 1,4α-linked galacturonic acid (GalA), in addition to rhamnogalacturonan I that contains a backbone of αGalA(1- > 2)-α-L rhamnose(1- > 4) disaccharide repeat carrying on the rhamnose residues, arabinan, galactan side chains and xylogalacturonan which is an HG substituted at O-3 with a ß-linked xylose. Comparison between D and all the other treatments upon the imbibition times tested (3, 15 and 24h), showed that no significant content change has been recorded (supplemental figure 1), suggesting that cell wall remodeling did not take place in the first 24 hours of imbibition.

Table 1. Monosaccharides content (%) in dormant sunflower cell wall axes (values are means of 3 replicates ± SD).

Sunflower Cell Wall Monosaccharides Content (%)	
Arabinose	43.5 ± 3.02
Xylose	15.15 ± 1.77
Galacturonic acid	11.38 ± 1.17
Galactose	9.54 ± 0.71
Rhamnose	8.16 ± 1.68
Mannose	7.17 ± 1.84
Fucose	1.4 ± 0.05
Glucuronic acid	0.7 ± 0.18

III.4. PME Activity Measurements

In order to study qualitative modification of cell wall of sunflower seed axes through the different treatments mentioned above, PME activity has been assessed at 24h of imbibition as compared to dry seeds (0h) and 48h which corresponds to almost 80 % of germinated seeds as a positive control of elongated growing seedlings.

PME activity kept relatively stable at 0 and 24 hours in ND (21.63 ± 0.23; 21.45 ± 2.83, respectively) (Fig. 3), but it peaked at 48 hours (31.51 ± 2.90) in elongated seeds, whereas it didn't change significantly in D (ρ=0.66>0.05) nor in D/ET (ρ=0.13>0.05) upon imbibition times and between each other (ρ =0.857>0.05). Surprisingly, at 24 hours PME activity was significantly (ρ =0.00<0.05) reduced in D/MV (3.37 ± 1.30) compared to D (15.63 ± 2.05) and it kept low at 48 hours (5.51 ± 0.81).

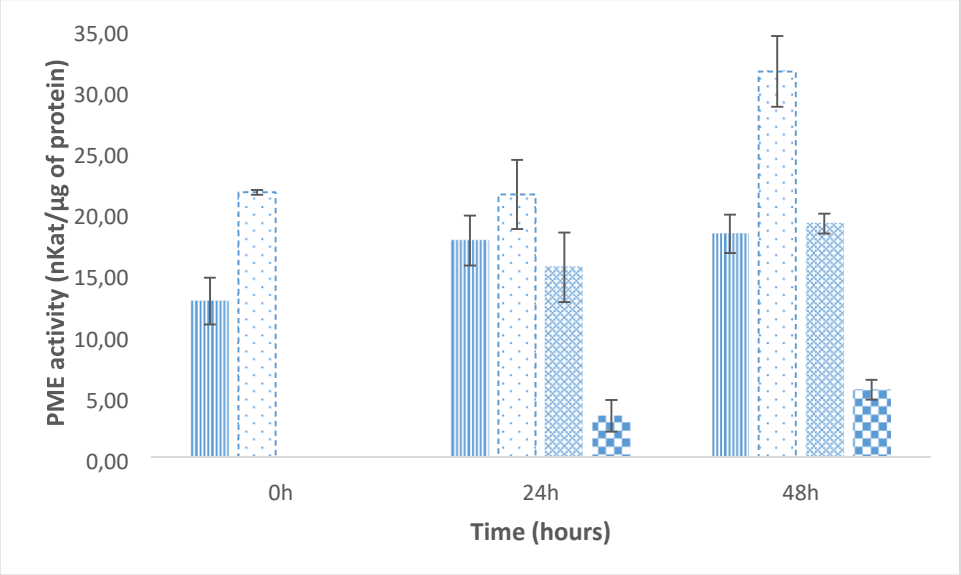

Fig. 3. Pectin methyl-esterase activity (nKat/µg of protein) of hulled sunflower seed axis at dry state, 24 and 48 hours of imbibition time on D (lines), ND (dots), D/MV (squares) and D/ET (cross). Values are means of three replicates ± SD.

III.5. In Situ Detection of Cell Wall Methylation

PMEs catalyze the specific demethylesterification of HG within plant cell walls and give rise to different degree of methylesterification (DM) in pectin backbones. In order to investigate

PMEs action on homogalacturonan, D, ND, D/MV and D/ET seed axes were analyzed by immunofluorescence using LM20, a monoclonal antibody which recognizes esterified epitopes present in pectin with DM above 50% and 2F4, a monoclonal antibody recognizing the dimeric association of pectic chain through calcium ion, thus recognizing HG stretches that are largely demethylesterified (up to 40% DM). At 24 hours of imbibition, LM20 epitopes are more abundant in the thickened cell walls, especially in the zones of cell adhesion at the corner with continuous but less pronounced fluorescence in the anticlinal walls in D compared to D/ET or D/MV (Fig. 4). Hence, less methylesterified domains highlighted by 2F4 epitopes, were more present in ND compared to the other treatments, D being the less labelled (Fig. 4). 2F4 epitopes was located mainly at the synclinal walls of the meristem in all germinating seeds (and ND) with continuous signal in ND versus interrupted one in D/ET and D/MV (Fig. 4). Lower signal has been recorded in D seeds where only few epitopes are present at the corners of cell-cell adhesion zones (Fig. 4).

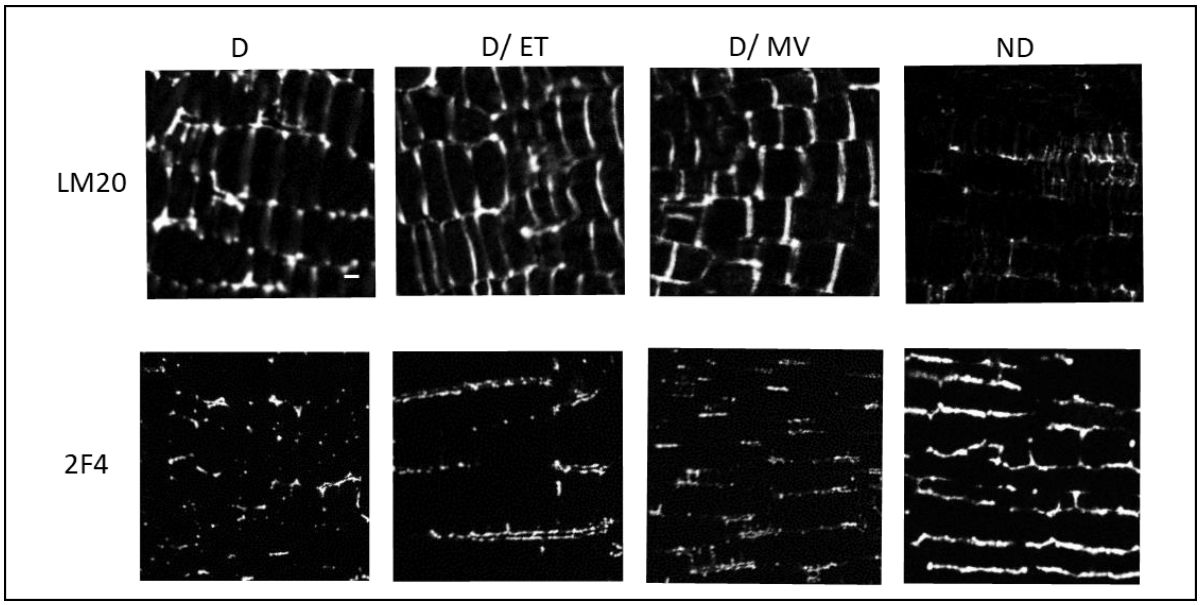

Fig. 4. Sunflower seed axis cell walls in meristematic zones immunolabeled with LM20 and 2F4 monoclonal antibodies in D, D/ET, D/MV and ND at 24 hours of imbibition. Bar = 30μm.

IV. Discussion

The completion of germination requires cell-wall weakening by decreasing in the mechanical restraint for radicle protrusion. To the best of our knowledge, AFM analyses were

performed for the first time to assess the degree of stiffness of germinating as compared to nongerminating sunflower seeds during germination *sensu stricto*, ie. before visible radicle

protrusion. AFM results have clearly shown the softening of cell walls occurring in germinating seeds as compared to dormant ones. Worth-noting that for AFM analyses, ACC treatment was used instead of ethylene as the latter is difficult to use due to its gaseous form. ACC induced dormancy breaking but not as much as ethylene. The low germination rate seen in D/ACC may be explained by the fact that being a precursor of ethylene, ACC may have a slower kinetic in seed germination response as its action depends on the activation of ACC oxidase (ACO) which catalyzes the latest reaction for ethylene biosynthesis. It is considered as a rate-limiting steps in ethylene biosynthetic pathway in dormant seeds. Indeed, after-ripening-induced dormancy decay is associated with an increase of ACO transcripts which is correlated with the enzyme activity (Corbineau et al., 2014). ACC induced germination was comparable to that of MV treatment (Fig. 1). Nevertheless, we showed that ACC and MV treatments as after-ripening (ND) enhance seed softness. Hence, at 24h, results have shown that ND presented a softer meristem texture than D (Fig. 2) which is in accordance with what Peaucelle et al. (2008) have suggested that the increase in elasticity both comes before and within organ emergence and correlated with an increase in pectin demethylesterification which was shown for ND case (Fig. 4). The soft texture seen in D/ACC underlines the possible ethylene action on PME and/or other cell wall hydrolases mentioned above or its action in synergy with ROS thus having cell wall loosening effect. Hence, it has been demonstrated that ethylene markedly enhanced ROS accumulation within dormant embryonic axes, probably through the activation of NADPH oxidase (El-Maarouf-Bouteau et al., 2015) or by its antagonist effect on the ABA inhibition of seed $^{\bullet}OH$ production (Graeber et al., 2010). Indeed, the latter authors have shown that ethylene promotes weakening of the micropylar endosperm by the expression of ROS that may cause cell wall loosening or cell separation of this tissue. Worth-mentioning that Peaucelle et al. (2011) have proposed a non-enzymatic action for plant cell-wall loosening which might be explained by the increase in cell wall hydration, facilitating the sliding of wall polymers or the mobility of wall modifying agents (ie: expansins, XTH) and increasing extensibility.

Sunflower seed cell wall composition analysis did not show any quantitative difference between germinating and non-germinating seeds (supplemental figure1) suggesting that polysaccharide metabolism did not take place during germination *sensu stricto*. In fact, the control of growth and development does not only require a precisely regulated biosynthesis of cell wall components, but also constant remodeling and modification after deposition of the polymers (Xiong et al., 2015). Moreover, the degree and pattern of methylesterification of

pectin are critical for biomechanical properties of cell wall, inducing change in porosity, permeability and elasticity mediating elongation and growth processes (Wolf et al., 2009;

