Regulatory role of hydrogen cyanide in dormancy removal of sunflower (Helianthus annuus L.) embryos

Krystyna Oracz

Warsaw University of Life Sciences – SGGW
Faculty of Agriculture and Biology
University Pierre and Marie Curie – Paris 6

Regulatory role of hydrogen cyanide in dormancy removal of sunflower (*Helianthus annuus* L.) embryos

Regulacyjna rola cyjanowodoru w usuwaniu spoczynku zarodków słonecznika (*Helianthus annuus* L.)

Krystyna Oracz

***Co-tutelle* doctoral thesis realized during Polish-French PhD study**

Supervisors:

Dr hab. Renata Bogatek-Leszczyńska –
Department of Plant Physiology WULS

Prof. Francoise Corbineau –
EA2388 Physiologie des Semences UPMC

Warsaw, 2008

Acknowledgements

This thesis is the result of four years of work during which I have been accompanied and supported by many people. It is a pleasure to have now the opportunity to express my gratitude for all of them.

Firstly, I would like to thank my Supervisors, Prof. **Francoise Corbineau** from EA2388 Physiologie des Semences at Université Pierre et Marie Curie (Paris 6) and Dr hab. **Renata Bogatek** from the Department of Plant Physiology at Warsaw University of Life Sciences, for theirs engagement to helping see this project through to its final completion, and their wise guidance during its development.

It is difficult to overstate my gratitude to Prof. **Christophe Bailly** from EA2388 Physiologie des Semences (UPMC-Paris 6), the best advisor and teacher I could have ever wished. With his enthusiasm, inspiration, and great efforts to explain things clearly and simply, he helped me to understand seed science. His profoundly optimistic attitude has encouraged me to carry on through these years.

I would like to thank Prof. **Sławomir Podlaski,** the Head of the Plant Physiology Department (WULS) for superior role during my PhD studies.

I sincerely thank Dr **Dominique Job** from Bayer CropSciences Laboratory (Lyon, France) for giving me advices, lab space and a possibility to work in his lab, and also Dr **Claudette Job** for invaluable help and support concerning proteomic analyses.

I would like to express my sincere thanks to Dr **Hayat EL-Maarouf Bouteau** and Dr **Juliette Leymarie** from EA2388 Physiologie des Semences (UPMC-Paris 6), for theirs constant and friendly support, encouragement and guidance, and for introducing me to the molecular biology methods.

I am grateful to Dr **Ilse Kranner** from The Royal Botanic Garden (Kew, UK) for giving me the possibility to perform glutathione measurements.

Special thanks to Dr **Tomasz Krupa**, Dr **Michał Godlewski** and MSc. **Christelle Monsempes** for sharing theirs time and knowledge to develop my scientific skills.

I would like also to thank Prof. **Zofia Starck** and Dr **Agnieszka Gniazdowska** from the Department of Plant Physiology (WULS), who took effort in reading and providing me with valuable comments on earlier versions of this thesis.

All my friends, especially **Emilia**, **Emmanuel**, **Nico**, **Carole**, **Nori**, **Julie** and **Loïc** are thanked for theirs cooperation and the friendly atmosphere, for many scientific and non-scientific discussions and other nice activities.

I wish to thank administrative and technical staff members of the Department of Plant Physiology (WULS) and of the EA2388 Physiologie des Semences (UPMC-Paris 6) which I have not mentioned before, for all their help and support.

Drogiej przyjaciółce **Agnieszce Jankiewicz**, za jej przyjaźń i wiarę we mnie.

Am schönsten danke ich Denen, die meinem Herzen so nah sind **Janine**, **Wojti** und **Turi**, für all das, dass ich jeden Tag auf Ihre Liebe und Unterstützung zählen konnte. Dank Euch habe ich den schwierigen Weg zu Ende gebracht.

Pragnę podziękować kochanym **Rodzicom**, dzięki którym rozpoczęła się moja przygoda z nauką, za ich miłość i wsparcie.

I also acknowledge the generous financial support from the **Government of France**, in the form of PhD fellowship.

Finally, I would like to thank everybody who was important to the successful realization of this thesis and theirs names were not mentioned here.

Oświadczenie promotorów pracy

Oświadczamy, że niniejsza praca została przygotowana pod naszym kierunkiem i stwierdzamy, że spełnia ona warunki do przedstawienia jej w postępowaniu o nadanie tytułu zawodowego.

Warszawa, ... Podpis promotora pracy.....

Paryż, ... Podpis promotora pracy.....

Oświadczenie autora pracy

Świadom odpowiedzialności prawnej oświadczam, że niniejsza praca doktorska została napisana przeze mnie samodzielnie i nie zawiera treści uzyskanych w sposób niezgodny z obowiązującymi przepisami.

Oświadczam również, że przedstawiona praca nie była wcześniej przedmiotem procedur związanych z uzyskaniem tytułu zawodowego w wyższej uczelni.

Oświadczam ponadto, że niniejsza wersja pracy jest identyczna z załączoną wersją elektroniczną.

Warszawa, ... Podpis autora pracy.....

Declaration of supervisors

We hereby declare that the dissertation supervised by us is submitted in fulfilment of the requirements for the degree of an academic degree.

Warsaw, ...□□.. Supervisor, ...

Paris, ... Supervisor, ...

Declaration of the author

To the best of my knowledge and belief, I certify that this thesis has been written by myself and does not contain materials previously published or written by another person, except where due reference has been made.

I declare, that this thesis does not contain materials which has been accepted for any earlier degree or diploma by another person in other University.

Moreover, I certify that this version of the manuscript is identical with added electronic copy of it.

Warsaw, ...□..... Author, ...

Statement of contribution of others

The results dealing with proteomics (presented Chapter *6.2.3.* and *6.2.4.* in the present manuscript) are issued from the following paper: Oracz K., El-Maarouf-Bouteau H., Farrant J., Cooper K., Belghazi M., Job C., Job D., Corbineau F., Bailly C. (2007) - ROS production and protein oxidation as novel mechanism of seed dormancy alleviation. *The Plant Journal*, 50: 452-465.

They were obtained within the frame of a joint project between University Pierre et Marie Curie (Paris, France), Centre National de la Recherche Scientifique/Bayer CropScience Joint Laboratory (Lyon, France) and University of Cape Town (Cape Town, South Africa) in which Krystyna Oracz was invited to participate.

The results of this thesis were presented on international conferences in the form of poster and oral presentations:

Oral presentations:

1. **Oracz K.**, Bailly C., Bogatek R., Corbineau F. (21 June 2006). "Hydrogen cyanide in the regulation of germination of sunflower embryos". Physiological and practical aspects of the yield and seed quality improvement by ecological methods. Seed Network Conference, Warsaw, Poland.

2. **Oracz K.**, El-Maarouf-Bouteau H., Bogatek R., Corbineau F., Bailly C. (1-4 July 2007). "Unrevealing the molecular basis of HCN effect on alleviation of sunflower embryos dormancy: possible interplay with ethylene signalling pathway". 2nd ISSS Workshop on Molecular Aspects of Seed Dormancy and Germination, Salamanca, Spain.

3. **Oracz K.**, El-Maarouf-Bouteau H., Bogatek R., Corbineau F., Bailly C., (26-30 August 2007). "Possible interplay of HCN and ROS with ethylene signalling pathway in alleviation of sunflower embryo dormancy. 3rd Conference of Polish Society of Experimental Plant Biology, Warsaw, Poland - ***award in the category of the very interesting results presented on the conference.***

Poster presentations:

1. Bailly C., Mamadou N., **Oracz K.**, Come D., Corbineau F. (24-28 May 2004). "Is there a role for cyanide in sunflower seed germination". 3rd International Symposium on Plant Dormancy, Wageningen, Netherlands.

2. **Oracz K.**, Bailly C., Corbineau F. (8-13 May 2005). "Effect of cyanide on the germination of dormant sunflower (*Helianthus annuus* L.) seeds". 8th International Workshop on Seeds - Germinating New Ideas, Brisbane, Australia.

3. **Oracz K.**, El-Maarouf-Bouteau H., Bogatek R., Corbineau F., Bailly C. (26-30 August 2007). "Possible interplay of HCN and ROS with ethylene signalling pathway in alleviation of sunflower embryo dormancy. 3rd Conference of Polish Society of Experimental Plant Biology, Warsaw, Poland - ***award in the category of the very interesting results presented on the conference.***

Abbreviations

ABA	abscisic acid
ACC	1-aminocyclopropane-1carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AFGC	*A. thaliana* functional genomic facility
AIB	aminoisobutyric acid
AMT	aminotriazole
AMV	avian myeloblastosis virus reverse transcriptase
AO	cooper amine oxidases
AOA	amino-oxyacetic acid
APX	ascorbate peroxidase
ASA	ascorbic acid
BSA	bovine serum albumin
CaM	calmodulin
β-CAS	β-cyanoalanine synthase
CAT	catalase
CGP EST	Compositae Genom Project, EST database
CHES	2-5N-cyclohexyl(amino)ethane-sulphonic acid
CM-H_2DCFHDA	5-(and-6)-chloromethyl-2□,7□-dichlorofluorescein diacetate
CS	cysteine synthase
CTR	constitutive triple response
DCF, H_2DCFH	2□,7□-dichlorofluorescein
degLOW	lower degenerated primer
degUP	upper degenerated primer
DELLA	the amino acid sequence DELLA in primary structure DELLA protein
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DM	dry matter
DMAB	3-dimethylaminobenzoic acid
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenylhydrazone
DNPH	2,4-dinitrophenylhydrazine
dNTPS	mix of deoxyribonucleotide triphosphates
DPI	diphenyleneiodonium
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF1α	elongation factor 1α
EIL	EIN3-like protein
EIN	ethylene insensitive protein
ERF	ethylene response factor
ERS	ethylene response sensor
EST	expressed sequence Tag ("single-pass" cDNA sequences)
ETR	ethylene response (receptor)
FAOs	formes actives de l'oxygène
FW	fresh weight
GA	gibberellic acid