Peaucelle et al., 2012). It was also supposed to occur during germination *sensu stricto* in seeds since several transcriptomic studies showed the up-regulation of PME genes (Morris et al., 2011; Dekkers et al., 2016). Transcriptomic data on sunflower seeds showed also the upregulation of PME3 gene in ND and D/ET and PME2 and 4 in D/ET as compared to D at 24h (Xia et al., 2018). The PME activity results presented in this study do not disagree with previous transcriptomic reports, they point out the delay existing between gene transcription and enzyme activity. In fact, in ND, no significant change in PME activity has been recorded at 24 h, while it increased markedly at 48h which corresponds to the completion of germination time for 80% of the seeds (Fig. 1). This phenomenon was not shown for D/ET as PME activity had remained stable comparing 24 and 48h but may have occurred in a time-shifted way proportionally to the difference between ND and D/ET in germination rate (Fig. 1). In fact, several papers have reported the induction of PME activity by ethylene treatment. Lohani et al. (2004) showed that ethylene treatment induced PME peak at day 2 of banana fruit ripening. Furthermore, Scheler et al. (2015) have shown that ACC treatment on after-ripened garden cress seeds had no effect on total activity of PME in the radicle but led to a strong increase in activity in the micropylar endosperm at the testa rupture time point in the cap only. Thus ethylene action might be compartment-specific and time specific or it may act on other cell wall hydrolases influencing other polysaccharides (i.e. hemicellulose: xyloglucan) or polysaccharides linkage (i.e. cellulose-xyloglucan, cellulose-pectin, xyloglucan-cellulose) leading to cell wall loosening. Indeed, in sunflower seeds, ethylene and after-ripening induced the up-regulation of beta D-xylosidase (Xyl 4), pectate lyase and xyloglucan endotransglucosylase/hydrolase (XTH) (Xia et al., 2018). Many studies have presented the effect of ethylene treatment on polygalacturonase (PG) mRNA accumulation in tomato and melon fruits (Brummell and Harpster, 2001; Nishiyama et al., 2007), β-Galactosidase (TBG4), XTH genes in kiwi fruit, and expansin gene LeExp1 mRNA in tomato fruit ripening (for review, Brummell and Harpster, 2001; Brummell et al. 2004; Merelo et al., 2017). Nevertheless, our results suggest that PME remodeling occur upon radicle elongation, after germination *sensu stricto* is completed. This is in accordance with previous work suggesting that PME activity reaches its maximum around the time of testa rupture in wild-type seeds but declined just before the completion of germination, ie. radicle protrusion (Müller et al., 2013). Surprisingly, at 24 hours of imbibition, PME activity was significantly

decreased in D/MV comparing to D or the other treatments. In addition, high methylesterified epitopes in pectin backbone were detected through immunofluorescence analyses in D/MV and at a lesser manner in D and D/ET. The lower PME activity found in D/MV and subsequently

their higher DM might be due to the fact that ROS may oxidize PME itself altering its activity and/or may act on an independent oxidative pathway for cell-wall loosening. Indeed, Fry (1998) has shown that hydroxyl radical (•OH), a short-lived highly reactive molecule, cleaves cell wall polysaccharides in a non-enzymatic reaction and can, in this way, cause wall loosening in isolated cell walls as well as in living tissues (Schopfer, 2001; Müller et al., 2009). Hence, Oracz et al. (2009) have shown that MV treatment on sunflower dormant embryos was always associated with an increase in hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) production, especially at 24 hours. These reactive oxygen species are the targets of a peroxidase-catalyzed reaction producing •OH in the cell wall (Schopfer, 2001; Schopfer et al., 2002; Rodriguez et al., 2002; Liszkay et al., 2004). Moreover, Xiong et al. (2015) have presented the effect of exogenous H_2O_2 in rice roots promoting cell expansion through modification of pectin biosynthesis and HG demethylesterification by acting as a downstream signaling molecule of auxin inducing PME activity, suggesting that ROS, hormonal and enzymatic cell-wall loosening might act synergistically in *planta*.

Interestingly, even ND as well as ROS and ethylene treatments induced tissue softening evaluated by AFM analysis, they showed different cytological detection of high and low esterified pectic groups. Indeed, Fig.4 showed a shift from high to low esterification state induced by after-ripening (comparison between D and ND), while treatments with ethylene and methylviologen induced an intermediate state, with significant decreased high-esterified epitopes in the corners which play a key role in cell-cell adhesion. Moreover, low esterified epitopes were localized in the growth axis probably facilitating cell volume increase for elongation in germinating seeds.

The seed compartment-specific regulation of mechanical properties of cell wall through the degree of methylesterification has been shown in endospermic seeds containing two layers surrounding the embryo, the endosperm and the testa (Scheler et al., 2015). Our AFM analyses showed that in sunflower, a non-endospermic seed, such differential regulation also exists but concerns the meristematic zone (Fig. 2). Interestingly, our previous work showed differential regulation of the key hormones involved in dormancy and germination (ABA, GA and ethylene)

in the meristem as compared to the whole embryo (personal data). The association of these hormones with cell wall resistance has been described. In fact, ABA reduce both the accumulation and the activity of β-1,3- glucanase and subsequent endosperm rupture

(Leubner-Metzger et al., 1995). ABA is also associated to the change in degree of methylesterification and change of the endosperm and radicle cell properties (Müller et al., 2013). GA was described as the major hormone responsible for the induction of genes corresponding to cell wall modifying enzyme in the endosperm cap (Nonogaki et al., 2000; Chen and Bradford 2000; Chen et al., 2002). *Xyl1*, corresponding to a α-xylosidase Arabidopsis mutant associated with altered endosperm resistance due to its cell wall composition, is able to germinate on paclobutrazol, an inhibitor of gibberellin biosynthesis (Sechet et al., 2016). Ethylene was reported to promote endosperm cap weakening of Lepidium (Linkies et al., 2009), and to be involved in transcriptional regulation of β-1,3- glucanase or several other endosperm cap-enriched genes have been shown (Martinez-Andujar et al., 2012) suggesting that endosperm cap weakening and rupture are promoted by ethylene. Thus, the association hormone-cell wall modifying enzyme- endosperm seems very credible in the regulation of endospermic seeds. Here we show that the same association can induce cell wall softening in meristematic cells in nonendospermic seeds suggesting a conserved process.

V. Conclusion

Germination is like a 100m race, to start exactly at the perfect moment, the seed in the starting blocks is fully ready for growth and elongation which means a massive water intake facilitated by cell wall softening. This work has supported the view that enzymatic action of pectin methyl-esterase takes place in wall-loosening of sunflower seeds during the completion of germination. Softening of the cell wall could also be achieved without demethylation of pectins but directly following oxidative stress as pointed out by methylviologen treatment through probably the increase of cell wall permeability and the facilitation of the mobility of other modifying cell wall agents.

Expansion of the embryonic root happens in a later stage and could be concomitant with apparent cell wall synthesis activity thus for the first time decoupling mechanical changes of

the cell wall which is a chemical change and cell wall expansion which requires cell wall synthesis.

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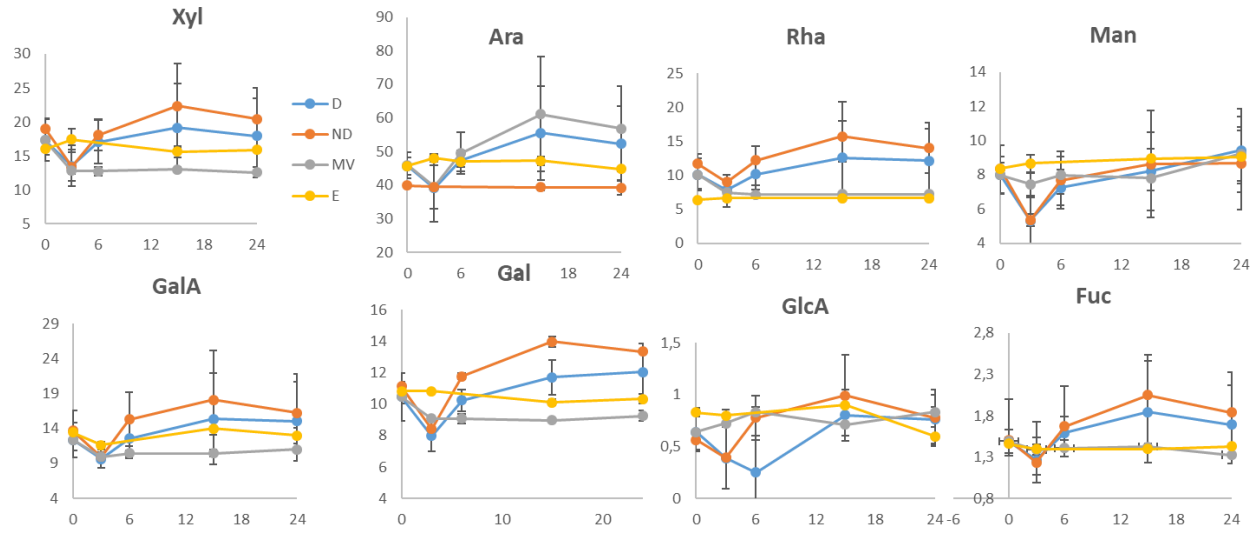
Supporting Information



Supplemental figure 1: monosaccharides content in sunflower seed embryos of dormant (D), non dormant (ND), dormant treated with ethylene (D/ET) and methylviologen (D/MV) at imbibition times (0, 3, 6, 15 and 24 hours). Values are means of 3 replicates with ±SD).

Chapter 3 Proteomics of protein-bound methionine oxidation in sunflower seed

Abstract

In order to understand the role of methionine oxidation on sunflower seed dormancy alleviation and germination completion, dormant and non-dormant sunflower seed axes at dry state and 24 hours of imbibition were subjected to diagonal chromatography LC-MS/MS analysis. This is a proteomic technique used to point out oxidized methionine residues in protein. Hence, a total of 113 unique peptide sequences containing at least one Met residue (oxidized or reduced) was found and 61 corresponding proteins were identified. Indeed, non-dormant seeds proteins were the most affected by methionine oxidation. Moving from dormant (D) to germinating seeds (ND), the major active pathways for dormancy release were: transcription and translation, protein metabolism folding and remodeling and stress response. Moreover, at 24 hour of imbibition, the time for sunflower seed germination completion, the dominant pathways were: energy and metabolism and oxidation-reduction process. The results indicate that the observed specific changes in protein Met oxidation patterns are probably required for counteracting and/or utilizing the production of reactive oxygen species caused by recovery of metabolic activity in the germinating seeds. To the best of our knowledge, this study is the first to assess the effect of Met oxidation on sunflower seed germination using proteomic approach.

Keywords: Diagonal chromatography, dormant, dormancy-alleviation, germination, methionine oxidation, non-dormant, proteomics, sunflower seed.

I. Introduction

Seed dormancy is characterized by the inability of the seed to germinate after harvest under favorable environmental conditions (Finch-Savage and Leubner-Metzger, 2006) without the help of dormancy breaking agents, such as the stratification (imbibition at low or warm temperatures), after-ripening (a period of dry storage) or phytohormones such as gibberellins (GAs) and ethylene (ET). Germination starts with water uptake and ends with radicle protrusion and initiation of cell division (Bewley, 1997). Hence, the pattern of imbibition is divided into three stages: an initial rapid period (phase I), followed by a lag phase with little change in water content (phase II) where metabolism is reactivated, and a subsequent increase in water content coincident with radicle emergence and growth resumption (phase III). Phase I and II are characterized as germination *sensu stricto* and phase III as post-germination (Bewley et al., 2013).

Throughout seed life cycle, reactive oxygen species (ROS), which are partially reduced or activated derivatives of oxygen, play a key role in various mechanisms depending on the seed moisture content. In orthodox seeds, ROS are produced from embryogenesis to germination, i.e., in metabolically active cells, but also generated in quiescent dry tissues during after ripening and storage (Bailly et al., 2008). Despite their potentially harmful reactivity toward most biomolecules, ROS also play key signaling roles in many biological processes in a wide range of organisms, such as signal transduction and gene expression, receptor activation, antimicrobial and aging (Suzuki et al., 1997). Proteins are the major cellular targets of the oxidative species, more than DNA and lipids. Nonetheless, it is surmised that oxidation of proteins is not necessarily a deleterious phenomenon. Protein oxidation occurs both at the peptide backbone and at amino acid side-chains (Davies, 2016). Depending on the oxidized amino acid, ROS damage of proteins can be irreversible such as carbonylation, thus triggering the degradation of the oxidized proteins by the cytosolic 20S proteasome or can be repaired such as methionine sulfoxidation through the action of methionine sulfoxide reductases. Amino acids vary in susceptibility to oxidative damage, methionine (Met) residue is the most vulnerable followed by cysteine (Cys) and tyrosine (Tyr) (Davies, 2005; Rinalducci et al., 2008; Kim et al., 2014). ROS, reactive nitrogen species (RNS) and reactive chlorine species (RCS) are responsible of Cys and Met oxidation (Drazic and Winter, 2014). Hydrogen peroxide (H_2O_2) oxidizes Met to Met sulfoxide (Met-SO) with a rather slow rate, while the most reactive species as hydroxyl radicals (•OH) and hypochlorous acid (HOCl) oxidize Met to MetSO with second

(Davies, 2005; Drazic and Winter, 2014). Met-SO can be reversed through the specific action of methionine sulfoxide reductases (MSRs), MSR A and B acting on S- and Rdiastereomers of Met-SO, respectively (Moskovitz, 2005). MSRs possess two redox active cysteines and function generally using a similar three step catalytic mechanism that involves the formation of a cysteine sulfenic acid intermediate, the subsequent formation of a disulfide bond and, lastly, the regeneration of reduced MSR by a reducing system, generally thioredoxin (Boschi-Muller and Branlant, 2014). MSR A reduces free Met-SO and Met-SO imbedded in a peptide or protein structure. In contrast, MSR B almost exclusively acts on peptidyl Met-SO (Drazic and Winter, 2014). Moreover, the activity of Msrs depends on the environment of the oxidized Met. Indeed, Ghesquière et al. (2011) have demonstrated a favorable docking of MSR A when methionine was preceded by Lys or Arg. Many studies have highlighted the major role of MSRs in many organisms (Drazic and Winter, 2014). Hence, despite the dogma of Met oxidation as inactivating post-translational modification (PTM), reversible Met oxidation is now being acknowledged as sacrificial antioxidants and powerful mode of triggering protein activity (Ghesquière et al., 2014). Interestingly, Tarrago et al. (2012) have shown that MSRs repair preferentially unfolded proteins highlighting their important action in the repair of damaged proteins caused by oxidative insult. Indeed, MSRs play a determinant role in the tolerance of oxidative stress and control lifespan in microorganisms, insects, and mammals, including human and thus the successiveness of physiological actions. Their involvement in ROS scavenging and redox signaling pathways could occur in seeds to limit the extent of damage resulting from the oxidative conditions reigning during maturation and imbibition. In sum, the enzymatic regeneration of Met-SO through MSR activity encompasses into three types of mechanisms (Oien and Moskovitz, 2008): the regulation of signaling pathways, the action of Met as an antioxidant for protecting proteins from higher oxidative events causing damage in protein function (Levine et al., 1996), and the subsequent formation of protein-carbonyl adducts (Moskovitz and Oien, 2010). Moskovitz and Oien (2010) have presented the fact that methionine sulfoxide modifications to proteins may precede the formation of protein-carbonyl adducts because of consequent structural changes that increase the vulnerability of amino acid residues to carbonylation. In addition, it was shown that the lack of or compromised MSR activities (mainly of MSR A) may cause faster accumulation of protein-carbonyls in yeast by ageing (Moskovitz and Oien, 2010). Thus, it is suggested that in the lack of MSRs, enhanced

protein–carbonyl accumulations may be due to an increased oxidation prompted by Met-SO which may cause conformational changes to proteins, leading to further irreversible oxidation.

Met oxidation can affect the other types of protein changes such as phosphorylation (Hardin et al., 2009). These authors have presented the fact that Met-SO can couple oxidative signals to changes in protein phosphorylation. Hence, oxidation of surface-exposed Met residues in kinase substrate proteins, can inhibit the phosphorylation of nearby sites. Thus, Met oxidation regulates the ability of kinases to bind the target protein, thereby directly linking oxidative signals to changes in protein phosphorylation.

Methionine is a strongly hydrophobic amino acid, so most Met residues were found in the hydrophobic core of proteins. While such Met residues are fairly protected from oxidation, surface exposed Met residues are susceptible to oxidation (Drazic and Winter, 2014). Hence, Met-SO is more hydrophilic than Met and this alteration may affect the protein structure and biological activity (Stadtman et al., 2003). However, Levine et al. (1996) have shown that surface-exposed methionine residues surrounding the entrance to the active site are preferentially oxidized without loss of catalytic activity, consistent with the hypothesis that methionine residues function as an endogenous antioxidant defense system. In fact, even when methionines are favorably exposed, it is important to take into account that they can be oxidized at different rates depending on the nature of their neighboring residues. Consequently, peptides having Lys-Met, Arg-Met, His-Met, and Pro-Met motifs have been shown to get oxidized at slower rates (Ghesquière et al., 2011). In addition, nearby alanines, threonines, and serines seemed to increase the oxidation susceptibility of methionines (Ghesquière et al., 2014).

A number of high-throughput, large scale-omics studies have been investigated to gain insight into molecular networks of seed dormancy and germination. Several transcriptomic analyses have provided gene expression profiles underlying dormancy-related differential gene expression (Gao et al., 2012; Dekkers et al., 2016). Recently, some reports showed the importance of translational regulation in the control of this process (Layat et al., 2014; Bassbous-Serhal et al., 2015). The absence of correlation between transcriptome and translatome reported suggests that translation regulation and consequently proteome change should be considered more than transcriptional regulation (Xia et al., 2018). PTMs represent another level of regulation in seeds. Several post-translational modifications have been characterized in seed dormancy, such as oxidation, protein redox thiol status or phosphorylation

(Rajjou et al., 2012; El-Maarouf-Bouteau et al., 2013). Henceforth, proteomics is an increasingly important tool for the study of several plant functions because it allows the investigation of the underlying molecular processes in plant physiology. Indeed, proteomic change investigated in dormant and non-dormant seeds pointed out the importance of metabolism, energy production, protein metabolism, cell growth and defense classes (Chibani et al., 2006; Wang et al., 2016; Chang et al., 2018; Xia et al., 2018). Thus, the emergence and development of proteomics technology provide a means to study the mechanisms underlying these phenomena. Given the high vulnerability of Met to oxidation, detection of Met in the reduced/oxidized state is of great importance. Drazic and Winter (2014) have listed the detection methods including mass spectrometry (Rosen et al., 2009; Ghesquière et al., 2011), chemical modification (ie. CNBr) of Met or Met-SO residues combined with amino acid analysis (Shechter, et al., 1975), radioactivity assays (Brot et al., 1982), high performance liquid chromatography coupled with SILAC-labeling (SILAC: stable isotope labeling by amino acids in cell culture (Minetti et al., 1994; Ong et al., 2002; Ghesquière et al., 2011), electrophoretic mobility shift assay on polyacrylamide gels (Saunders and Stites, 2012) and immunological methods using antibodies specific to the Met-oxidized forms (Oien et al. 2009; Wang et al., 2009; Liang et al., 2012). Combined Fractional Diagonal Chromatography (COFRADIC), first introduced by Gevaert et al. (2002), is a gel-free proteome approach which starts from a protein cell lysate that was digested with trypsin. Briefly, a subset of peptides fractions, which is highly representative of the parent proteins originally present in the lysate is selected from a first run of reversed-phase liquid chromatography. In between two consecutive identical runs, a chemical intervention is applied. Originally, when talking about methionine-COFRADIC, hydrogen peroxide (H_20_2) was introduced as the reagent that specifically oxidizes the side-chain of methionine to a methionine sulfoxide under well-controlled reaction conditions. The net result was that peptides carrying the latter amino acid became less hydrophobic. Thus, the subset of altered peptides will change elution times in the second run, while the non-modified peptides will elute at the same predictable positions. The shifted peptides can be collected for analysis. Such strategy, in which a modification is carried out between two identical runs is called diagonal chromatography. The number of secondary runs, which in principle should be equal to the number of fractions collected in the primary run, can be reduced by combining primary fractions. Thus, the shifting peptides of a given fraction do not overlap with the non-shifting

peptides of neighboring fractions and the sorted peptides elute in a less compressed manner, so facilitating their identification by further LC-MS/MS analysis. The unaltered peptides are mostly discarded, while the sorted peptides are either on-line analyzed by mass spectrometry, or collected for identification in a ternary liquid chromatography-mass spectrometry (LC-MS) coupled system. The procedure, in which fractions of the first chromatographic step are combined, modified and run in a diagonal chromatographic manner, is therefore called COmbined FRActional DIagonal Chromatography (COFRADIC).

A variant of the original COFRADIC was subsequently used by many authors, using enzymatic reduction instead of oxidation (Ghesquière et al., 2011; Jacques et al., 2015) and sometimes using the method of labeling with stable isotopes after protein digestion in order to correct for spontaneous and thus artificial methionine oxidation that may occur during sample handling (Ong et al., 2002; Ghesquière et al., 2011). Here, the reverse of the original COFRADIC sorting step was established by reducing peptides carrying methionine-sulfoxide using both MsrA and B enzymes to sort out peptides carrying in vivo formed methionine-sulfoxide and quantify their degree of oxidation by a LC-MS/MS analysis (without applying any labeling method). By this, avoiding the exogenous application of hydrogen peroxide, making the model system well suited for studying physiological methionine oxidation (Walton et al., 2016). Altering Met-SO back to methionine through a mixture of recombinant MSR A and MSR B, in-between consecutive, identical RP-HPLC peptide separations will create a hydrophobic shift of the affected peptides, leading to their specific enrichment.

In this study, proteomic approach was used on dormant and non-dormant sunflower seeds at dry state and 24 hours of imbibition (late phase II of germination *sensu stricto*). Briefly, after digestion by trypsin of proteins, peptides were separated by high pH reverse phase fractionation (HpRP). Sequential elution with an increasing ACN concentration was performed. Fractions were collected, split equally in two, F1 as control and F2 as MSR treated fraction. Fractions were then analyzed by nanoLC-ESI-MS/MS for protein identification. Thus, the aim was to investigate, at both times, the major proteins containing Met-SO and their relation with seed dormancy alleviation and germination completion in order to establish the first "Metoxomes" of seeds and find new elements involved in dormancy and germination.

II. Materials and Methods

II.1. Seed Materials and Treatment Conditions

Three batches of sunflower (*Helianthus annuus*, "LG5665") seeds, harvested in 2015 near Montlémar (Drôme, France) and purchased from Limagrain (www.limagrain.com), were used in this study. At harvest, dormant seeds (D) were stored at -20ºC until use in order to maintain their dormancy. After-ripening (ND) was performed by placing seeds at 25ºC and 75% relative humidity (moisture content of approximately 0.05 g water/g dry weight) for at least 3 to 4 months to break their dormancy. Germination tests were performed with seeds without pericarp (embryos) in darkness, in 9-cm Petri dishes (25 seeds per dish, three replicates) on a layer of cotton wool moistened with distilled water. Petri dishes were placed at 10ºC, a suboptimal temperature for sunflower seed germination (Corbineau et al., 1990). An embryo was considered as germinated when the radicle had elongated to 2-3 mm. Germination counts were made daily for 7 days and germination percentage means are the results of six replicates ± SD. Dry and imbibed (24h in darkness at 10ºC) D and ND sunflower seeds were used for the following experiments.

II.2. NAD(H), NADP(H), Glutathione and Ascorbate Pool Measurement

NAD(H), NADP(H), glutathione and ascorbate (ASC) contents were measured using a plate reader method of Queval and Noctor (2007). 100 mg of seed axes were ground by a mortar and a pestle using liquid nitrogen and was stored at -80˚C till the time of experiment. Extraction was done by adding 1 ml of 200 mM HCl for NAD(P) and 1 ml of 200 mM NaOH for NAD(P)H measurements. The supernatant was collected by centrifugation at 16000 g for 10 min at 4 °C. 200 µl of the supernatant was incubated in boiling water (100 ˚C) for 1 min and neutralized by adding 50 µl of 200 mM NaH2PO4 (pH 5.6) and a certain amount of 200 mM NaOH to the final pH=6-7 for NAD(P) and a certain amount of 200 mM HCL to the final pH=6-7 for NAD(P)H. To assay ASC and glutathione, supernatant aliquot of 0.5ml was neutralized as above, without heating, with approximately 0.4ml of 0.2 M NaOH in the presence of 100µl of 0.2 M NaH2PO4 (pH=5.6) with a final pH of the neutralized acid extracts between 4.5 and 5. For the plate-reader, 20 µl of the neutralized supernatant was introduced into the plate wells with 100 µl of 0.1 M Hepes (pH 7.5), 2 mM EDTA, 20 µl of 1.2 mM DCPIP, 10 µl of 20 mM

PMS, 25 µl water, 10 µl ADH for NAD/NADH and 10 µl G6PDH for NADP/NADPH. The reaction was started by adding 15 µl of 100% ethanol for the former and 10 µl of 10 mM G6P for the latter. Readings were recorded at A600. For all assays, the reaction mix was homogenized by programmed shaking. Following initiation of each reaction as stated below, the mix was shaken twice and then readings were taken at the appropriate wavelength every 23s with programmed mixing by shaking between each reading. For the quantification middle time at 2.5 min was chosen to choose the best results. Contents were calculated by reference to standards run concurrently. To assay ASC, triplicate aliquots of 40 µl neutralized supernatant were introduced into plate wells containing 0.1ml of 0.2M NaH2PO4 (pH 5.6) and 55 µl water. Readings were recorded at A265 before and after addition of 5 µl AO. To assay total ascorbate, 0.1ml neutralized supernatant was first added to 0.14ml of 0.12M NaH2PO4 (pH 7.5) and 10 µl of 25 mM DTT, and solutions were incubated for 30min at room temperature unless stated otherwise. Triplicate aliquots of this solution were then assayed as described for ASC. Without pretreatment of extracts, GSSG was achieved by pretreatment of 0.2 ml neutralized extract with 2-vinylpyridine (VP) for a 30min incubation at room temperature to complex GSH. To measure total glutathione, triplicate aliquots of 10 µl neutralized extract were added to plate wells containing 0.1ml of 0.2 M NaH2PO4 (pH 7.5), 10 mM EDTA, 10 µl of 10 mM NADPH, 10 µl of 12 mM DTNB, and 60 µl of water. The increase in A412 was monitored after addition of 10 of GR. After removing excess VP, GSSG was measured by the same principle after, triplicate 20µl aliquots of the final supernatant were assayed as described above with 50 µl of MQ water added. Principal Component Analysis (PCA) was performed by using MetaboAnalyst 4.0 (Chong et al., 2018) to compare the treatments among and the metabolites clustering tendencies. Two-way ANOVA was used to check the significance between treatments and metabolites through SPSS 20.0 software.