GC	gas chromatography
ga-1	GA-deficient mutant
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
JA	jasmonic acid
LOF	loss-of-function
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
mBBr	monobromobiamine
MBTH	3-methyl-2-benzothiazolidone hydrazone
MC	moisture content
MDA	malondialdehyde
MDHAR	monodehydroascorbate reductase
ME	menadione
MSA	mercaptosuccininc acid
MV	methylviologen
2′-NADPH	β-nicotinamide adenine dinucleotide 2′phosphate, reduced form
2,5-NBD	2,5-norbornadiene
NADP	oxidized nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBT	nitro blue tetrazolium chloride
NCBI	National Center for Biotechnology Information
NEM	N-ethylmaleimide
2PG	2-phosphoglycerate
P450	cytochrome P_{450} linked enzyme
PEP	phosphoenolopyruvate
PCR	polymerase chain reaction
PLP	pyridoxal 5-phosphate
POX	cell wall peroxidase
PPDK	pyruvate orthophosphate dikinase
PPP	pentose phosphate pathway
PTKs	protein tyrosine kinases
PTPs	protein tyrosine phosphatases
PVP	polivinylpyrrolidone
qRT-PCR	quantitative reverse-transcription PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
SA	salicylic acid
SAM	S-adenosyl-methionine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser/ThrPK	Ser/Thr protein kinase
SHAM	salicylhydroxamic acid
ss-cDNA	"single-pass" cDNA sequences

SOD	superoxide dismutase
T_{50}	the time to obtain 50 % of germination
TBA	2-thiobarbituric acid
TCA	trichloroacetic acid
TMV	tobacco mosaic virus
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride, Tris (hydroxymethyl) aminomethane hydrochloride

2. Summaries

2.1. Summary in English

Embryonic dormancy of freshly harvested sunflower (*Helianthus annuus* L.) seeds is expressed by their poor germination at low temperatures (10-15 °C). The research topic of the present work concerns the involvement of different endogenous factors such as hydrogen cyanide (HCN), reactive oxygen species (ROS), ethylene (C_2H_4) and abscisic acid (ABA) in the regulation of dormancy of sunflower embryos.

The aims of the present work were: (i) to identify the effect of gaseous HCN on breaking of sunflower embryo dormancy, (ii) to determine whether HCN improving action is related with the ethylene synthesis or signaling pathways, and (iii) to investigate the mechanisms of action of HCN, particularly whether ROS are involved in this phenomenon.

A short pre-treatment (3 hours) of dormant sunflower embryos by gaseous HCN released their dormancy and allowed their subsequent germination at 10-15 °C, temperatures which prevented germination of dormant embryos. This stimulatory effect did not result from an activation of the pentose phosphate or the cyanide-insensitive pathways, since other respiratory inhibitors such as sodium azide (NaN_3) and salicylhydroxamic acid (SHAM), did not allow the germination of dormant embryos at 10-15 °C. In addition, β-cyanoalanine synthase (β-CAS) activity measurements excluded the role of HCN in stimulating amino acids synthesis (as alanine, serine, asparagine) during dormancy alleviation, by improving the activity of β-CAS, since no significant difference in the activity of this enzyme was observed in axes isolated from control non-treated and HCN or MV treated embryos.

To identify the process by which dormancy of sunflower (non-cyanogenic) embryos is broken by HCN, the present work was focussed on its possible interaction with reactive oxygen species in this phenomenon. After HCN treatment, ROS (H_2O_2 - hydrogen peroxide and $O_2^{\cdot -}$ - superoxide anions) progressively accumulated in cells of embryonic axis. However, application of HCN had only a slight effect on the *in vitro* activity of the main

enzymes involved in the antioxidant defence system, such as catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), since these enzymes were potentially active. Imbibition of embryos in the presence of methylviologen (MV), as well as menadione (MD), ROS generating compounds, had the same stimulatory effect on germination as HCN, suggesting that HCN probably improved germination through accumulation of ROS. Increase in H_2O_2 and $O_2^{\cdot -}$ concentration in the embryonic axis occurred concomitantly with an oxidation (carbonylation) of specific proteins. Obtained data allow us to propose a novel explanation for cyanide mode of action in alleviation of sunflower embryo dormancy, which involves ROS production and targets changes in protein oxidation patterns. In the group of carbonylated proteins specifically associated with breaking of dormancy by HCN and MV, some are identified as epoxide hydrolase and alcohol dehydrogenase (Oracz *et al*. 2007).

In order to determine whether the stimulatory effect of HCN during germination of sunflower embryo might be related to ethylene biosynthesis and ethylene signaling pathway, transcription of genes involved in these proceses was studied simultaneously to HCN and MV treatments. The stimulatory effect of HCN and MV on germination of sunflower embryos, is not associated with a modification of transcription of genes involved in ethylene production such as *ACS (ACC synthase)* and *ACO (ACC oxidase)*, and does not result from a stimulation of ethylene production. It was also demonstrated, that HCN and MV markedly increased the transcription of *ERF1 (ethylene response factor 1)* but not that of *ETR2 (ethylene response 2)* and *CTR1 (constitutive triple response 1)*. This suggests that the transduction pathway of cyanide and MV, initiates the transcription factor *ERF1*, but through different mechanism of that involved by ethylene.

The putative molecular mode of action of HCN on sunflower embryo germination is discussed, and its possible relationships with ROS and ethylene synthesis and signaling pathways are also evoked. The first time are presented the direct experimental data concerning HCN effect on the expression of genes related to (i) ROS production [*NADPHox (NADPH oxidase), AO1 and AO2*

(amine oxidase 1 and 2), POX (peroxidase)], (ii) ROS signaling [*Ser/ThrPK (Ser/Thr protein kinase), MAPK6 (mitogen activated protein kinase 6), PTP (protein tyrosine phosphatase), CaM (calmodulin)*], (iii) ethylene formation *(ACS, ACO)* and (iv) signal transduction induced by ethylene *(ETR2, CTR1, ERF1*). It is demonstrated, that HCN did not change the transcription of genes involved in ROS production *(NADPHox, AO1, AO2, POX)*, and also did not seem to require the typical elements of ROS signaling pathway *(MAPK6*, *SerThrPK*, *PTP*, *CaM)*, but can induce the physiological response, using some elements of other signal transduction pathways (e.g. *ERF1* in ethylene signaling pathway).

This work proposes HCN as a signal molecule, which *via* ROS as a second messenger, regulates many pathways leading in consequence to alleviation of embryo dormancy and stimulation of germination. These pathways probably do not operate independently, but rather are linked together in a network of interactions.

2.2. Résumé

La dormance embryonnaire des semences de tournesol (*Helianthus annuus* L.) fraîchement récoltées se caractérise par une germination difficile à des températures basses (10-15 °C). Le sujet de cette étude concerne la régulation de la dormance embryonnaire du tournesol par différents facteurs tels que le cyanure d'hydrogène (HCN), les formes actives de l'oxygène (FAOs), l'éthylène (C_2H_4) et l'acide abscissique (ABA).

Les objectifs de ce travail de recherche ont été: (i) de caractériser les effets du cyanure gazeux sur l'élimination de la dormance des embryons de tournesol, (ii) de déterminer si l'action stimulatrice du cyanure sur la germination était liée à la synthèse de l'éthylène ou à ses voies de signalisation, et (iii) de rechercher si les mécanismes d'action du HCN mettaient en jeu les voies de signalisation des FAOs.

Un prétraitement de courte durée (3 h) des embryons dormants de tournesol par du HCN gazeux élimine leur dormance et permet leur germination à 10-15 °C, températures qui empêchent la germination des embryons dormants. Cet effet stimulateur ne résulte pas d'une activation de la voie des pentoses phosphates ou de la voie insensible au cyanure, car d'autres inhibiteurs respiratoires comme l'azoture de sodium (NaN_3) et l'acide salicylhydroxamique (SHAM) ne permettent pas la germination des embryons dormants à des températures fraîches. En outre, les mesures de l'activité de la β-cyanolanine synthase (β-CAS) permettent d'exclure le rôle du cyanure dans la stimulation de la synthèse d'acides aminés, comme l'alanine, la sérine ou l'asparagine, au cours de la levée de dormance.

Afin d'identifier les mécanismes mis en jeu dans l'élimination de la dormance des semences de tournesol en présence de HCN, nos recherches ont porté en grande partie sur l'analyse du rôle possible des FAOs dans ce processus physiologique. Après traitement par du HCN, les FAOs (H_2O_2 - peroxyde d'hydrogène et $O_2^{\cdot-}$ - anions superoxydes) s'accumulent progressivement dans les cellules des axes embryonnaires. Toutefois, l'application de HCN n'a qu'une légère incidence sur l'activité *in vitro* des

principales enzymes impliquées dans le système de défense antioxydant, comme la catalase (CAT), la glutathion réductase (GR) et la superoxyde dismutase (SOD). L'imbibition des embryons dormants en présence de méthylviologène (MV) ou de ménadione (MD), composés générateurs de FAOs, a le même effet stimulateur sur la germination que le HCN, ce qui suggère que l'accumulation des FAOs joue un rôle dans la germination. L'augmentation de la teneur en H_2O_2 et $O_2^{\cdot -}$ dans l'axe embryonnaire s'accompagne d'une oxydation (carbonylation) de protéines spécifiques. Ces résultats permettent de proposer un nouveau mode d'action du cyanure dans l'élimination de la dormance des semences de tournesol, qui implique la production de FAOs et l'oxydation de protéines. Parmi les protéines spécifiquement carbonylées au cours du traitement des embryons en présence de HCN et de MV, ont été identifiées une époxyde hydrolase et une alcool déshydrogénase (Oracz *et al*. 2007).

Dans le but de déterminer si l'effet stimulateur du HCN sur la germination des embryons dormants de tournesol pouvait être lié à l'éthylène et à sa voie de signalisation, la transcription des gènes responsables de la synthèse de l'éthylène et de la réponse à ce composé a été étudiée dans les axes d'embryons traités par du HCN et du MV. Les résultats montrent que l'effet stimulateur du cyanure et du MV sur la germination des embryons dormants n'est pas associé à une modification de la transcription des gènes impliqués dans la biosynthèse de l'éthylène, *ACC synthétase* (*ACS*) et *ACC oxydase* (*ACO*), et ne résulte pas d'une augmentation de la production d'éthylène. Il a également été démontré que le HCN et le MV augmentent nettement la transcription de *ethylene response factor 1* (*ERF1*), mais n'ont pas d'effet sur la transcription de *ethylene response 2 (ETR2*) et de *constitutive triple response 1* (*CTR1*). Ces résultats suggèrent que la voie de transduction du cyanure initie le facteur de transcription *ERF1*, mais par un mécanisme différent de celui de l'éthylène.

Le mode d'action moléculaire putatif du cyanure sur la germination des embryons de tournesol est discuté et ses éventuelles relations avec l'éthylène, les FAOs et leurs voies de signalisation sont également évoquées. C'est la première fois que des données expérimentales concernant directement le

cyanure et son effet sur l'expression de gènes liés à (i) la production des FAOs N*ADPH oxydase* (*NADPHox*), *amines oxydase 1* et *2* (*AO1* et *AO2*), *peroxydase (POX*) et (ii) leur voie de signalisation *Ser/Thr protein kinase* (*Ser/ThrPK*), *mitogen-activated protein kinase 6* (*MAPK6*), *protein tyrosine phosphatase* (*PTP*), *calmodulin* (*CaM*), (iii) la synthèse d'éthylène (*ACS*, *ACO*) et (iv) la transduction du signal induit par l'éthylène (*ETR2*, *CTR1*, *ERF1*) sont présentées. Il est démontré que le cyanure ne modifie pas la transcription des gènes codant pour les enzymes impliqués dans la production de FAOs (*NADPHox*, *AO1*, *AO2*, *POX*) et de ceux codant pour les éléments de leur voie de signalisation (*MAPK6*, *SerThrPK*, *PTP*, *CaM*), mais qu'il peut induire une réponse physiologique, en utilisant certains éléments d'autres voies de signalisation (par exemple *ERF1*).

L'ensemble de ce travail permet de proposer que le cyanure d'hydrogène est une molécule signal qui, en utilisant les FAOs comme messagers secondaires, régule de nombreuses voies menant à l'élimination de la dormance et la stimulation de la germination des semences dormantes de tournesol. Ces voies ne sont vraisemblablement pas indépendantes, mais interviennent probablement au sein d'un réseau plus large d'interactions cellulaires.

2.3. Streszczenie

Zarodki słonecznika (*Helianthus annuus* L.) wyizolowane ze świeżo zebranych nasion charakteryzują się spoczynkiem, który wyraża się niemal całkowitym brakiem ich zdolności kiełkowania w niskich (10-15 ^{o}C) temperaturach. W niniejszej pracy podjęto próbę określenia regulacyjnej roli wybranych czynników endogennych takich jak cyjanowodór (HCN), reaktywne formy tlenu (ROS), etylen i kwas absysynowy (ABA), w usuwaniu spoczynku zarodków słonecznika.

Celem tej pracy było: (i) poznanie efektów sygnałowego oddziaływania gazowego cyjanowodoru na kiełkowanie spoczynkowych zarodków słonecznika, (ii) określenie czy działanie HCN jest związane z modyfikacją biosyntezy etylenu i transdukcji sygnału inicjowanego przez ten hormon, a także (iii) poznanie mechanizmu działania HCN, a w szczególności jego możliwe współdziałanie z ROS w regulacji spoczynku i kiełkowania zarodków słonecznika.

Krótkotrwałe (3 godz.) traktowanie spoczynkowych zarodków słonecznika gazowym HCN (1 mM) przełamuje spoczynek i umożliwia ich kiełkowanie w temperaturze 10-15 °C. Wykazano, że stymulujący efekt HCN nie wynika z aktywacji cyklu pentozo-fosforanowego, czy też drogi oddychania niewrażliwej na cyjanowodór, gdyż zastosowanie innych inhibitorów takich jak azydek sodu (NaN_3) i kwas salicylohydroksamowy (SHAM), nie umożliwiało kiełkowania zarodków w temperaturze poniżej 10 °C. Ponadto wykluczono udział HCN w stymulacji syntezy aminokwasów (takich jak alanina, seryna, asparagina) poprzez stymulację aktywności β-CAS, gdyż we wszystkich analizowanych próbach (bez względu na rodzaj traktowania) zaobserwowoano tylko nieznaczne różnice w aktywności tego enzymu.

W kolejnym etapie badań podjęto próbę poznania mechanizmu współdziałania HCN z ROS w regulacji spoczynku zarodków słonecznika. Stwierdzono, że traktowanie HCN powodowało sukcesywny wzrost stężenia ROS (H_2O_2 - nadtlenek wodoru i $O_2^{\cdot -}$ - rodnik ponadtlenkowy) w komórkach osi zarodkowych wyizolowanych z zarodków słonecznika. Jednocześnie, aplikacja HCN miała nieznaczny wpływ na aktywność *in vitro* głównych enzymów

systemu antyoksydacyjnego, takich jak katalaza (CAT), reduktaza glutationowa (GR), dysmutaza ponadtlenkowa (SOD). Ponadto, imbibicja zarodków w obecności metylviologenu (MV), jak również menadionu (MD), substancji generujących ROS, miała podobny do HCN efekt na kiełkowanie, co wskazuje na to, iż HCN stymuluje kiełkowanie poprzez akumulację ROS. Wykazano, że wzrostowi stężenia H_2O_2 oraz $O_2^{\cdot -}$ w osiach zarodowych towarzyszy utlenienie (karbonylacja) specyficznych białek. Wsród tych specyficznie związanych z przerywaniem przez HCN i MV spoczynku zarodków słonecznika, zidentyfikowano między innymi hydrolazę epoksydową oraz dehydrogenazę alkoholową (Oracz *et al.* 2007). Na podstawie tych wyników, zaproponowano nowy mechanizm działania cyjanowodoru w ususwaniu spoczynku zarodków słonecznika, który związany jest ze wzrostem produkcji ROS oraz utlenieniem określonych białek.

W celu poznania molekularnych mechanizmów działania HCN na kiełkowanie zarodków słonecznika po raz pierwszy zbadano wpływ HCN na ekspresję genów związanych z (i) produkcją ROS tj. *oksydaza NADPH (NADPHox)*, *oksydaza aminowa 1 i 2* (*AO1* i *AO2*), *peroksydaza* (*POX*), (ii) transdukcją sygnału indukowanego przez ROS tj. *serynowo/treoninowa kinaza białkowa (Ser/ThrPK)*, *kinaza aktywowana mitogenami 6* (*MAPK6*), *fosfataza tyrozynowa* (*PTP*), *kalmodulina (CaM)*, (iii) syntezy etylenu *(ACS, ACO)* oraz (iv) transdukcji sygnału indukowanego przez etylen *(ETR2, CTR1, ERF1*).

Wykazano, iż HCN nie zmienia transkrypcji genów związanych z produkcją ROS *(NADPHox, AO1, AO2, POX)*, jak również nie zmienia transkrypcji elementów sygnału indukowanego przez ROS *(MAPK6*, *SerThrPK*, *PTP*, *CaM)*. Przeprowadzone badania wykazały także, iż stymulacyjny efekt HCN i ROS (MV) na kiełkowanie zarodków słonecznika nie jest związany z regulacją transkrypcji genów uczestniczących w produkcji etylenu, takich jak *ACC syntaza* (*ACS*) i *ACC oksydaza* (*ACO*), oraz nie wynika ze stymulacji syntezy etlenu. Natomiast HCN i ROS znacząco zwiększają transkrypcję jednego z elementów szlaku transdukcji indukowanego przez etylen tj. *ERF1*, nie modyfikując jednocześnie transkrypcji innych elemantów tj. *ETR2 i CTR1.* Świadczy to, iż HCN indukuje szlak sygnałowy związany ze stymulacją czynnika

transkrypcyjnego *ERF1*, lecz w sposób odmienny od szlaku inicjowanego przez etylen.

Wyniki badań przedstawionych w niniejszej pracy wskazują, że cyjanowodór pełni wiele regulacyjnych, a nawet sygnałowych, funkcji w roślinie. Wydaje się, że cyjanowodór można uznać za ważną sygnałową cząsteczkę, która *via* ROS jako wtórne przekaźniki, uruchamia wiele szlaków prowadzących do usunięcia spoczynku i stymulacji kiełkowania zarodków słonecznika. Szlaki te prawdopodobnie nie funkcjonują niezależnie, lecz raczej są wzajemnie powiązane, tworząc sieć współdziałających ścieżek sygnałowych.

3. Introduction

3.1. Source of HCN in the environment, microorganisms and plants

Cyanide played an important role in the evolution of live on Earth and remains an important form of nitrogen for microorganisms, fungi and plants (Ebbs 2004).

Cyanide can be present in the environment as simple cyanides (e.g. HCN, CN^-, NaCN), metal cyanide complex, cyanates and nitriles. Free cyanide, especially HCN gas at pH 9.31 and lower, is the primary toxic agent in the environment. The toxicity of metal complex is related to their ability to release cyanide ions in solution with relatively small fluctuations in pH (Gurbuz *et al.* 2004). Cyanide can complex with Fe, Au, Cd, Co, Cu and Ni (with the Fe and Au complexes being the most stable). Iron cyanides are the dominant CN species in soil and ground water. As Fe is a ubiquitous element in soils and aquifers, equilibrium favors the formation of complex such as ferrocyanide $[Fe(CN)_6^{-4}]$ and ferrycyanide $[Fe(CN)_6^{-3}]$. These metal-cyanide complexes show greater resistance to biodegradation than simple cyanides (Ebbs 2004).

Microbial cell wall contains functional groups of protein, lipopolysaccharides and lipids, which are capable of adsorbing ions in cyanide solutions (Gurbuz *et al.* 2004). The rhizosphere is inhabited by a diversity of organisms, including a component known as the rhizobacteria, which are characterized by aggressive colonization and subsequent establishment on plant roots (Kremer and Souissi 2001). Concentrations of HCN produced by rhizobacteria were similar to exogenous concentrations inhibiting seedling growth in bioassays, suggesting that cyanogenesis (the ability of living organisms to produce HCN) by rhizobacteria in the rhizosphere can adversely affects plant growth. Growth inhibition of lettuce and barnyardgrass by volatile metabolites of the cyanogenic rhizobacteria confirmed that HCN was the major produced inhibitory compound. Results of Kremer and Souissi (2001) suggest that HCN production in the rhizosphere of seedlings by selected rhizobacteria is a potential and environmentally compatible mechanism for biological control of weeds. About 50 % of rhizobacteria on potato (*Solanum tuberosum* L.) roots

produced cyanide, which was implicated in measurable inhibition of potato growth (Kremer and Souissi 2001).

Numerous of plants are also cyanogenic, utilizing cyanide in the soil. Cyanogens, such as cyanogenic lipids and glycosides are found in more than 3000 species of higher plants. Cyanogenesis, has been known for several centuries in apricots, peaches, almonds and other important food plants (Nahrstedt 1993). Hydrogen cyanide, as a product of cyanogenic compound hydrolysis was firstly isolated from plant in 1802 by Scrade (from bitter almond and from the leaves of peach) (Vetter 2000). The term intrinsic bound up with circulation of cyanide is its potential, a reflection of the concentration of cyanogenic glycosides in the plant which, upon degradation, leads to the release of HCN (Miller and Conn 1980). The simple scheme of the biosynthesis and catabolism of cyanogenic glycosides is presented on Figure 1.