II.3. Protein Extraction

Thirty axes of sunflower seeds were grind in liquid nitrogen using a mortar and pestle until getting a fine powder. Powder was mixed in a cold eppendorf tube (2mL) with 800 µL solubilization buffer (Urea (8M), ammonium bicarbonate (50mM), CHAPS (2.5% (w/v)), antiprotease cocktail sigma (1‰ (v/v), β-mercaptoethanol (0.07% (v/v)) then mixed and briefly centrifuged (30 sec, 10 000g). Resulting supernatant was filtered through a syringe equipped with a polytetrafluoroethylene (PTFE) membrane filter unit (Ministar Sartorius; 25mm, 0.22µm

pore size). Protein concentration were measured using 2-D Quant Kit (GE Healthcare Life Sciences).

II.4. Protein Digestion

Based on protein concentration measurements, 1 mg of protein samples were digested. Samples were diluted by 2 in a buffer containing ammonium bicarbonate (final concentration 50 mM) and urea (final concentration 4 M). Cysteine residues were reduced (2.3 mM dithiothreitol (DTT) final, 56°C for 30 min) and alkylated (5 mM iodoacetamide final, room temperature for 30 min, in the dark), followed by addition of DTT to a final concentration of 2.3 mM. Samples were diluted by 4 in ammonium bicarbonate 50 mM. Trypsin (Promega, gold, mass spectrometry grade) was added to a ratio protein/enzyme of 30/1. Incubation was carried out overnight at 37°C. Samples were acidified with formic acid (FA) (final concentration 5 % vol), desalted using Sep-Pak C18 cartridges (Waters) and eluted with 80 % acetonitrile (ACN)/FA(2 %). Samples were dried down using Eppendorf Speed Vac (Concentrator Plus 5305) and resuspended in 10 % FA.

II.5. Diagonal Chromatography

Figure 1 summarizes strategy used. For high pH reverse phase fractionation (HpRP), Sep-Pak C18 cartridges were used (1cc, 50 mg, Waters). Cartridges were washed with 1 mL of ACN and conditioned with 2 times 1 mL of LB (NH4OH 10 mM (adjusted to pH 10)). 150 µg of protein digests were resuspended in 1 mL of LB and loaded on the cartridge. Sequential elution with 200 µL of solutions containing 10 mM NH4OH and increasing ACN concentration (10 % (v/v), 12.5 % till 40 %) was performed. Fractions were collected, split equally in two (F1 and F2) and dried down. Fractions were resuspended in 40 µL of NH4HCO3 (50 mM), DTT (10 mM). In the F2 fractions, 3.5 µL of methionine sulfoxide reductase MSRA and 3.5 µL of MSRB (Jena Bioscience) were added. All fractions were incubated for 2 h at 37°C. Fractions were then acidified (FA 10 %) and analyzed by nanoLC-ESI-MS/MS.

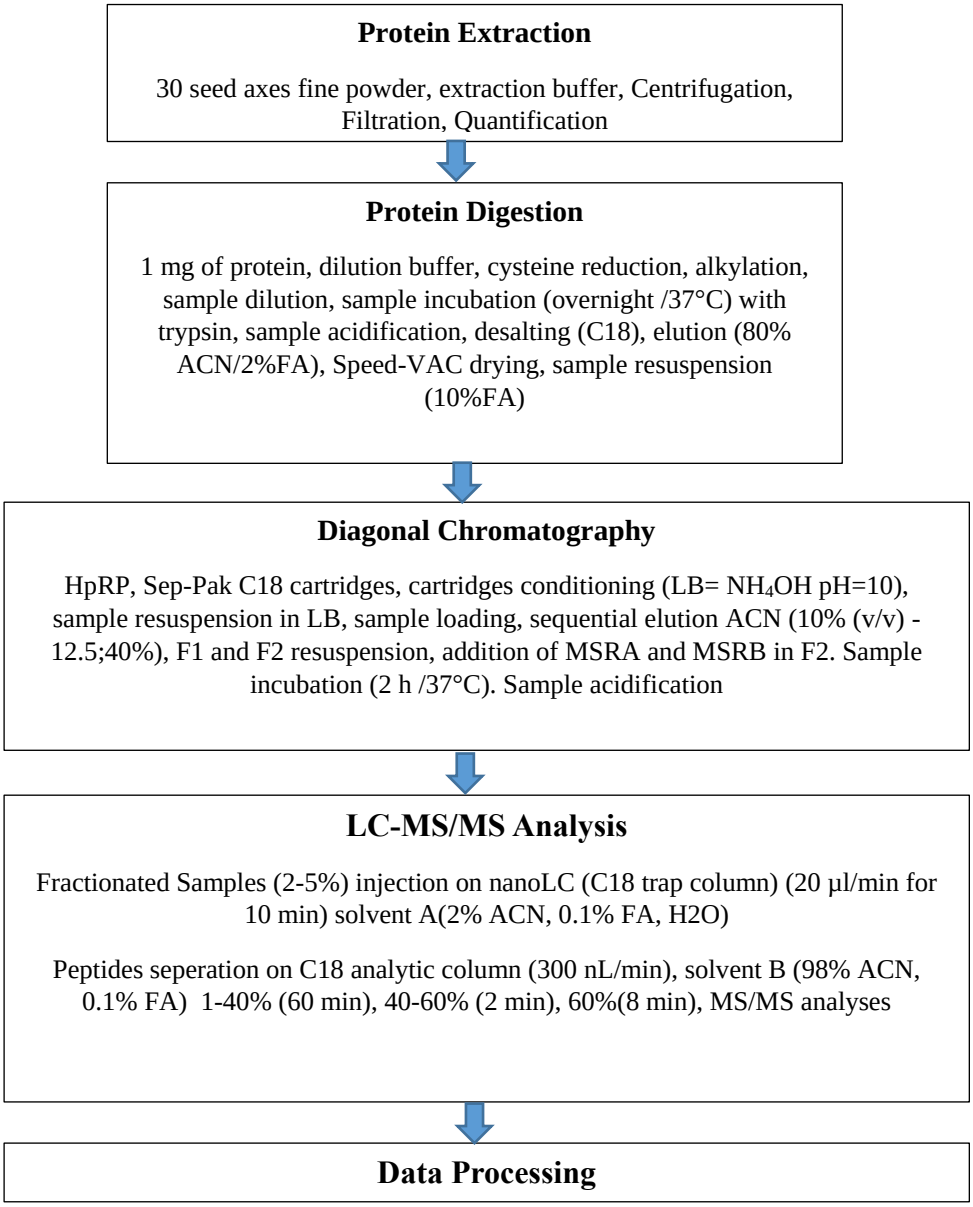


Fig. 1. Isolation of methionine sulfoxide containing peptides from a complex mixture of sunflower seed axes proteins using diagonal chromatography. ACN: acetonitrile, FA: formic acid, F1: fraction 1, F2: fraction 2, HpRP: high pH reverse phase fractionation, MSR: methionine sulfoxide reductases.

II.6. LC-MS/MS Analysis

2-5 % of the fractionated samples were injected on a nanoLC Ultimate 3000 (Dionex) and trapped on a C18 trap column (5 µm, 100 A° pore, 300 µm i.d., LC Packings, France) at a flow rate of 20 µl/min for 10 min using solvent A (2% ACN, 0.1% formic acid in H2O). Subsequently, the peptides were separated on a 50 cm analytical column packed with C18 phase (3 µm, 75 µm i.d.) at 300 nL/min with the following gradient: 1% to 40% of solvent B (98% ACN, 0.1% formic acid) in 60 min, 40% to 60% B in 2 min, 60% B for 8 min. The nanoLC was

coupled to a LTQ Orbitrap XL (Thermo Fisher Scientific) operated in positive ion mode. All the MS spectra were acquired on the Orbitrap and the MS/MS spectra on the LTQ. The survey scans were performed with the following parameters: m/z range 300 - 2000, resolution 30 000, AGC target 2.105, maximum injection time 100ms. The 3 most intense precursors were selected for data dependent CID fragmentation scans with the following parameters: minimum intensity 500, isolation window 2 Da, normalized collision energy 35 %, AGC target 5000, maximum injection time 100ms. Dynamic exclusion was enabled (repeat count 1, duration 60 sec).

II.7. Data Processing

Peak lists were generated from the raw files using Proteome Discoverer (version 1.3, Thermo Fisher Scientific). Peak lists were searched against sunflower data base (https://www.heliagene.org/HA412.v1.1.bronze.20141015) using Mascot (version 2.4, Matrix Science) search engine software. The following parameters were used: mass tolerance of 30 ppm for the precursors and 0.6 Da for fragment ions, enzyme specificity Trypsin with 2 missed cleavages allowed, carbamidomethylation of cysteines as fixed modification, oxidation of Met as variable modification. PSMs were validated with the following parameters: < 1% false discovery rate (peptide validator node), minimal peptide Mascot score of 20, and search engine rank 1. Peptide areas were calculated by Proteome Discoverer. All reports were opened together to allow for homogeneous protein grouping.

Peptides containing an oxidized Met were validated according to the following criteria: (i) the peptide must exist in both F1 (non-treated) and F2 (MSR-treated) fractions; (ii) in F2, Met residues must be reduced at least partially; (iii) retention times must be consistent throughout the same series, and peptides containing reduced Met residues must elute later then oxidized ones. For 1 fraction (a single LC-MS/MS run), an abundance percentage was calculated for each peptide by dividing this peptide area by the sum of the areas of all forms of this peptide.

III. Results and Discussion

III.1. Germination Assay

Release of sunflower seeds dormancy occurs during after-ripening (storage in dry conditions) allowing the transition from dormant (D) to non-dormant (ND) state. Figure 2 shows the germination behavior of D and ND sunflower seeds at 10˚C. D seeds reached a maximum of 20% of germination after 7 days, whereas ND became able to fully germinate within 3 days.

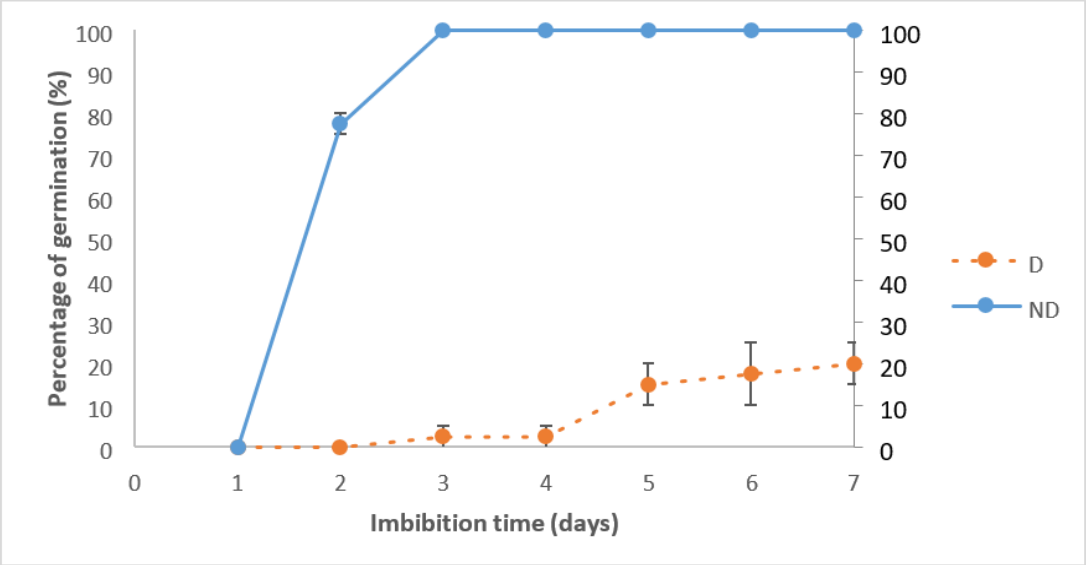


Fig. 2. Germination of dormant (D) (dots) and non-dormant (ND) (perfect line) sunflower seeds upon imbibition time (days). Seeds were imbibed with distilled water at 10 °C in darkness, an embryo was considered as germinated when the radicle had elongated to 2–3 mm. Values are means of six replicates ± SD.

III.2. Redox Metabolite Status

As an attempt to check the redox metabolites profile in D an ND seeds at 0 and 24h of imbibition, a spectrophotometrical approach was applied using the method of Queval and Noctor (2007).

III.2.1. Redox treatments profile

Using data (not shown except for GSH and GSSG) of quantification of ascorbate(Asc), dehydroascorbate (DHA), glutathione (GSH), glutathione disulfide (GSSG), nicotinamide

adenine dinucleotide (NAD), NAD reduced (NADH), NAD phosphate (NADP) or NADP reduced (NADPH), 3D-Principal component analysis (3D-PCA) was used to search for global patterns among treatments upon redox metabolite changes (Fig. 3). The PC1 indicated a separation between two groups according to imbibition times: D and ND at dry state and D and ND at 24 hours of imbibition clustered together. PC2 and PC3 have shown a separation of treatments: D at 0 and 24h and ND at 0 and 24h. Worth-mentioning that a significant difference ($\rho<0.05$) was seen between ND0 and D24/ND24.

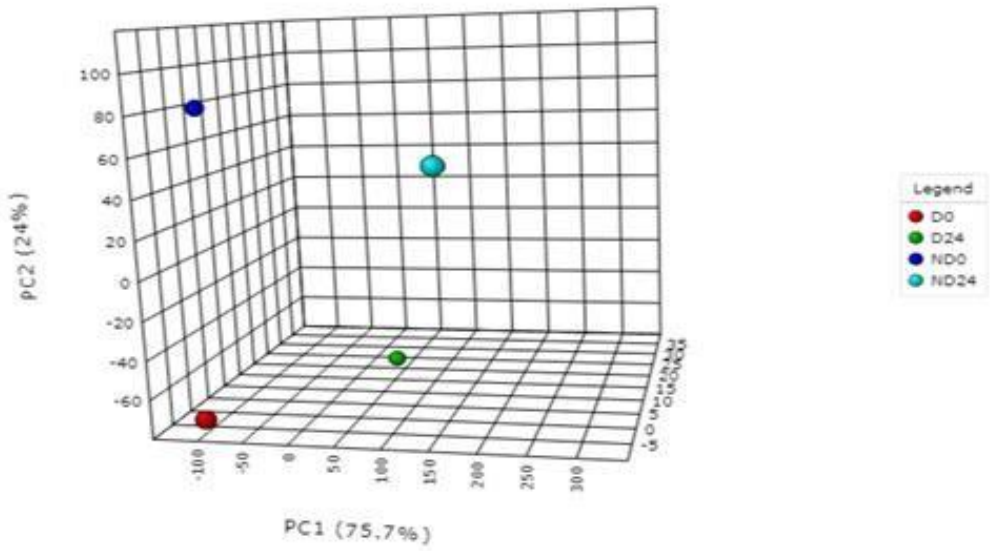


Fig. 3. 3D-Principal Component Analysis (3D-PCA) showing global trends among treatments upon their redox metabolites changes. The treatments are: D: dormant, ND: non-dormant and 2 imbibition times: 0 and 24 hours. PC1, PC2 and PC3 accounted for 75.7, 24% and 0.4% of the variance among treatments respectively.

III.2.2 Redox metabolites profile

Global impacts of the treatments and imbibition times on the metabolic pools were displayed by the Principal Component Analysis (PCA) (Fig.4). The PCA showed themaximal variance across the first two components (PC1 and PC2) which together explain 99.7% of variability where GSH presented a distinct cluster.

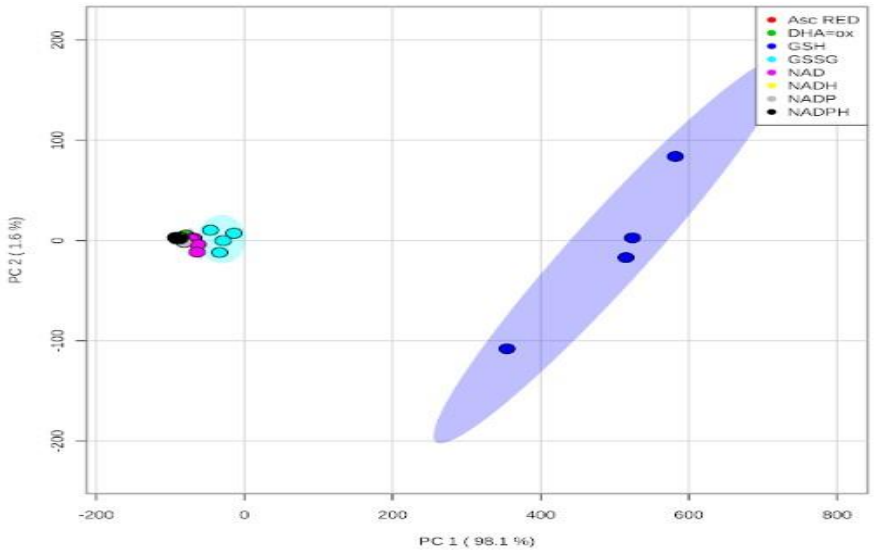


Fig. 4. Principal Component Analysis (PCA) was applied to the expression of all metabolites trends. PCA has shown the different metabolites (Asc, ascorbate; DHA, dehydroascorbate; GSH, glutathione; GSSG, glutathione disulfide; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADP, NAD phosphate; NADPH, reduced NADP) grouping according to tendency upon treatments (D and ND) and imbibition times (0 and 24 hours). PC1, PC2 accounted for 98.1% and 1.6% of the variance among treatments respectively.

This complete and significant ($\rho<0.05$) separation of GSH comparing to the other metabolites showing a moving tendency of this latter upon treatment and imbibition times (Fig. 5).

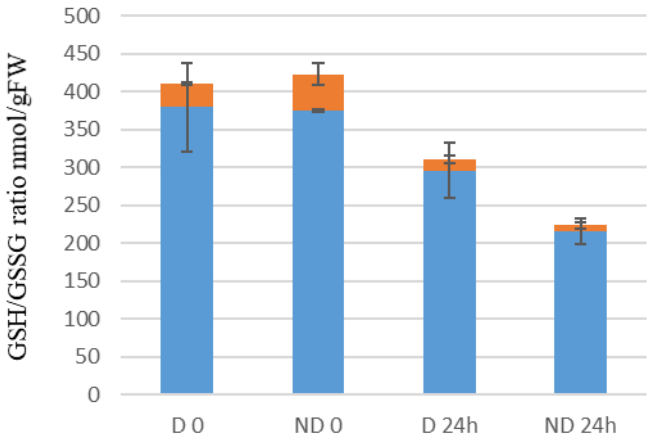


Fig. 5. GSH, glutathione (blue) and GSSG, glutathione disulfide (orange), contents in D and ND at dry state (D 0 and ND 0) and during 24 hours of imbibition (D 24h and ND 24h).

Hence, GSH was the most expressed metabolite among the others. Indeed, GSH is the major intracellular thiol, water soluble antioxidant which also participates in the control of the cellular redox status (Foyer and Noctor, 2005; Kranner et al., 2006). Redox changes of GSH play a central role in the onset of metabolism during germination (Kranner and Grill, 1993; Buchanan and Balmer, 2005). Moreover, Foyer and Noctor (2005) have presented the fact that in metabolically active tissues, millimolar concentrations of GSH act as a key redox buffer, forming a barrier between protein Cys groups and ROS. In addition, the activities of ascorbate–glutathione cycle enzymes are well known to be responsible for scavenging ROS (Ma et al., 2017). Indeed, GSH is used as an antioxidant to reduce oxidized ascorbate in the ascorbate-glutathione cycle which is proved to act as well on cell wall loosening by stimulating H+ATPase activity thus leading to cell wall loosening and giving rise to oxalate which promotes wall loosening by binding calcium (Smirnoff, 1996). Nevertheless, as a result, the reduction of GSSG to GSH back release NADP+ thus stimulating the NADPH-oxidase associated in ROS production so triggering germination in seeds (Müller et al., 2009). As for GSSG content, Bahin et al. (2011) have presented that GSSG accumulates during dry storage of Barley grains. Hence, Tommasi et al. (2001) have shown that higher GSSG content in dry *Pinus pinea* L. seed could contribute to inhibit germination process since it blocks protein synthesis. During germination, the level of GSSG decreases due to the continuous activity of glutathion reductase as an attempt to protect other protein from oxidation. The small variance seen for NAD may be due to its remobilization probably to allow a restart of metabolism and to respond to germination needs such as the production of energy and ROS which intervenes in signaling processes (Oracz et al., 2009, El-Maarouf-Bouteau et al., 2015). All together these data highlight the active redox status for the sake of the “oxidative window” for an optimum balance thus having a full circle of the germination process.

III.3 Protein Identification and Expression Profiles among Different Treatments

Comparative proteomics using the diagonal chromatography LC-MS/MS technique (Fig.1) on D and ND sunflower seed axes at dry state and late phase II (24h) of germination *sensu stricto* was performed, and the results are discussed based on functional classifications of differentially Met-oxidized or reduced containing proteins in both treatments at the same imbibition time. Protein oxidation is in direct relation with dormancy release and germination

completion. To show the global features of the specific functional categories, only protein showing Met-SO in one treatment and Met in the other at the same imbibition time at once, were shown in Table 1 (whole list of identified proteins containing Met is shown in Table S1). A preliminary analysis allowed the identification of 2881 peptides corresponding to 1221 proteins. Among these peptides, 113, corresponding to 78 proteins, contained at least one Met residue (Table S1). In order to study the relation between the regulation of proteins and methionine oxidation, the oxidation state of identified peptides was compared in D and ND with the hypothesis that this regulation is involved in the germination process, thus showing different trends (only oxidised or only reduced) between these two physiological conditions. Venn Diagrams were drawn to select peptides with different oxidised behaviour. In figure 6, the red circles show 18 peptide sequences which were found containing Met-SO in ND and Met in D at dry state, whereas 7 were found in the reversed case. In addition, at 24h of imbibition, 23 peptide sequences were found containing Met-SO in ND and Met in D, conversely 10 were found in the opposite case, it has been noted that some of them were found in both imbibition times. In sum, among the 113 identified peptides, 41 peptides were selected as differentially oxidised in D vs ND allowing the identification of 34 proteins (Table 1).

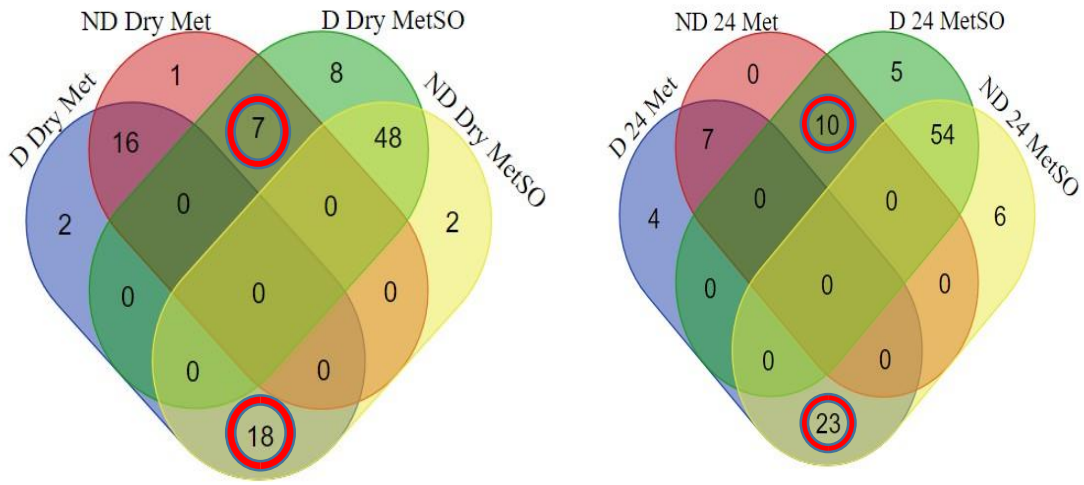


Fig. 6. Venn diagrams showing the number of sunflower seed peptides containing methionine differentially modified (oxidized and reduced) between conditions: dormant (D) and non-dormant (ND), and at different imbibition times (0 and 24 hours).

Many of the identified proteins were involved in transcription and translation, stressinduced process, protein metabolism, binding and remodeling, oxido-reduction process,

energy and metabolic pathways, cytoskeleton and cell growth and division. Hence, the corresponding proteins containing Met/Met-SO are listed in Table 1 and discussed below according to their functional pathways. To investigate a possible role for the primary sequence of proteins in the oxidation sensitivity of Met, an iceLogo sequence alignment (Colaert et al., 2009) of neighboring amino acids was generated for the 41 peptides whose oxidized Met were selected. Methionine was fixed at position 6 (P6) and the five surrounding amino acids both N and Cterminal to this site were retrieved (P1–P11). Interestingly, Met-SO peptides revealed a motif of hydrophobic amino acids such as glycine, valine and alanine prior to the oxidation site (Fig. 7). This hydrophobic motif was already observed by Jacques et al. (2015) who had emitted the hypothesis of an increased susceptibility of oxidation due to the hydrophobic environment.