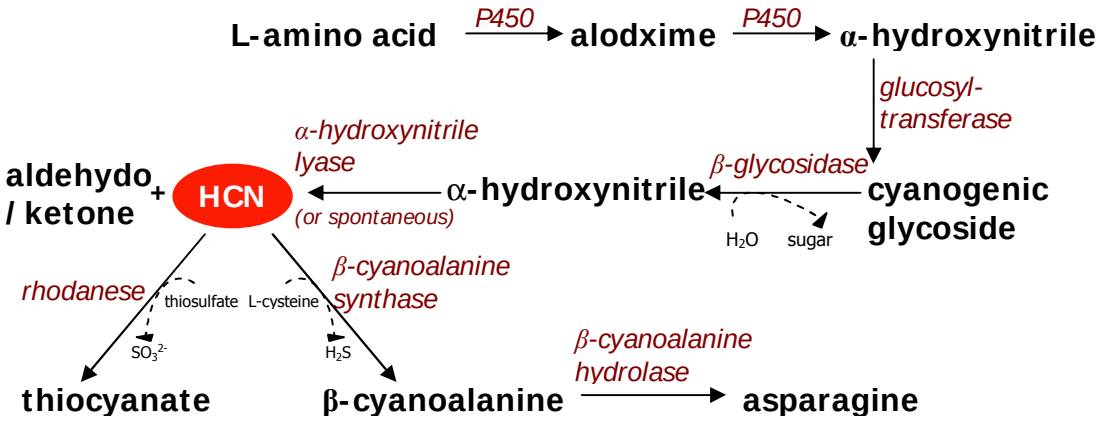


Figure 1. Biosynthesis and catabolism of cyanogenic glycosides. Enzymes involved in are in *italic*. *P450,* cytochrome P_{450} linked enzyme. (Siegień and Bogatek 2006, modyfied).

The genetic control of cyanogenesis has no unique mechanism. The plants show variation in the amount of the produced HCN. This variation occurs within individual plants or genotypes, depending on different physiological factors (Vetter 2000). The production of HCN depends on both the biosynthesis of cyanogenic compounds and on the existence (or absence) of its degrading enzymes (Vetter 2000). The tissue level compartmentalization of cyanogens

and their hydrolyzing enzymes prevents large-scale hydrolysis in intact plant tissue. Plants which exhibit this phenomenon usually contain one or more compounds as precursors, which liberate HCN upon hydrolysis. The cyanogenic glycosides belong to the products of secondary metabolism (Vetter 2000).

In plant tissue, hydrolysis of cyanogenic glycosides is initiated by cleavage of the glucosidic linkage(s) by one or more soluble β-glucosidases. The resulting cyanohydrins are relatively unstable and decompose either spontaneously or enzymatically, in reaction catalyzed by a α-hydroxynitrile lyase to yield HCN and an aldehyde or a ketone (Jones *et al.* 2000). In intact plant tissue, continuous releasing of cyanide from cyanogens is prevented by separation of the substrates and hydrolytic enzymes at different compartments at the cellular level (Seigler 1991). Usually, cyanogenic glycosides are localized in apoplast while α-hydroxynitrile lyase mainly exists in the cell wall (Selmar 1993). During disruption of the tissues (e.g. by herbivore attack), the glycosides are brought into contact with β-glycosidases and α-hydroxynitrile lyase, which release the toxic HCN. On the contrary to the processes involving injury, many plant cyanogenic glycosides are also metabolized in intact cells and transported within the plant, as it occurs during seed germination of rubber tree (*Hevea*) species (Selmar 1993).

Cyanogenesis is not restricted to cyanogenic lipids and glycosides containing plant species. Peiser *et al.* (1984) were among the first to show that cyanide was a co-product of the ethylene biosynthesis pathway, where it is produced in stoichiometrically equal amounts to ethylene (Grossman 2003). The other routes of cyanide formation in plant tissues are from glyoxylate, the product of photorespiration, and hydroxylamine, the possible intermediate of nitrate assimilation (Hucklesby *et al.* 1982). The existence of several potential sources of cyanide in plants supports the hypothesis that this gaseous molecule is present in almost all plant tissues and may contribute to the control of many important physiological events (Siegień and Bogatek 2006).

3.2. Role of HCN in plant tissues

3.2.1. Toxic effect of HCN and its detoxification in plant tissues

It is well known that cyanide is very toxic to all living cells. Treatment of *Arabidopsis* by cyanide resulted in a marked growth inhibition, a reduction in plant size, and a decrease in chlorophyll content (Smith and Arteca 2000). It caused many necrotic spots on tobacco (*Nicotiana tabacum*) leaves (Siefert *et al.* 1995), as also induced nucleus degradation in pea (*Pisum sativum*) leaves (Samuilov *et al.* 2000). Cyanide is a common inhibitor of various intracellular enzymes involved in many important metabolic pathways. The most vulnerable enzymes include Cu/Zn superoxide dismutase, catalase, peroxidase, cytochrome-c oxidase, nitrate/nitrite reductase and nitrogenase (Grossmann 2003). In green plant tissues, ribulose-bisphosphate carboxylase is quite sensitive to cyanide as well. Liang (2003) demonstrated the occurrence of cyanide interaction with the Cu-protein plastocyanin which is involved in photosynthetic electron transport. The concentrations of endogenous cyanide required to cause 50 % inhibition of sensitive enzymes are mostly in the range of 5-10 μM (Grossmann 1996). Therefore, in order to protect these systems, the concentration of HCN in plant tissues is tightly regulated by different enzymes that may quickly detoxify and re-metabolize this toxic compound.

The crucial role in detoxification of cyanide is played by β-cyanoalanine synthase (β-CAS, EC 4.4.1.9), which catalyzes the formation of the non-protein amino acid β-cyanoalanine from cysteine and cyanide (Blumenthal *et al*. 1968) (Figure 1). The activity of β-CAS was found to be localized predominately if not entirely in the mitochondria - the organelle most sensitive to HCN (Wurtele *et al.* 1985, Meyers and Ahmad 1991). This is not surprising if a function of this enzyme is detoxification of HCN which block respiration in mitochondria and is irreversible under physiological conditions. The activity of β-CAS in barley was dependent on the developmental stage of the tissue (Wurtele *et al.* 1985). The older sections of barley had up to 3.5-fold higher activity of β-CAS than younger sections. β-Cyanoalanine synthase appears to be ubiquitous in higher plants, as it plays a general role in fixation of HCN formed from cyanogenic

compounds (Grossmann 1996), and also cyanide produced concomitantly during ethylene biosynthesis (Yip and Yang 1988). It is interesting that activity of β-CAS may be enhanced itself by cyanide (Liang 2003), and also by ethylene (Maruyama *et al.* 2001). In addition, β-CAS allows also the recycling of the reduced nitrogen of cyanide into amino acid synthesis, since in blue lupine (*Lupinus angustifolius*) *β*-cayanoalanine, the product of β-CAS is subsequently metabolized to asparagine by a β-cyanoalanine hydrolase (Castric *et al*. 1972).

In plants, two classes of β-CAS seem to exist, based on differences in amino acid composition and protein structure (Ikegami *et al.* 1988). In blue lupine, β-CAS is a monomeric enzyme, with a molecular mass of about 52 kDa, and contains 1 mol pyridoxal phosphate mol^{-1} protein, which is essential for the catalytic activity (Akopyan *et al.* 1975). In spinach (*Spinacia oleracea*) the enzyme contains two identical subunits of 28 to 30 kDa, each containing 1 molecule of pyridoxal phosphate (McAdam and Knowels 1984, Ikegami *et al*. 1988). The structure of the second class of β-CAS is very close to that of cysteine synthase (CS; EC 4.2.99.8), which is a homodimer of about 30- to 35-kDa subunits, each containing 1 molecule of pyridoxal phosphate (Droux *et al.* 1992). Compartment-specific CS isoforms have been purified from cauliflower (*Brassica olerucea* L.) cytosol, chloroplast and mitochondrial fractions (Lunn *et al.* 1990, Rolland *et al.* 1992). CS catalyzes cysteine formation from *O*-acetyl-L-serine and sulfide, but also possess β-CAS activity (Maruyama *et al.* 2001). However, CS may lose its activity during tissue disruption, due to the enzyme high sensitivity to oxidation, which often happens under different stress conditions (Liang and Li 2001). Therefore the main role in HCN detoxification in living organisms seems to be played by β-CAS.

3.2.2. Biological activity of HCN in plant responses to stress

HCN at non-toxic concentration may play a role of signaling molecule involved in the control of physiological processes, such as nitrate assimilation (Solomonson and Barber 1990) or in plant responses to some environmental stimuli (Grossmann 1996). Therefore, HCN may play a dual role in plants, depending on its concentration (Siegień and Bogatek 2006).

The content of cyanide in stressed tissue may be regulated not only by control of activity of β-CAS, but also by different intracellular compartmentation of cyanide release (ACC oxidase exists in the cytoplasm) and cyanide detoxification (β-CAS is mainly located in the mitochondria) (Grossmann 1996). Thus, cyanide removal in cell compartments other than mitochondria may be less efficient, and consequently could result in its transiently elevated levels in cell compartments such as cytoplasm, chloroplasts and peroxisomes (Grossmann 1996). On the other hand, most cyanide released during the ACC oxidase reaction exists in the undissociated form, and could therefore diffuse easily through membranes, to reach different compartments of the cell (Meyer *et al.* 2003). The transient increase in HCN concentration in a small region of plant tissue may allow cyanide acting as a signaling cellular molecule, which triggers the events consequently leading to acquisition of stress resistance. It was shown that non-lethal concentration of cyanide enhances the resistance of tobacco leaves to tobacco mosaic virus (TMV) (Chivasa and Carr 1998).

There is highly probable that cyanide is involved in alleviation of stress through its effect on synthesis of ethylene. In 2000, Smith and Arteca showed that in *A. thaliana*, *ACS* gene (*ACS6*) is rapidly activated after treatment with cyanide. Moreover, Northern blots analyze shown that the level of *ACS6* transcript is dependent not only upon the concentration of cyanide, but also upon duration of the stress (Smith and Arteca 2000). Recently, was reported that at least part of the growth regulatory action of ethylene is mediated *via* its effects on the DELLA proteins which act as repressors of growth in response to ethylene (Achard *et al*. 2003). Reduction of growth is one of the strategies which plants have adopted under stress conditions. The ability to reduce cell growth under unfavorable conditions may not only allow conservation of energy for defense purpose, but also may limit the risk of heritable damage (May *et al.* 1998).

There exist some data demonstrating that sub-lethal levels of cyanide produced from ACC together with ethylene can play a role in acclimation of plants to biotic and abiotic stresses. It was reported, that the preconditioning of

mammalian cells with sub-lethal concentration of sodium cyanide (NaCN) protected the neurons against subsequent NaCN-induced damages (Jensen *et al.* 2002). Thus, cyanide may trigger many events which lead to the acclimation of plants growing under adverse conditions. However, more experimental data are needed to confirm that these events are common for many biotic and abiotic stresses accompanied with the co-evolution of ethylene and cyanide.

3.3. Complexity of seed dormancy phenomenon

3.3.1. What is seed dormancy?

The seed is the structure in which a usually fully developed embryo is dispersed, and which enables the embryo to survive the period between seed maturation and seedling establishment, thereby ensuring the initiation of the next generation. The dry seed is well equipped to survive extended periods of unfavorable conditions (Koornneef *et al.* 2002).

Seed dormancy is an important stage in the life cycle of many plants. Recent scientific progress has been made in understanding the physiological, biochemical and molecular changes which take place in seed dormancy. Despite of that fact, there is still no unambiguous definition of that phenomenon, perhaps because it is a complex state, manifested and broken in different ways in different species. Bewley (1997) defined dormancy as the failure of an intact viable seed to complete germination under favorable conditions and is controlled by several environmental factors, such as light, temperature and the duration of seed storage (after-ripening). Inability to germinate has evolved differently across species through adaptation to prevailing environment, so that germination occurs when conditions for establishing a new plant generation are likely to be suitable (Bewley 1997). Therefore, a diverse range of blocks (dormancy mechanisms) have evolved, in keeping with the diversity of climates and habitats in which they operate. A more sophisticated and experimentally useful definition of dormancy has recently been proposed by Baskin and Baskin (2004): a dormant seed have not the capacity to germinate in a specified period of time under any combination of normal physical environmental factors

that are otherwise favorable for its germination, i.e. after the seed becomes non-dormant. Dormancy should not just be associated with the absence of germination but rather, it is a characteristic of the seed that determines the conditions required for germination (Vleeshouwers *et al.* 1995).

Mentioned above condition is distinct from quiescence, which is a state of arrested development in non-dormant seeds imposed by unfavorable environmental conditions. Seeds can possess a coat-imposed dormancy or true embryo dormancy (or both). Seeds shed from the mother plant in a dormant state are in primary dormancy. Induced dormancy in mature, partially or fully after-ripened (non-dormant) seeds is termed secondary dormancy. The transition of many seeds from a dormant to non-dormant state (after-ripening) is accomplished by exposing the seed for a period of time to specific environmental conditions. The duration of after-ripening is governed by the degree of dormancy and environmental factors. Degree of dormancy specifies the germination phenotype and is influenced by genetic and environmental factors during seed development. The environmental factors involved in after-ripening include temperature, moisture, oxygen, and the type and level of these that are required differ between species. For example, apple (*Malus domestica*) seeds require cool, moist conditions (stratification), whereas other species, such as sunflower (*Helianthus annuus* L.), after-ripen under warm, dry conditions. A low dormancy level is characterized by a wide range of environmental conditions permissive for seed germination, while seeds presenting a high dormancy level show a narrow range of environmental conditions permissive for seed germination. For example, changes in dormancy status of weed seed populations are associated with changes in the range of temperatures and water potentials permissive for seed germination (Benech-Arnold *et al.* 2000). As dormancy is released, the range of temperatures and water potentials permissive for germination widens until it reaches a maximum. Conversely, as dormancy is induced, the range of temperatures and water potentials over which germination can proceed narrows, until germination is no longer possible at any temperatures or water potential (Batlla and Benech-Arnold 2005).

Many hypotheses have been proposed to explain the mechanism of seed dormancy, including the hormone balance theory and the metabolic deficiency theory (Bewley and Black 1994). However, because of its complexity, we still cannot answer two fundamental questions: how does the embryo block the germination to maintain dormancy and how does the embryo emerge from dormant state to complete germination?

3.3.2. HCN, ROS and hormones (ABA, GA and ethylene) in seed dormancy

Seed dormancy and germination are known to be regulated by a wide range of hormones. There is general agreement that abscisic acid (ABA) is the primary mediator of seed dormancy (Koornneef *et al.* 2002). The role of ABA in dormancy onset during seed development has been well documented by genetic and physiological studies. Analysis of mutant and transgenic plants has provided strong evidence that ABA biosynthesis and responses to this phytohormone are clearly involved in the onset and maintenance of dormancy. Reciprocal crosses and grafting experiments between ABA biosynthetic mutants and wild type plants have showed that dormancy in developing seeds is dependant on ABA that is synthesized in the embryo and not on maternal sources of ABA (Karssen *et al.* 1983).

Several early studies have reported that ABA sensitivity, but not ABA content, is correlated with the level of dormancy in mature seeds, leading to speculation that changes in ABA signaling are at least in part responsible for dormancy breakage (Bewley and Black 1994).

Recent studies have shown, however that breaking of dormancy by after-ripening, stratification, dark, and smoke is strongly correlated with changes in ABA content during imbibition and with a corresponding increase in germination capacity (Gubler *et al.* 2005). It is clear from inhibitor studies that *de novo* synthesis of ABA is required to maintain dormancy in imbibing seeds. Fluridone, an ABA biosynthesis inhibitor, is very effective in breaking of dormancy of several species (Ali-Rachedi *et al.* 2004). The inactivation of ABA by its conversion to phaseic acid is a crucial step in dormancy release in different species (Gubler *et al.* 2005).

The breaking of dormancy is characterized by changes in numerous physiological parameters that affect the subsequent germination response. Several studies have shown that ethylene and gibberellic acid (GA) promote the germination of dormant seeds. There is strong evidence that GA plays a role in germination by promoting the growth potential of the embryo and by mediating the weakening of tissues which surround the embryo (Finch-Savage and Leubner-Metzger 2006). Generally, accumulation of active GA in the embryos occurred only when ABA content decreased. In imbibed dormant barley embryos, ABA content remained high and active GAs remained very low. ABA represses the expression of *GA20-oxidase* gene in isolated sorghum embryos, providing an enticing clue which suggests that ABA might block germination process in dormant seeds by repressing GA biosynthesis (Perez-Flores *et al.* 2003).

Phytohormone such as ethylene (C_2H_4), has been implicated in the breaking of dormancy in several species such as sunflower (Corbineau *et al.* 1990, Borghetti *et al.* 2002), peanut, apple or cocklebur (Kępczyński and Kępczyńska 1997) and also some seeds have been shown to synthesize ethylene at the beginning of seed germination (Matilla 2000). Non-dormant *Chenopodium album* seeds do not require ethylene for radicle protrusion, although they produce the gas.

Recent data indicate that the site of ethylene perception is localized at the endoplasmic reticulum and emphasize an important role of protein complexes in mediating the initial steps in ethylene signal transduction. Elements of the pathway for ethylene signal transduction were identified by genetic approaches in *Arabidopsis*. A brief outline on these signaling components is shown in Figure 2. C_2H_4 is perceived by a family of five ER membrane-bound receptors (ETR1, ETR2, ERS1, ERS2, EIN4) that are similar to bacteria two-component hisitdine kinase receptors (Bleecker 1999). Such a localization is compatible with the ready diffusion of ethylene in both aqueous and lipid environments (Abeles *et al.* 1992).

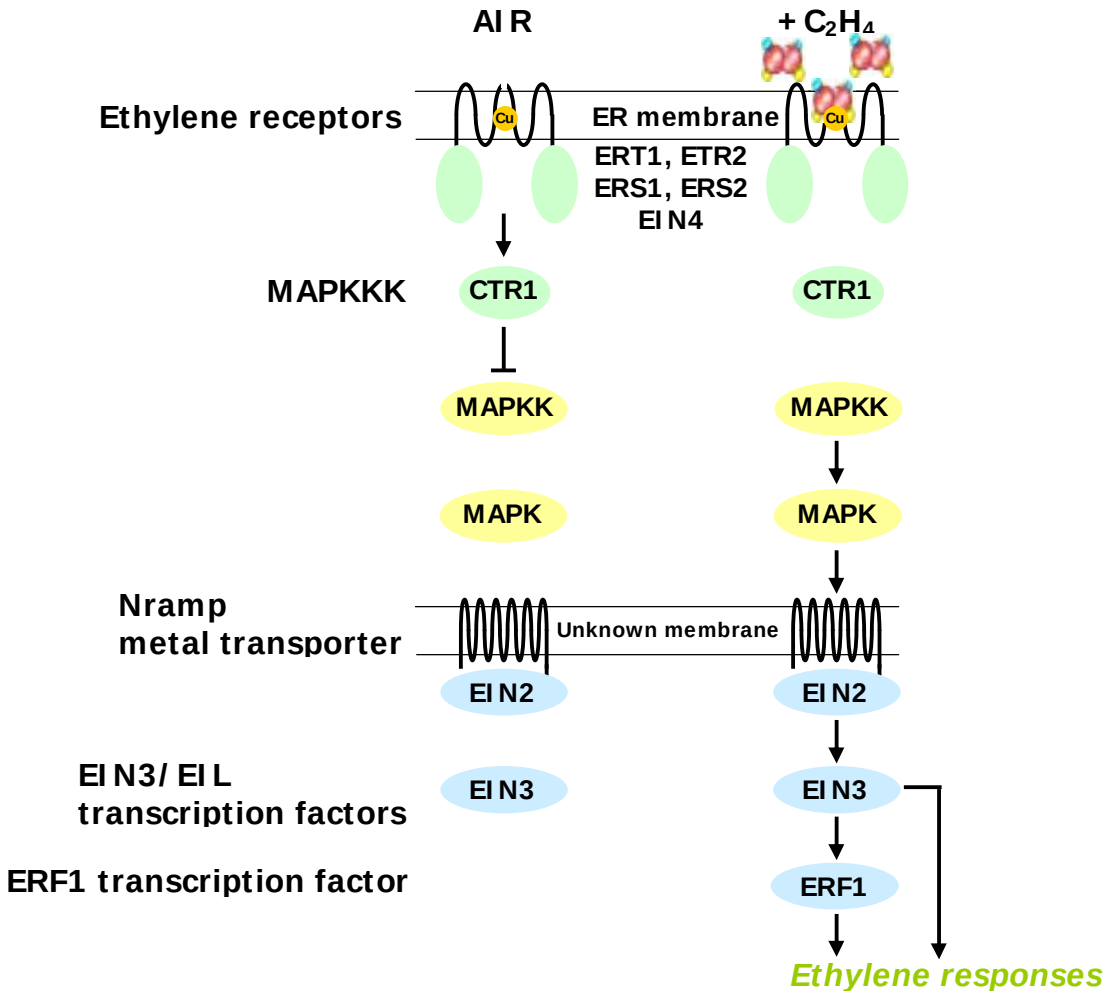


Figure 2. Model for ethylene signal transduction that incorporates biochemical features of the pathway components. Soluble protein domains are shown as color circles and transmembrane structures are shown as black, wavy lines. ETR1, ethylene response 1; ETR2, ethylene response 2; ERS1, ethylene response sensor 1; ERS2, ethylene response sensor 2; EIN2, ethylene insensitive 2; EIN3, ethylene insensitive 3; EIL, EIN3-like protein; CTR1, constitutive triple response; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; ERF1, ethylene response factor 1. (Chen *et al.* 2005, modified).