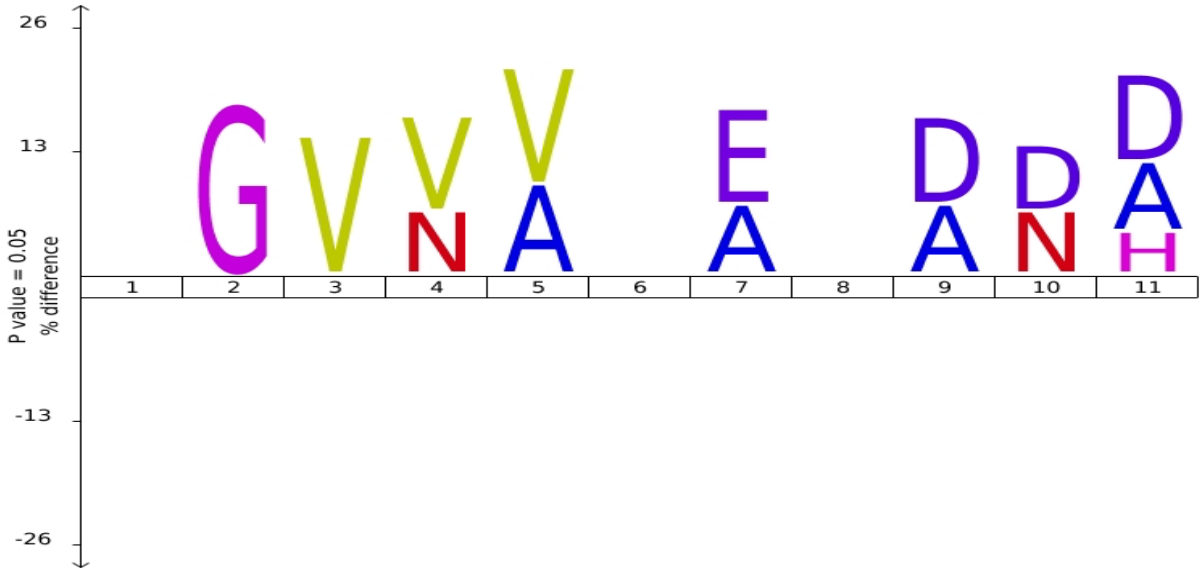


Fig. 7. An iceLogo of the amino acid context of the oxidized methionine. The sequence context of proteins (n 41) reveals a preference ($\rho \leq 0.05$) for hydrophobic amino acids prior the site of methionine oxidation follow by acidic amino acids. Methionine was fixed at P6 and the five surrounding amino acids; both N-terminal (P1–P5) and C-terminal amino acids (P7–P11) are shown.

Table 1. Proteins containing methionine being oxidized in one condition and reduced in the other and vice versa, in dormant (D) and non-dormant (ND) sunflower seed axes at dry or 24 hours of imbibition, with specified position of Met (M). The Heliagene accession accession number, protein name, identified peptide sequences and Met position in protein sequences are indicated. The different position statuses of oxidized Met in the protein sequence are presented as follow: E: exposed, M: medium, B: bury, they were identified by using http://raptorx.uchicago.edu/StructurePropertyPred/predict/

Heliagene Accession	Protein Name	Short Name	Identified Peptide Containing Oxidized Met	Met Position	Position Status of Met
D dry MetSO/ND dry Met					
Ha412v1r1_14g028420	Leucine-rich repeat, typical subtype	Leu-rich_rpt typical-subtype	LPENMEGLR	M47	B
Ha412v1r1_00g085610	Actin-related protein	Actin	YPIEHGIVSNWDDMEK	M67	B
Ha412v1r1_13g024520	Armadillo-type fold	ARM-type_fold	IMTHLATSEDAEVR	M195	B
Ha412v1r1_05g023550	Ribosomal protein L5 domain	Ribosomal_L5_dom_sf	AMQLLESGLK	M69	M
Ha412v1r1_12g005440	Heat shock protein 70 family	Hsp_70_fam	DAGAISGLNVMR	M173	M
Ha412v1r1_05g032250	Late embryogenesis abundant-3	LEA-3	EVAAESGEESMEWAEWAK	M344	M
D dry Met/ND dry MetSO					
Ha412v1r1_01g037770	Ribosomal protein L1, conserved site Ribosomal protein L1, superfamily	Ribosomal_L1-like	VCMLGDAQHVEEAQK	M66	B
Ha412v1r1_13g032980	Ribosomal protein S13, H2TH 30s ribosomal protein S13, C-terminal	Ribosomal_S13-like_H2TH 30s_Rbsml_prot_S13_C	YSQVTSNALDMK	M61	E

Ha412v1r1_05g024240	Ribosomal protein S25	Ribosomal_S25	MVSAHASQQIYTR	M68	M
Ha412v1r1_06g009560	Seed maturation protein	SMP	NQPEMATYPGGVAASMAAAAR	M163	B
Ha412v1r1_03g003500	Seed maturation protein	SMP	GGPAAVMQSAASVNR	M34	B
Ha412v1r1_15g000960	Transcription factor TGA like domain	TGA_domain	IVSASVSDMSEEQVER	M109	M

Ha412v1r1_03g020300	Peptidase A1/Saposin-like/Aspartic peptidase	Aspartic_peptidase_A1/Saposin-like/Peptidase_aspartic_dom_sf	GYWQFDMGDVLIGDK	M272	B
Ha412v1r1_10g048900	Cyclophilin-like peptidyl-prolyl cis-trans isomerase domain	Cyclophilin-type_PPIase_dom	IVMELYADTTPR	M22	B
			HVVFGKVVEGMDVVK	M143	M
Ha412v1r1_05g049460	Phosphoglycerate kinase	Phosphoglycerate_kinase	MADDCIGPDVEK	M37	M
Ha412v1r1_05g000840	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain	Bifunc_inhib/LTP/seed_store	RGQFGGQEMDIAR	M260	M
Ha412v1r1_10g049450	L-lactate/malate dehydrogenase	L-lactate/malate_DH	SEVVGYMGDENLGK	M87	E
Ha412v1r1_11g034310	Caleosin	Caleosin-related	VMAMADGNALTPEADCQPLTHER	M6 M8	E E
Ha412v1r1_16g013830	CBS domain	CBS_dom	TDNIHPTMEPQVHLK	M17	M
Ha412v1r1_03g033550	Pyruvate,phosphate dikinase/Pyruvate/Phosphoenol pyruvate kinase-like domain	Pyruvate_phosphate_dikinase Pyrv/PenolPyrv_Kinaselike_dom	VMANADTPNDALTAR	M606	B
Ha412v1r1_10g001660	Haem peroxidase	Haem_peroxidase	MGQLSVLTGTQGEIR	M313	B
Ha412v1r1_16g052300	Universal stress protein A	Universal_stress_UspA	ATCADYGITAEGMTEIGDPK	M104	M

D24 MetSO/ND24 Met					
Ha412v1r1_14g028420	Leucine-rich repeat Leucine-rich repeat, typical subtype	Leu-rich_rpt Leu-rich_rpt_typical-subtyp	LPENMEGLR	M47	B
Ha412v1r1_13g024520	Armadillo-type fold	ARM-type_fold	IMTHLATSEDAEVR	M195	B
Ha412v1r1_03g033550	Pyruvate, phosphate dikinase Pyruvate/Phosphoenolpyruvate kinase-like domain	Pyruvate_phosphate_dikinase Pyrv/PenolPyrv_Kinaselike_dom	FLDMFGNVVMGIPHALFEEK	M215	B
Ha412v1r1_09g053620	Glucose/ribitol dehydrogenase	SDR_fam	IMNMTGAQGVLYPTSIQSSEVK	M214	M
Ha412v1r1_17g029260	Late embryogenesis abundant-3	LEA-3	KADQMSGQAFNDVGVMDEDDDAK	M373	E
Ha412v1r1_17g030380	Cyclophilin-like peptidyl- cis-trans prolyl isomerase domain	Cyclophilin-type_PPIase_dom	IVMELFADTTPR	M22	B
Ha412v1r1_11g000420	Ribosomal protein L3	Ribosomal_L3	AHLMEIQVNGGTVAQK	M211	M
Ha412v1r1_11g034310	Caleosin	Caleosin-related	VMAMADGNALTPEADCQPLTHER	M6	E
D24 Met/ND24 MetSO					
Ha412v1r1_00g082090	Ribosomal protein S4, bacterial-type Ribosomal protein S4/S9	Ribosomal_S4_bac-type Ribosomal_S4/S9	IFEGEALMR	M79	B
Ha412v1r1_13g032980	Ribosomal protein S13-like, H2TH 30s ribosomal protein S13, C-terminal	Ribosomal_S13-like_H2TH 30s_Rbsml_prot_S13_C	YSQVTSNALDMK	M61	E

Ha412v1r1_05g024240	Ribosomal protein S25	Ribosomal_S25	VNNMVLFDK	M16	M
			MVSAHASQQIYTR	M68	M
Ha412v1r1_05g023550	Ribosomal protein L5/Ribosomal protein L5 domain	Ribosomal_L5/Ribosomal_L5_dom_sf	AMQLLESGLK	M69	M
Ha412v1r1_12g039630	ATPase, F1 complex, beta subunit/ ATP synthase, F1 beta subunit/ ATPase, F1 complex beta subunit/V1 complex, C-terminal	ATP_synth_F1_beta_su/ATPase_F1/V1_b/a_C	TIAMDGTEGLVR	M138	B
Ha412v1r1_00g069190	Alcohol dehydrogenase superfamily, zinc-type L-threonine 3-dehydrogenase GroES-like NAD(P)-binding domain	L-Threonine_3-DHase/GroESlike_sf/NAD(P)-bd_dom	EAGKPLVMEEVEVAPPQK	M22	M
Ha412v1r1_10g048900	Cyclophilin-like peptidyl-prolyl cis-trans isomerase domain	Cyclophilin-type_PPIase_dom	IVMELYADTTPR	M22	B
Ha412v1r1_05g049460	Phosphoglycerate kinase	Phosphoglycerate_kinase	IVPASSIPDGWMGLDIGPDSIK	M239	M
Ha412v1r1_13g032940	RmlC-like jelly roll fold	RmlC-like_jellyroll	KMVGECHQSCQGSQGEQQHPQCFQSCIQK	M157	E
Ha412v1r1_03g033550	Pyruvate, phosphate dikinase Pyruvate/Phosphoenolpyruvate kinase-like domain	Pyruvate_phosphate_dikinase Pyrv/PenolPyrv_Kinaselike_dom	LSEVNPMLGFR	M733	M
			VMANADTPNDALTAR	M606	B
Ha412v1r1_03g027340	Aconitase/iron regulatory protein 2 Aconitase/isopropylmalate dehydratase	Aconitase/IRP2	MFVDYSEPQQEK	M429	B
Ha412v1r1_10g001660	Haem peroxidase	Haem_peroxidase	MGQLSVLTGTQGEIR	M313	B
Ha412v1r1_12g005440	Heat shock protein 70 family	Hsp_70_fam	NQVAMNPQNTVFDAK	M63	E
			DAGAISGLNVMR	M173	M

Ha412v1r1_01g023910	11-S seed storage protein, plant	11S_seedstore_pln	QESVLFMPGGSYTGSR	M461	E
Ha412v1r1_13g015610	Poly [ADP-ribose] polymerase 3 isoform X1	PARP	SDAMWSDFSQR	M517	M
Ha412v1r1_13g028340	Translation elongation factor EF1A, eukaryotic/archaeal/Translation elongation factor EFTu/EF1A, bacterial/organelle/P-loop containing nucleoside triphosphate hydrolase	Transl_elong_EF1A_euk/arc/Transl_elong_EFTu/EF1A_bac/org/P-loop_NTPase	VETGVIKPGMVVTFGPSGLTTEVK	M264	B
Ha412v1r1_09g053500	Glyceraldehyde/Erythrose phosphate dehydrogenase family	GlycerAld/Erythrose_P_DH	VVDLIVHMASVAK	M333	B
Ha412v1r1_05g032250	Late embryogenesis abundant-3	LEA-3	EVAAESGEESMEWAEWAK	M344	M
Ha412v1r1_05g027000	Annexin	Annexin	YEGSEVNMTLA	M165	M
Ha412v1r1_06g009560	Seed maturation protein	SMP	NQPEMATYPGGVAASMAAAAR	M163	B

III.4. Functions and Pathways Analysis of Differentially Expressed Proteins

The oxidation of proteins at the methionine level can have several consequences, one of which may be the regulation of the activity of modified proteins (ie. activation or inhibition). The results obtained are discussed in the context of dormancy and germination of sunflower seed, complex phenomena combining activation and/or inhibition of cellular processes (ie: transcription, translation, metabolism, stress response…).

III.4.1 Transcription and translation

Several translational factors were found to be oxidized mainly in non-dormant seeds at 0 and 24h Ribosomal protein L1(60S ribosomal protein L10a-1); L5; S4; S13(40S ribosomal protein S18) ; S25(40S ribosomal protein S25) and for L3 and L5 at 24h and 0h respectively in dormant seeds. Regulation of ribosomal proteins (r-proteins) gene expression can occur at transcriptional, post-transcriptional, translational and post-translational level depending on the organism (McIntosh and Bonham-Smith, 2006). Indeed, the most described r-protein posttranslational modification are phosphorylation and abiotic stress related phosphorylation suggesting that r-protein modification is an important part in translation regulation in plant cell (Pérez et al., 1990). Henceforth, plants have a r-protein translational control mechanism as for the interactions of kinase and phosphorylation which increase in recruitment of r-protein mRNA into polysome in Arabidopsis cell culture (Turck et al., 2004). Beltrán-Peña et al. (1995) have demonstrated the storage of r-protein mRNA in maize embryos for active translation during germination. Indeed, dormancy releasing treatments motivate translation activities (Lu et al., 2018). Several studies highlighted the importance of the translation machinery in conditioning germination, seed longevity and vigor. Thus, mRNA translation is critical for Arabidopsis (Rajjou et al., 2004), rice (Sano et al., 2013) and sunflower (Layat et al., 2014) seed germination. Nevertheless, Job et al. (2005) have presented a range of translation factors undergoing carbonylation during Arabidopsis seed germination which can be another level of regulation.

The elongation factor 1-alpha (EF1A, Ha412v1r1_13g028340), which is responsible for the selection and binding of the cognate aminoacyl-tRNA to the A-site (acceptor site) of the ribosome, was the target of Met264 oxidation in ND24. Interestingly, a previous study conducted in the laboratory on Arabidopsis seeds had shown that EF1A3 (AT1G07920, Q0WL56), which has 97% of identity with sunflower EF1A, contained an oxidized Met264 in Arabidopsis ND seeds at dry state (unpublished data). Moreover, Chang et al. (2018) have

underlined its role during germination by mentioning that an abundance of EF1A is a prerequisite for rapid cell division, which is necessary for *Gnetum parvifolium* seed germination. Thus, specific methionine oxidation in EF1A (M264) might play a role in regulating the function of the protein and in fine tuning of the translation of mRNAs within the cell.

The transcription factor TGA like domain (Ha412v1r1_15g000960), which is a transcriptional activator, was found to be oxidized at dry state in ND (M109). Indeed, the TGA like domain, by its binding to the as-1-like cis elements, mediates auxin-inducible transcription (Niggeweg et al., 2000). Auxin, which is known as a plant hormone, it acts on cell cycling, growth and development, formation of vascular tissues, and pollen and development of other plant parts (Miransari and Smith, 2014). Many studies have shown the interplay between auxin and ABA signaling pathway in Arabidopsis seed dormancy and that dormancy is dramatically released when having auxin receptor or biosynthetic mutants (Liu et al., 2007; 2013).

III.4.2 Protein metabolism, binding and remodeling

The seed mobilizes proteins such as seed maturation and desiccation-associated proteins that are degraded concurrent with the imbibition to provide primary amino acids, and, therefore, storage proteins are downregulated after seed germination (Yang et al., 2007; Nakabayashi et al., 2005). Hence, seed maturation proteins (Ha412v1r1_06g009560 (M163), Ha412v1r1_03g003500 (M34)) were found to be oxidized in ND, at dry state and at 24 hours of imbibition. In fact, late embryogenesis abundant proteins (LEA) which are seed maturation proteins accumulate after maturation drying and remain at high levels in the mature seeds (LePrince et al., 2017). They are thought to act by stabilizing protein and membrane structure, removing ROS, confering desiccation tolerance to plant seeds, replacing water and sequestering ions (Tunnacliffe and Wise, 2007; Battaglia et al., 2008). In maize seeds, LEA proteins decreased in amount during germination when the seed lost its desiccation tolerance (Huang et al., 2012). In addition, analysis of the proteome changes during pea seed germination revealed that the abundance of SBP65 (belonging to the group 3 of LEA proteins) continuously decreased during germination, coinciding with the loss of desiccation tolerance (Wang et al., 2012).

Furthermore, aspartic peptidase A1 (Ha412v1r1_03g020300), proposed to degrade seed storage proteins providing amino acids for seed germination (Simões and Faro, 2004), were found to be oxidized (M272) at ND0. Müntz et al. (2001) have mentioned that several

proteinases such as aspartic proteinase and carboxypeptidases were found to target storage protein breakdown in seeds and this can start by globulin mobilization since dry state in embryonic axis.

One of cystathionine beta-synthase (CBS) family protein (Ha412v1r1_16g013830, (M17)) was found to be oxidized in ND at dry state. CBS domains may act as autoinhibitory regulatory units in some proteins and activate or further inhibit protein function upon binding to adenosine nucleotides (AMP, ADP, ATP, S-adenosyl methionine (Ado-Met), NAD, diadenosine polyphosphates) so CBS domain-containing proteins can sense cell energy level (Baykov et al., 2011).

III.4.3 Energy and metabolism

For this category, almost all enzymes related to metabolism and energy were found to be oxidized in ND and reduced in D (Table 1). Indeed, the germination of seeds requires efficient use of energy via metabolism. Thus, Phosphoglycerate kinase (PGK, Ha412v1r1_05g049460, (M37, M239)) which is enrolled in both glycolysis and neoglucogenesis,Glyceraldehyde/erythrosephosphate dehydrogenase (Ha412v1r1_09g053500, (M333)) involved in glycolysis pathway, L-lactate/malate dehydrogenase (MDH1, Ha412v1r1_10g049450, (M87)), which participate in the glycolysis and Krebs cycle, Aconitase/iron regulatory protein 2 (Ha412v1r1_03g027340, (M429)), an enzyme that catalyzes the isomerization of citrate to isocitrate in the TCA cycle and Pyruvate, phosphate dikinase (PPDK, Ha412v1r1_03g033550 M606, M733) were found to be oxidized in ND. The latter was also oxidized in D24 on M215 (Table 1). An RmlC-like jelly roll fold (deoxythymidine diphosphates-4-dehydrorhamnose 3,5-epimerase, Ha412v1r1_13g032940, (M157)), which is a protein domain found in different enzymes related to glycolysis and neoglucogenesis, hormonal regulation, redox defense and others, was found to be oxidized in ND24.

Interestingly, in sunflower Xia et al. (2018) have presented the regulation of such enzymes in seed dormancy alleviation and germination. Hence, Gallardo et al. (2002) have shown that aconitase accumulated prior to radicle protrusion during germination of the Arabidopsis seeds. Moreover, Job et al. (2005) have underlined the fact that this enzyme appears to be particularly susceptible to oxidase damage presumably because of the presence of a [4Fe-4S]$^{2+}$ cluster at its active site, which can result in the release of free iron and, in turn, trigger a

cascade of oxidative modifications in macromolecules via the Fenton reaction. Interestingly, PPDK, which is involved in photosynthesis in C4 plants, has been shown to decrease during germination *sensu stricto* in rice seeds (Xu et al., 2016) and to be carbonylated in ND sunflower seeds (Oracz et al., 2007).

In sum, oxidation of proteins related to energy and metabolism has been already underlined during seed germination. These enzymes are involved in glycolysis/gluconeogenesis pathways and TCA cycle which are the most predominant biochemical pathways during seed germination. Specific oxidation of glycolytic enzymes has also been in response to oxidative stress. Hence, it has been proposed previously that blocking glycolysis could be beneficial during conditions of oxidative stress since it would result in an increased flux of glucose equivalents through the pentose phosphate pathway leading to the generation of NADPH which could provide the reducing power for antioxidant enzymes (Job et al., 2005) or triggering other oxidative modifications through Fenton reaction for the sake of dormancy alleviation and germination process. These authors have shown that many of these proteins were the target of carbonylation reported in *Arabidopsis thaliana* seeds which could highlight again the link between the Met sulfoxidation as a prerequisite for protein carbonylation (Moskovitz and Oien, 2010).

Furthermore, generation of energy from oxidative phosphorylation associated with ATPase F1 complex/ATP synthase, F1 beta subunit (Ha412v1r1_12g039630) can also be involved as the protein was found to be oxidized in ND24 on M138. The ATPase family act as proton pump, which is the main mechanism responsible for reducing the internal pH of the cell, altering the membrane potential and being used as power source for the hydrolysis of proteins stored within the cellular tissues of seeds. Moreover, in tomato seeds, an increase in the expression of genes encoding ATPase in response to treatment with gibberellic acid promoted cell wall acidification, facilitating cell elongation and consequently longer roots (Trindade et al., 2018). However, Job et al. (2005) have shown the sensitivity of ATP synthase to oxidation during Arabidopsis seed germination. In sunflower seeds, recent data suggest that ABA dependent inhibition of plasma membrane (PM) H^+-ATPases could participate in dormancy maintenance and that ethylene- and ROS-dependent PM H^+-ATPases stimulation could participate in dormancy release in sunflower seeds (De Bont et al., unpublished data).

III.4.4 Stress response

Stress induces many unfavorable conditions as the aggregation of newly synthesized protein. Yet as a remedy comes the role of heat shock protein (HSP). Indeed, HSPs act as molecular chaperones by repairing and aiding the renaturation of proteins damaged by stress (Sun et al., 2002). In maize, HSPs can also act within the first days of rehydration (Huang et al., 2012) and are associated in Arabidopsis with seed development, reserve synthesis and mobilization, protein translocation (Forward et al., 2002). Moreover, Xu et al. (2016) have found 2 HSPs increased in embryo during germination process of rice seeds. Hence, HSP70 were found to be mainly oxidized in ND24 (Ha412v1r1_12g005440 (M63, M173)) in addition to D0 (Ha412v1r1_12g005440 (M173); thus, showing their importance in maintaining the stability of proteins required during germination. Nevertheless, Oracz et al. (2007) and Job et al. (2005) have shown that, a large number of molecular chaperones like HSPs were found to be carbonylated during sunflower and Arabidospis seed germination, respectively. In fact, they suggested that by their oxidation, HSP may act as shields protecting proteins from oxidative damage.

Other stress related proteins have been characterized such as Universal stress protein A (Ha412v1r1_16g052300, (M104)), which confers oxidative stress resistance and dehydration to plant, was found to be oxidized in ND at dry state. In *Arabidopsis thaliana* seeds, this protein has shown to be involved in germination (Gorshkova et al., 2018). Glucose/ribitol dehydrogenase (Glc/RibDH) (Ha412v1r1_09g053620, (M214)), also found to be oxidized in D24, belongs to a class of short alcohol dehydrogenases, most of which are known to be NAD- or NADP-dependent oxidoreductases against abiotic stress and salinity tolerance. It was shown that Glc/RibDH transcripts and protein were found in developing barley embryos, and the level of transcript was observed to decrease during germination (Witzel et al., 2010). Indeed, in rice, Glucose/ribitol dehydrogenase homolog is known to change (Sano et al., 2013) and to be phosphorylated (Han et al., 2014) during germination. Thus, its oxidation in D and reduction in ND at this imbibition time might underline its essential role in ND as a stress tolerance against abiotic factors limiting seed germination completion.

III.4.5 Oxidation–reduction-related proteins

Finnie and Svensson (2009) have shown in Barley that several proteins involved in redoxregulation were observed to change in abundance during germination. This process is

presented here by the action of peroxidases which are important proteins in cellular detoxification. These haem-containing enzymes use hydrogen peroxide as the electron acceptor to catalyze a number of oxidative reactions. They were found to be oxidized in ND (Ha412v1r1_10g001660, M313) at both imbibition times.

Annexin D2-like, which are multifunctional soluble proteins capable of Ca^{2+}-dependent or independent association with membrane phospholipids, have many function in plants including peroxidase activity (Mortimer et al., 2008; Laohavisit and Davies, 2011). Annexin D2-like (Ha412v1r1_05g027000, (M165)), was found to be oxidized in ND24.

Alcohol dehydrogenase (ADH, Ha412v1r1_00g069190), which is a group of dehydrogenase responsible for maintaining the NAD+ level in cells, was found to be oxidized (M22) at 24 hours in ND. Indeed, Oracz et al. (2007) have shown that ADH was being carbonylated in after-ripening imbibed sunflower seeds. However, alcohol dehydrogenase transcripts were found to be up-regulated in barley and Arabidopsis seeds during early imbibition (Sreenivasulu et al., 2008; Weitbrecht, et al., 2011). In addition, ADH was shown to increase during germination of rice seeds (Yang et al., 2007).

In sum, these results showed the possible interplay between the oxidized proteins characterized in our study with hormones or central metabolism, several of them being regulated by redox metabolites. Many of them have been already related to dormancy or germination. Their respective role in this process remain however to be refined.