Loss-of-function (LOF) mutations in any single ethylene receptor have little or no effect upon seedling growth, consistent with functional overlap within the receptor family (Hua and Meyerowitz 1998). Plants with multiple LOF mutations in the receptors show a constitutive ethylene response, indicating that the receptors are negative regulators of ethylene signaling (Hua and Meyerowitz 1998). This effect of LOF mutations in the receptors is probably due to their

interaction with the downstream component CTR1 (constitutive triple response). CTR1 is a Raf-like ser/thr kinase with similarity to a mitogen-activated protein kinase kinase kinase (MAPKKK). LOF mutations in CTR1 result in a constitutive ethylene-response phenotype, indicating that CTR1 is a negative regulator of ethylene signaling. The next component of the ethylene signal transduction pathway, EIN2 (ethylene insensitive 2) has similarity with members of the Nramp metal-ion transporter family (Alonso *et al.* 1999). EIN2 plays a major role in the ethylene response as LOF mutations result in complete ethylene insensitivity for all ethylene responses tested, indicating that EIN2 is a positive regulator of the pathway. In addition, although EIN2 is predicted to be membrane-localized, the specific membrane system has not yet been determined. Thus the actual function of EIN2 in the pathway is still a mystery. Functioning downstream of EIN2 is a small family of transcription factors that includes EIN3 and various EIN3-like (EIL) proteins (Roman *et al.* 1995, Chao *et al.* 1997). LOF mutations in EIN3 cause partial ethylene insensitivity. This insensitivity can be rescued by expression of EIN3, EIL1 or EIL2 indicating that, along with EIN3, at least these two EILs can mediate an ethylene response (Chao *et al.* 1997). The EIN3/EIL family is involved in a regulatory cascade and stimulates the transcription of other transcription factors such as ERF1 (ethylene response factor 1) (Solano *et al.* 1998), a member of the ERF family of transcription factors (Fujimoto *et al.* 2000). These factors have been shown to act as activators or repressors of additional downstream ethylene-responsive genes (Ohme-Takagi and Shinshi 1995). An *ein3/eil1* double mutant eliminates virtually all the transcriptional response to ethylene, indicating the key role this family to transcription factors plays in the immediate response of plants to ethylene.

According to the basic working model (Figure 2), in the air (absence of C_2H_4) the ethylene receptors maintain CTR1 in an active state that serves to repress downstream responses. In the presence of ethylene, the repression is relieved. Binding of ethylene via a cooper co-factor inactivates the receptors, thereby inactivating CTR1. As a result, EIN2 is activated and a transcriptional cascade involving the EIN3/EIL and ERF transcription factors is indicated.

Both families of transcription factors are involved in regulating ethylene responses. An important feature of the ethylene signaling pathway is that it contains both positive and negative regulators, some proteins thereby serving to induce the responses while others suppress them.

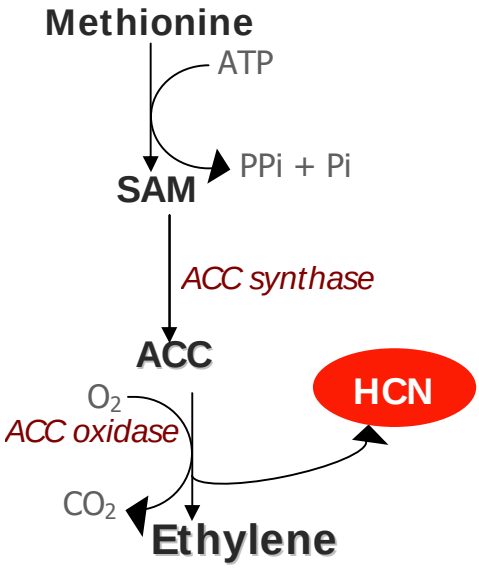


Figure 3. Production of ethylene and HCN from methionine. SAM, S-adenosyl-methionine; ACC, 1-aminocyclopropane-1carboxylic acid.

There exist a cross-talk between the ethylene signaling pathway and other hormone signaling pathways, particularly with auxin, whose effects are often mediated by ethylene, but also with ABA, cytokinins, gibberellins and brasinosteroids (Chen *et al.* 2005). Both GA and ethylene affect gene expression (Kieber 1997) and in particular were shown to induce proteinase synthesis during seed imbibition and germination (Asano *et al.* 1999). Ethylene can also fully rescue the germination defect of the Arabidopsis GA-deficient mutant *ga-1* (Karssen *et al.* 1989, Koornneef and Karssen 1994), suggesting that some components of ethylene signaling are involved in the effects of GAs on this process. Recently, Achard *et al.* (2003) reported that at least a part of the growth regulatory action of ethylene is mediated via its effects on the DELLA proteins, which act as repressors of growth in response to GA as well as ethylene and auxin signals.

Since Peiser *et al.* (1984) demonstrated that the putative product of C-1 and amino moieties of 1-aminocyclopropane-1carboxylic acid (ACC) during its

conversion to ethylene is hydrogen cyanide (HCN) (Figure 3), many questions have been raised about the physiological significance of this metabolite in plants. Cyanide presents some similar biochemical properties as ethylene, including the ability to permeate membranes as a gas and to bind certain metalloproteins. One of the most known properties of cyanide appears to be its involvement in dormancy removal and effect on seed germination. From literature it is known that cyanide at millimolar concentrations stimulates the germination of seeds of different species including rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.) (Roberts 1969), lettuce (*Lactuca sativa*) (Zagórski and Lewak 1985), apple (*Malus domestica*) (Dziewanowska *et al.* 1979a, Bogatek and Lewak 1991), cocklebur (*Xanthium pennsylvanicum* Wallr.) (Maruyama *et al.* 1996) and *Arabidopsis* (Bethke *et al.* 2006). Dziewanowska *et al.* (1979b) demonstrated that early stages of cold stratification (breaking of dormancy) of apple seeds are associated with releasing of the relatively large amount of HCN from cyanogenic glycosides. This situation could occur as a result of damage of the tonoplast during rehydration, which allows the direct contact of cytoplasmic glycosidase with the vacuolar cyanogen □ amygdaline. In addition, the concentration of cyanogenic glycosides seems to fluctuate diurnally, and varies greatly during the growing season (Niedźwiedź-Siegień 1998, Niedźwiedź-Siegień and Gierasimiuk 2001). Esashi *et al.* (1996) established that some nitrogenous inhibitors of respiration (KCN, NaN_3, etc.) which promoted the germination of *Xanthium pennsylvanicum* seeds enhanced the accumulation of cyanogens compounds. Part of the exogenously applied KCN was converted to cyanogenic glycosides and it is suggested that endogenous cyanogens might be involved in the germination of *Xanthium pennsylvanicum* seeds. The promoting effect of KCN on germination was confirmed by Maruyama *et al.* (1996). It was reported that during germination of seeds of rubber tree species (Selmar *et al.* 1988), cyanogens stored in endosperm are transported into the cotyledons as also primary leaves and subsequently metabolized to non-cyanogenic nitrogen-containing compounds functioning as mobilisable form of nitrogen for the growing seedlings. Production of HCN by both rice and cocklebur (*Xanthium pennsylvanicum*) increased during seed germination and preceded ethylene

(C_2H_4) production (Hasegava *et al.* 1994). Its concentration declined abruptly when the radicle emerged before the peak in ethylene emission. HCN production during initial imbibition may be derived from cyanogenic reserves and controlled by both preexisting and subsequently developing β-cyanoalanine synthase.

Data concerning about an effect of HCN on seed germination obtained in experiments with dormancy breaking of apple embryos indicated several putative modes of action of this compound:

1. One of the postulated mechanisms of cyanide mode of action is that it accelerates the shift from the oxidative pentose pathway (PPP) to glycolysis (Bogatek and Lewak 1988, Lewak *et al.* 2000). Glycolysis is the main sugar catabolism pathway during the first days of imbibition of dormant embryos in culture and it is operating mainly in embryonic axis, whereas PPP activity is restricted to cotyledons (Bogatek *et al.* 1989, Bogatek and Lewak 1991). Activities of several enzymes related to glycolysis (PP_i-dependent phosphofructokinase, pyruvate kinase, kinase 2) were stimulated in apple embryos as result of HCN pre-treatment, what supports this assumption (Bogatek *et al.* 1999).

2. It is also hypothesized that stimulatory effect of HCN on germination of dormant apple embryos may contributes to the regulation of reserved sugar catabolism (Bogatek *et al.* 1999, Lewak *et al.* 2000). The evidence supplying this hypothesis is the observation that HCN pre-treatment resulted in a gradual decrease in sucrose level, through the stimulation of alkaline invertase activity in upper (not being in contact with water medium) cotyledon of isolated dormant apple embryo. Sucrose hydrolysis in the upper cotyledon was stimulated by HCN to the level observed in a lower cotyledon, resulting in synchronic growth of both cotyledons (Bogatek *et al.* 1999).