IV. Conclusion

In this study, diagonal chromatography LC-MS/MS analysis was used as a proteomic technique to identify the role of methionine oxidation on seed dormancy alleviation and germination completion of dormant and non-dormant sunflower seed axes at dry state and 24 hours of imbibition. In general, the level of protein containing methionine oxidation was higher in nondormant axes than in dormant ones which is as well in accordance with what Oracz et al. (2007) have found as carbonylated protein in non-dormant sunflower seeds. Moving from dormant (D) to germinating seeds (ND), the major active pathways for dormancy release were: transcription and translation, protein metabolism folding and remodeling and stress response. Moreover, at 24 hour of imbibition, the time for sunflower seed germination *sensu stricto*, the dominant pathways were: energy and metabolism and oxidation-reduction process. Indeed, oxidation was not randomly distributed but targeted specific proteins such as: translation factors

which inhibition may provide a means of preventing continued gene expression under potentially error-prone conditions; Proteins folding and remodeling which oxidation may act for keeping the ROS level optimal for the germination process and/or limiting oxidative damage on other seed components, providing primary amino acids for other *de novo* synthesis or even facilitating protein mobilization during germination; Energy and metabolism which oxidation may play a role in triggering a cascade of oxidative modifications thus reducing power for antioxidant enzymes or triggering other oxidative modifications; Stress response and oxido-reduction enzymes which may be acting as shields protecting proteins from oxidative damage. It will be interesting to test other imbibition points during germination *sensu stricto*; thus tackling direct changes during seed germination especially at 6 hours and 15 hours, two imbibition times which have been already underlined as important in sunflower seed germination cycle (Xia et al., 2018). Nevertheless, it has to be taken into account that these results are qualitative and further quantitative experiments are in progress. In addition, since reversible Met oxidation is now being acknowledged as sacrificial antioxidants and powerful mode of triggering protein activity (Ghesquière et al., 2014), the major role of MSRs *in vivo* should be as well pointed out to check the kinetic response of these enzymes on the oxidized protein found above.

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Supporting Information

Table S1. Proteins containing methionine being oxidized or reduced in dormant (D) and nondormant (ND) sunflower seed axes at dry or 24 hours of imbibition. Identified peptide sequences, corresponding samples, oxidized Met position (modification), peptide mass (MH+), Heliagene accession number (Acc.), gene name, corresponding chromosome (Chr), protein description, number of Met affected, number of peptide-to-spectrum matches (PSMs), number of proteins, number of protein groups and number of miscleavage are indicated. (Check attached file)

Chapter 4 General conclusion

Seed dormancy and germination are very complex traits. Studying the effects of oxidative modifications on dormancy alleviation and the germination process comes in great importance for a better understanding and control of these mechanisms. The present work tackles on cell wall loosening, one major aspect in seed germination process, and the possible hormonal (ethylene) and oxidative (ROS) effects on this phenomenon, along with the relation of protein oxidation, methionine sulfoxidation, on dormancy alleviation and germination completion.

Our work brought the evidence that cell wall remodelling occurs prior to radicle protrusion suggesting that it is part of cellular mechanisms preparing for germination. We showed pectin demethylation, especially in the cell-cell adhesion zones in the meristem when moving from dormant to germinating seeds. PME activity was not shown to be increased in our treatments at 24h, so to improve its involvement, a time course analyses can be performed. Moreover, spatial analysis can be of high interest using embryo microdissection. Indeed, increasing works have shown the importance of studying seed compartments because of differentiated regulation of cellular processes. Furthermore, exploring the regulation of different polysaccharides modifying enzymes, such as XTHs or expansins acting on polysaccharides links (hemicellulose-pectin-cellulose) is needed to complete the overview of enzymes involved in wall remodelling. We have shown the effect of methylviologen and ethylene on cell wall loosening through a softer texture of the meristematic zone passing by the activity of PME. Indeed, many studies have proposed a non-enzymatic action for plant cell-wall loosening which might be explained by the increase in cell wall hydration, facilitating the sliding of wall polymers or the mobility of wall modifying agents (ie: expansins, XTH) and increasing extensibility. Thus, the likelihood of direct non-enzymatic action of ROS, specifically •OH, tracking its production kinetic and action along with other ROS on cell wall as well as the interaction between ROS and ethylene and their effect on different cell wall enzymes in cell wall loosening are to be explored.

In the second part of this study, we deciphered seed biological pathways affected by dormancy alleviation and germination response to methionine sulfoxidation (Met-SO), a reversible type of post-translational modifications. We found that methionine oxidation targeted mostly germinating seed proteins both at dry state and at the end of the germination *sensu stricto* as a positive attempt to reach germination completion. Indeed, oxidation was not randomly

distributed but targeted specific proteins of different biological processes as transcription and translation pathways through several proteins like (TGA, EF1A, ribosomal proteins). These proteins which role is in providing optimal elements for germination and growth, were shown to be oxidized which may provide a means of preventing continued gene expression under potentially error-prone conditions. Moreover, proteins metabolism and folding pathway was affected too by the changes through Met-SO of proteins including LEA, SSP and proteinases which role is in providing primary amino acids for seed germination and the CBS proteins that are involved in the fine tuning of other protein and hormone activity. Their oxidation might reveal a determinant role in limiting oxidative damage on other seed components during germination process and/or providing primary amino acids for other de novo synthesis or even facilitating protein mobilization during germination. Proteins related to stress response were also affected by the oxidation of HSP70, CLD, universal stress protein along with oxidoreduction related proteins (ADH, caleosin, annexin, PARP, haem-peroxidase) which are important proteins in cellular detoxification. Thus, their oxidation may act as shields protecting other proteins from oxidative damage. At last, several key enzymes of central metabolism were identified to be oxidized like: PGK, MDH, PPDK, aconitase, RmlC-like jelly roll fold and ATPase F1 showing that there is a regulation of TCA cycle, glycolysis and pentose phosphate pathway (PPP) activities. These pathways provide the energy for cell elongation but also all the co-factors and intermediates for biological reactions, such as PTMs and reserves or hormones metabolism. Being oxidized might be for them to play a role in triggering a cascade of oxidative modifications thus reducing power for antioxidant enzymes or triggering other oxidative modifications through the PPP. Moreover, PTMs can be intensified by co-factors like NAD or gluthation intermediates which regulate proteins involved in gene transcription, protein synthesis and degradation via the proteasome, hormone metabolism, perception and trafficking and cell wall modification. Indeed, this was underlined by the fact that GSH, GSSG and NAD were the redox metabolites the most expressed in D and ND at different imbibition times. Thus, studying these phenomena in quantitative analysis by completing conditions repetitions is of great importance. Furthermore, It will be interesting to test different imbibition points during germination *sensu stricto*; thus tackling direct change especially at 6 and 15 hours, two imbibition times which have been already underlined as important in sunflower seed germination cycle (Xia et al., 2018a). Hence, targetting the key proteins in these pathways and so analysing their content and activity through imbibition times and dormancy alleviation treatments will widen the view. To have the full circle, the reversible action of MSRs can’t be

forgotten as powerful reparing system. *A. thaliana* was found to have five MSR A and nine MSR B genes (Tarrago et al., 2009). These MSRs can be present in the cytosol, chloroplasts, endoplasmic reticulum, and may be routed to the secretory pathway (Tarrago et al., 2009; Rey and Tarrago, 2018). In sunflower (*Helianthus annuus),* 5 putative MSRA and 3 putative MSRB genes were found from genome analysis (Table 1). It will be of great importance to check their genetic expression and to tackle their kinetic response on the oxidized protein found among these pathways. Finally, by using mutants of targeted oxidized proteins, we can study the relationship between ROS and hormones in dormancy alleviation mechanism as a whole.

Table 1. MSR genes family of *Helianthus Annuus* L.

MSRA				
Accession	Gene name	Chromosome	#amino acids	Name*
Ha412v1r1_05g003820	Ha5.0382	Ha5	200	MSRA1
Ha412v1r1_16g033960	Ha16.3396	Ha16		
Ha412v1r1_08g022230	Ha8.2223	Ha8	205	MSRA3
Ha412v1r1_08g030700	Ha8.3070	Ha8	137	
Ha412v1r1_08g022250	Ha8.2225			
Ha412v1r1_16g000780	Ha16.0078	Ha16	152	
Ha412v1r1_16g000420	Ha16.0042	Ha16	109	
Ha412v1r1_02g010060	Ha2.1006	Ha2	248	MSRA5
MSRB				
Accession	Gene name	Chromosome	#amino acids	Name*
Ha412v1r1_15g044640	Ha15.4464	Ha15	197	MSRB1
Ha412v1r1_15g023380	Ha15.2338	Ha15	190	MSRB2
Ha412v1r1_14g024590	Ha14.2459	Ha14		
Ha412v1r1_16g032460	Ha16.3246	Ha16		
Ha412v1r1_14g024820	Ha14.2482	Ha14	127	

*Names were based on "Needleman-Wunsch Global Align Protein Sequences" scores obtained with Arabidopsis MSR genes

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Résumé

Les modifications oxydatives des macromolécules dans la graine de tournesol au cours de la germination

Nos travaux ont porté sur la caractérisation des propriétés de la paroi cellulaire et de l'état d'oxydation des protéines, ainsi que sur leur relation avec la levée de dormance et le processus de germination. Ainsi, nous avons étudié le changement de résistance de la paroi cellulaire en utilisant différents traitements de levée de dormance: la post-maturation au sec, les espèces réactives de l'oxygène (reactive oxygen species, ROS) et une hormone (l'éthylène). L'étude du changement de résistance de la paroi cellulaire a montré une diminution de rigidité chez les graines traitées, capables de germer, par rapport à celles dormantes. La quantification des monosaccharides de la paroi n'a pas mis en évidence de différence significative, ce qui suggère l'absence de phénomènes associés au métabolisme des polysaccharides à ce stade de la germination *sensu stricto*. Seules les graines dormantes traitées avec les ROS ont présenté une diminution significative de l'activité de la pectine méthylestérase, une enzyme de la paroi connue pour son rôle dans l'élongation et la croissance. Une analyse immunocytologique a permis de décrire une paroi cellulaire plus estérifiée chez les graines dormantes. Ainsi, les ROS pourraient avoir une action non-enzymatique alors que l'éthylène pourrait agir en synergie avec les ROS afin d'obtenir le relâchement nécessaire de la paroi cellulaire pour la croissance. En outre, une analyse protéomique combinant la technique chromatographie à une analyse LC-MS / MS a été utilisée sur des graines de tournesol dormantes et non dormantes à différents temps d'imbibition afin d'identifier des protéines contenant des méthionines sulfoxydées, une oxydation protéique réversible. Des analyses préliminaires suggèrent un niveau d'oxydation des protéines au niveau de la méthionine plus important dans les graines non dormantes. De façon intéressante, de nombreuses protéines cibles de l'oxydation de la méthionine sont liées à de différents processus biologiques (traduction, métabolisme des protéines, énergie et le métabolisme, processus d'oxydo-réduction) connus pour être des acteurs impliqués dans la germination, suggérant ainsi l'importance de l'oxydation de la méthionine dans la régulation de différentes voies cellulaires impliquées dans la levée de dormance et la germination.

Dormance, espèces réactives de l'oxygène, ethylène, germination, oxydation, protéomique, tournesol.

Abstract

The oxidative modifications in the macromolecules of sunflower seed during germination

Our work focused on the relationship of cell wall properties and protein oxidation state on dormancy alleviation and germination process. Different dormancy alleviation treatments have been used: afterripening, reactive oxygen species (ROS) and hormone (ethylene). Thus, the study of the change in resistance of the cell wall has shown a decrease in rigidity in treated seeds, able to germinate, compared to dormant ones. Quantification of the identified cell wall monosaccharides did not show any significant difference suggesting the absence of phenomena related to the metabolism of polysaccharides at this stage of germination *sensu stricto*. Only dormant seeds treated with ROS showed a significant decrease in pectin methylesterase activity, a cell wall enzyme known for its role in elongation and growth. An immunocytological analysis has described a more esterified cell wall in dormant seeds. Thus, the ROS could have a non-enzymatic action while ethylene could act in synergy with the ROS to fulfil the needed relaxation of the cell wall for growth. In addition, a proteomic analysis combining the diagonal chromatography technique with an LC-MS / MS analysis was used on dormant and non-dormant sunflower seeds at different imbibition times in order to identify proteins containing methionine sulfoxyde, a type of reversible protein oxidation. Preliminary analyzes suggest a higher level of protein oxidation in non-dormant seeds. Interestingly, many proteins affected by the oxidation of methionine are related to different biological processes (translation, protein metabolism, energy and metabolism, oxidation-reduction processes) known to be involved in germination, thereby suggesting the importance of methionine oxidation in the regulation of different cellular pathways involved in dormancy emergence and germination.

Dormancy, ethylene, germination, oxidation, proteomics, reactive oxygen species, sunflower.