3. The other possibility explaining the stimulatory effect of HCN is related to the regulation of ethylene synthesis pathway since this compound is known for releasing sunflower embryo dormancy, and that HCN is also a co-product of its biosynthesis, through ACC oxidase (ACO) activity. Bogatek and Sykała (2005)

demonstrate that HCN markedly enhanced ethylene production in dormant embryos by the stimulation of expression of *ACC synthase* gene (*ACS6*).

4. It is proposed also that removal of embryonic dormancy in apple embryos by HCN induces an oxidative stress (accumulation of H_2O_2) and increases the activity of antioxidant enzymes, especially glutathione reductase (GR), which in consequence may lead to induction of the PPP through oxidation/reduction of glutathione and NADP (Bogatek *et al.* 2003).

In the last decade the role of ROS in plant and seed physiology was examined by several authors. ROS derivate from the reduction of oxygen which gives rise to superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$). Plant cells have a diverse array of enzymatic mechanisms responsible for ROS production (Apel and Hirt 2004). Among those possible cellular mechanisms, the plasma membrane NADPH oxidase has been suggested to play a major role. In consequence ROS produced by that enzyme can be used for controlled polymer breakdown leading to wall loosening during extension growth. Backbone cleavage of cell wall polysaccharides can be accomplished by hydroxyl radicals produced from hydrogen peroxide and superoxide in a reaction catalyzed by cell wall peroxidase (POX) (Schopfer and Liszkay 2006). From the other hand, it was reported that cooper amine oxidases (AO) catalyse the oxidative de-amination of polyamines, which subsequent oxidation deriving to H_2O_2 production, has been correlated with cell maturation and lignification during development as well as with cell wall reinforcement during pathogen invasion (Cona *et al.* 2006). Moreover, as a signal molecule, H_2O_2 derived from polyamine oxidation mediates cell death, the hypersensitive response and the expression of defense genes. Furthermore, aminoaldehydes and 1,3-diaminopropane from polyamine oxidation are involved in secondary metabolite synthesis and abiotic stress tolerance (Cona *et al.* 2006).

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

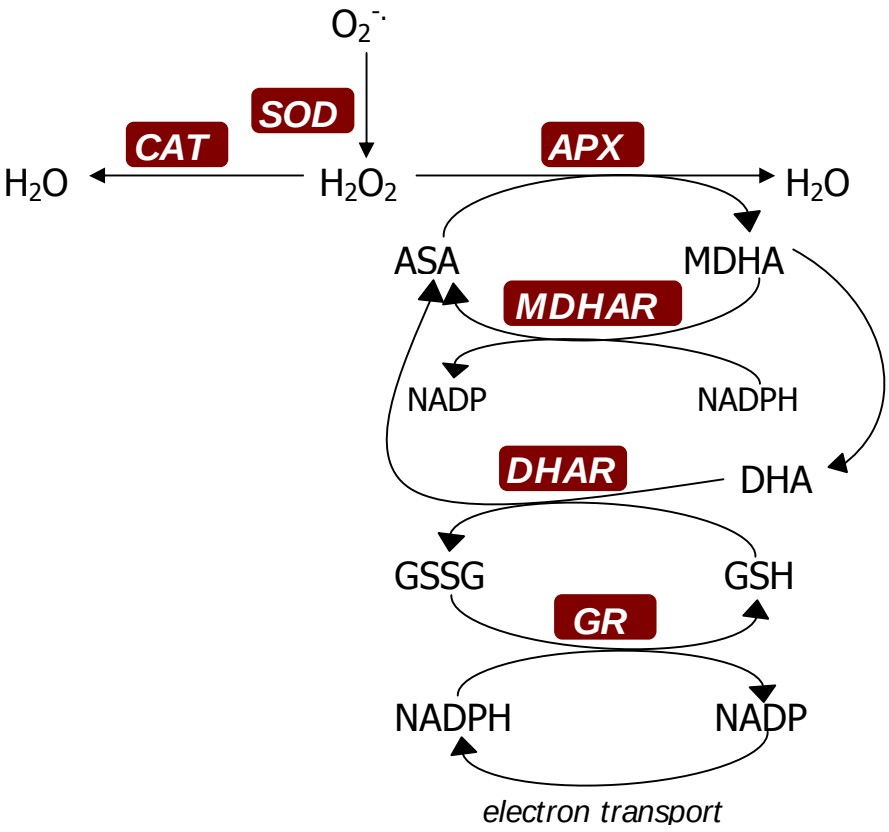


Figure 4. Main detoxifying systems in plants. CAT, catalase; SOD, superoxide dismutase; APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; ASA, ascorbate; MDHA, monodehydroascorbate; DHA, dehydroascorbate; GSSG, oxidized glutathione; GSH, reduced glutathione; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP, oxidized nicotinamide adenine dinucleotide phosphate. (Bailly 2004, modified).

seed moisture content. The uncontrolled accumulation of ROS is highly toxic for the cell. They can react with the majority of biomolecules, thus resulting in oxidative stress that can become irreversible and cause cellular damage (Bailly 2004). To avoid deleterious effects of ROS, antioxidant mechanisms which tightly control ROS concentration. Various enzymatic and nonenzymatic mechanisms play these roles in plant (Figure 4). Superoxide dismutase, which can be mitochondrial (MnSOD), cytosolic (Cu/ZnSOD) or chloroplastic (CuZnSOD, FeSOD), dismutates superoxide radicals into H_2O_2 and oxygen (Bowler *et al*. 1992). Hydrogen peroxide is eliminated by the action of catalase (CAT), which is located in glyoxysomes and peroxisomes (Willekens *et al.* 1995). The ascorbate-glutathione cycle may also take part in H_2O_2 scavenging

and it involves ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). The enzymes of this cycle, which are present in chloroplasts, the cytoplasm, mitochondria, peroxisomes and the apoplast (Mittler 2002), participate in the regeneration of the powerful antioxidants such as ascorbic acid, reduced glutathione and α-tocopherol. The antioxidant machinery also includes various compounds, such as polyphenols, flavonoids and peroxiredoxins (Aalen 1999).

Having simple chemical structure and able to diffuse over short distances, ROS are ideally suited to act as signaling molecules. Among different ROS, only hydrogen peroxide can cross plant membranes and might therefore directly function in cell-to-cell signaling. Plant cells possess still-unidentified specific ROS sensors that process and translate this information into respective biological output programs (Pitzschke and Hirt 2006). In several systems, various pathways, particularly those involving mitogen-activated protein kinases (MAPKs), are modulated by ROS. Several lines of evidence from biochemical and genetic studies of plant stress signaling indicate that reversible protein phosphorylation plays an important role in the regulation of physiological status and gene expression in response to various environmental stresses (Xiong and Yang 2003). Ser/ThrPK, MAPK, or others protein kinases transfer the terminal phosphate from ATP to a specific serine, threonine or tyrosine residues on protein substrates. The MAPK cascade is one of the major pathways by which extracellular stimuli are transduced into intracellular responses in eukaryotic cells (Tena *et al.* 2001). Activation of the MAPK can facilitate its translocation to the nucleus where it can phosphorylate and activate transcription factors, thereby modulating gene expression (Neill *et al.* 2002). It has been shown that MAPKs are involved in plant signal transduction in response to pathogens, drought, salinity, cold, wounding, hormone stimuli, O_3 and ROS (Tena *et al*. 2001, Zhang and Klessig 2001, Jonak *et al*. 2002, Lu *et al*. 2002, Mittler 2002, Samuel and Ellis 2002, Moon *et al*. 2003, Xiong and Yang 2003, Mittler *et al*. 2004). In *Arabidopsis*, there seems to exist multiple ways to activate MAPK3 and MAPK6 in response to ROS (Pitzschke and Hirt 2006). Other strategy employed by a cell to control the cellular responses to redox change is

reversible inactivation of protein tyrosine phosphatases (PTPs) under oxidative conditions. PTPs work antagonistically with protein tyrosine kinases (PTKs) to regulate signal transduction. PTKs phosphorylate tyrosine residues on a substrate protein and PTPs remove these phosphates from substrate tyrosines (dephosphorylation). Since the phosphorylation status of a protein can modulate its function, PTKs and PTPs work together to regulate protein function in response to a variety of signals, including hormones. Studies performed on soybean (*Glycine max*) have shown that, compared with its mammalian counterparts, the plant enzyme is relatively insensitive to inactivation by H_2O_2 but hypersensitive to S-glutathionylation (thiolation) promoted by the presence of oxidized glutathione (GSSG).

One of the universal second messengers, acting as a mediator of stimulus-response coupling in the responses to environmental stresses is calcium (Ca^{2+}), which transient elevations are sensed by several sensors such as calmodulin (CaM) (Hu *et al.* 2007). CaM has no catalytic activity of its own but, upon binding Ca^{2+}, activates numerous downstream target proteins. Ca^{2+}-CaM complex binds to target proteins and modulates their activities, and finally results in physiological responses as cell growth differentiation, stress tolerance or cell death (Hu *et al.* 2007). Study of Hu *et al.* (2007) indicate that CaM is involved in ROS signaling in plants.

A recent advance in plant physiology signaling pathways has lead to reconsider role of ROS from a new point of view. ROS accumulation can be also beneficial for seed germination and seedling growth by regulating cellular growth, ensuring a protection against pathogens or controlling the cell redox status (Bailly 2004). It is known also that they interact with abscisic acid and gibberellins transduction pathway and are likely to control numerous transcription factors (Beligni *et al.* 2002).

It is highly probable that cyanide may interfere with these mechanisms since this compound is also known to stimulate ROS production and to affect antioxidant enzymes during removal of seed dormancy (Bogatek *et al.* 2003). Some papers suggest that cyanide may lead to an imbalance between

antioxidant defenses and increase the amount of ROS resulting in oxidative stress (Gunasekar *et al.* 1998). ROS have been invoked to play a role in cellular signaling (Bailly 2004), raising the hypothesis that these compounds can facilitate the shift from a dormant to a non-dormant status in seeds.

3.3.3. Sunflower as a model for dormancy study

There are few favored models in dormancy studies such as *Arabidopsis*, *Rosace* and cereals. *Helianthus* also provides important models for dormancy research. Freshly harvested seeds of the most popular sunflower *Helianthus annuus* L. (cv. LG 5665) present physiological dormancy localized at the embryo axis which prevents germination at low temperatures and is progressively broken during dry storage (Corbineau and Côme 2003). Their dormancy results from both seed coat- and embryo- imposed dormancy, the latter being involved in the failure to germinate at 10-15 °C. Application of abscisic acid and methyl jasmonate were found to inhibit germination of sunflower seeds and growth of seedlings (Corbineau *et al.* 1988). LePage-Degivry and Garello (1992) demonstrated that *in situ* ABA synthesis in the sunflower embryo is necessary for the imposition of dormancy. Authors suggested also that applied ABA prevented germination of isolated developing sunflower embryos. This inhibition was overcome upon their transfer to water. Moreover, only ABA synthesized within the embryo imposed lasting sunflower seed dormancy (LePage-Degivry and Garello 1992). As with other species, such as lettuce (*Lactuca sativa* L.) (Abeles and Lonski 1969), amaranth (*Amaranthus caudatus* L.) (Kępczyński and Karssen 1985), ethylene and etephon strongly stimulate the germination of dormant sunflower seeds (Corbineau and Côme 1987). Bagniol (1987) shows that application of gibberellic acid and cold stratification had no significant effect on the breaking of sunflower seed dormancy.

In spite of many studies performed on this seed species, the molecular mechanisms of sunflower embryo dormancy and of its release during after-ripening and/or involvement of different compounds regulating that process (HCN and ROS) are still largely unknown.

4. The aims of this work

Internal factors such as hormones, HCN and ROS act through specific, overlapping signal transduction pathways to regulate dormancy. Since is known that HCN is the putative product of ACC conversion to ethylene and it stimulates the germination of seeds of many species (Roberts 1969, Zagórski and Lewak 1985, Dziewanowska *et al.* 1979a, Bogatek and Lewak 1991), there exists circumstantial evidence that this molecule is an important controlling factor involved in regulation/removal of seed dormancy. Data concerning about a role of HCN in dormancy breaking of cyanogenic apple embryos (chapter *3.3.2.* in an *Introduction)*, indicated several putative modes of action of this compound. However, how this regulation is achieved in non-cyanogenic seeds, as sunflower, is still not well understood.

In this work, was attempted to determine and describe the understanding of the control of seed germination by gaseous cyanide, focusing principally on its possible effect on dormancy removal and its putative mechanisms of action. To identify the process by which dormancy is broken by HCN treatment, this work was focused on the analyse of few basic hypothesis describing the possible mechanism of action of those compound in germinating sunflower embryos. Based on the common properties and functions of HCN, it was of a particular interest to investigate whether (i) this molecule might increase the pool of the amino acids by changing the activity of β-CAS, (ii) its mode of action was related to modification of the respiratory pathway by inhibition of cytochrome oxidase or/and stimulation of alternative oxidase, as also (iii) whether ROS were generated by HCN and (iv) if ROS might play a role in dormancy removal by participation in regulation of ethylene production and/or signal transduction pathways. In order to assess a putative causal association between HCN, ROS and dormancy alleviation, sunflower embryos were imbibed in the presence of hydrogen cyanide, a compound that breaks dormancy (Taylorson and Hendricks 1973, Bogatek and Lewak 1991), or in the presence of methylviologen (MV), a ROS-generating compound (Slooten *et al.* 1995).

5. Material and Methods

5.1. Plant material

Sunflower *(Helianthus* spp.) is an annual plant, native to the Americas in the *Asteraceae* family, which contain around 80 species. The typical fruit of the sunflower family (*Asteraceae*) is the achene. It is a small (4 mm wide, 8 mm length) fruit containing a single seed. The seed is attached by a pericarp, but the seed coat is free from the inner wall of it. About 50 % of the dry matter in cotyledons isolated from sunflower seeds is lipid. The predominant fatty acids are linoleic and oleic acids (Walters *et al.* 2005).

Three batches of sunflower (*Helianthus annuus* L., cv LG5665) seeds, harvested in 2004, 2005 and 2006 near Montélimar (Drôme, France) and purchased from Limagrain (www.limagrain.com), were stored at -30 °C until use. The pericarp and seed coat were removed for the experiments and only naked embryos were used. Seeds were dormant at harvest and their moisture content was about 4 % on a fresh weight (FW) basis.

5.2. Physiological study

5.2.1. Germination assays

Germination assays were performed with naked embryos (i.e. without pericarp and seed coat) in darkness, in 9 cm Petri dishes (25 embryos per dish, 4 to 8 replicates) placed on a layer of cotton wool moistened with deionized water (control) or various solutions (depending on the treatment) (Table 1).

Germination assays were carried out at 10 °C (seeds harvested in 2004) or 12 °C (seeds harvested in 2005 and 2006), in darkness. An embryo was considered as germinated when the radicle had elongated to 2-3 mm. Germination counts were made daily for 10 days.

5.2.2. Chemical treatments

Treatment of sunflower embryos by gaseous 1 mM HCN was carried out by the method described by Bogatek and Lewak (1988). Isolated dry embryos were placed in a glass jar (500 ml) containing sterile cotton moistened with

deionized water (50 embryos per one box). Inside the receptacle a glass tube with 5 ml of 0.1 M KCN solution was placed. In the tightly closed container gaseous HCN was produced by acidifying KCN solution by 5 ml of lactic acid (10 % v/v). After various durations of treatment in darkness (at 10 °C for seeds harvested in 2004 and at 12 °C for seeds harvested in 2005 and 2006) cyanide was released, by opening the glass jar in the hood. After 15 min embryos were rinsed carefully 3 times with deionized water and transferred into Petri dishes containing sterile cotton moistened with deionized water.

To identify the effect of gaseous hydrogen cyanide on breaking of dormancy and to investigate the possible mechanisms of action of HCN, several compounds were used. The short characteristic of those chemicals is presented in Table 1.

Table 1. Characteristic of chemicals used in germination assays determining the possible mode of action of HCN in alleviation of sunflower embryo dormancy.

Chemical	Abbreviation	Role
Sodium azide (1 mM)	NaN_3	An inhibitor of cyanide sensitive respiratory pathway
Salicylhydroxamic acid (1 mM)	SHAM	An inhibitor of the cyanide insensitive respiratory pathway
Ethylene (50 μM)	C_2H_4	A stimulatory hormone of germination
Abscisic acid (0.1 mM)	ABA	An inhibitory hormone of germination
Amino-oxyacetic acid (1 mM)	AOA	An inhibitor of ethylene synthesis (ACS activity)
Aminoisobutyric acid (1 mM)	AIB	An inhibitor of ethylene synthesis (ACO activity)
Cobalt chloride (1 mM)	$CoCl_2$	An inhibitor of ethylene synthesis (ACO activity)
2,5-norbornadiene (1 mM)	2,5-NBD	An inhibitor of ethylene action
Methyl viologen (0.1 mM)	MV	A ROS generating compound
Menadione (1 mM)	MD	A ROS generating compound
Diphenyleneiodonium (0.1 mM)	DPI	An inhibitor of NADPHox activity
Aminotriazole (1 mM)	AMT	An inhibitor of CAT activity
Hydrogen peroxide (0.5 mM)	H_2O_2	Reactive oxygen species

For treatment with ethylene, uncovered glass Petri dishes (containing sterile cotton moistened with deionized water) with sunflower naked embryos (25 per dish) were placed in 6000 ml plastic containers. Ethylene (final concentration 50 μM) was injected into tightly closed containers. The amount of ethylene was verified by using a Hewlett Packard (Hewlett Packard 5890 series II) gas chromatograph. Embryos were incubated in the presence of ethylene during duration of germination, in darkness, at 10 °C (seeds from 2004) and 12 °C (seeds from 2005 and 2006).

For the experiments with 2,5-norbornadiene treatment, uncovered glass flask (volume 10 ml) with 35 μl of 2,5-NBD was placed in tightly closed 500 ml glass jar containing 50 sunflower embryos laid on sterile cotton moistened with deionized water. 2,5-NBD evaporated completely into the headspace of the receptacle. Embryos germination was determined in the presence of NBD during 10 days at 10 °C (seeds harvested in 2004) and 12 °C (seeds harvested in 2005 and 2006).

During embryos treatment by gaseous HCN (1 mM) in the presence of 2,5-NBD, inside the receptacle (500 ml) prepared as above (glass jar containing 50 embryos laid on sterile cotton moistened with deionized water), next to a glass tube with 5 ml of 0.1 M KCN solution, a small 10 ml flask with 2,5-NBD (35 μl) was placed. After that, in tightly closed glass jar (volume 500 ml) 5 ml of lactic acid (10 % v/v) was added to KCN solution (with syringe) and the gaseous cyanide and 2,5-NBD was diffused out of the solutions into the headspace of the receptacle. The incubation was carried out during next 3 h (cyanide in the presence of 2,5-NBD), at 10 or 12 °C (as described in chapter *5.2.1.*), in darkness. After the 3 h of treatment HCN and 2,5-NBD were released, embryos were rinsed carefully 3 times with deionized water and transferred to Petri dishes (containing sterile cotton moistened with deionized water) placed in the presence of 35 μl NBD in tightly closed 500 ml containers for germination continuation at the same temperature and light conditions as above.

All results correspond to the means ± SD of three-four experiments.

5.2.3. Estimation of the moisture content and dry weight

Ten whole embryos, isolated embryonic axes or cotyledons were weighed and then dried by oven drying at 105 °C, for 48 h for determination of the DM and calculation of water content. Water content was calculated on a DM basis. Results expressed as g H_2O g^{-1} DM correspond to the mean of the values obtained in three replicates of 10 whole embryos or 10 organs ± SD.

5.3. Biochemical study

5.3.1. Determination of superoxide anion content

Superoxide content was determined according to the method developed by Elstner and Heupel (1976). Axes (0.2 g FW) were ground in 4 ml of sodium phosphate buffer (pH 7.8, 50 mM) at 4 °C. The extracts were centrifuged at 16 000 x *g* for 15 min, and the resulting supernatants were used for $O_2^{-\cdot}$ determination. The supernatant (1 ml) was first incubated at 25 °C for 30 min in the presence of 1 ml hydroxylamine hydrochloride (1 mM) in 50 mM sodium phosphate buffer (pH 7.8). A volume (0.5 ml) of this reaction mixture was then incubated with 0.5 ml of 17 mM sulfanilamide and 0.5 ml of 7 mM 2-naphtylamine at 25 °C for 30 min. The absorbance was measured at 540 nm after centrifugation at 13 000 x *g* for 30 min. A calibration curve was established using sodium nitrite. The results are expressed as µmol g^{-1} DW and correspond to the means of measurement carried out on five extracts ± SD.

5.3.2. Determination of hydrogen peroxide content

The H_2O_2 content of excised axes was determined according to the method described by O'Kane *et al.* (1996). Axes (0.5 g FW) were ground with a mortar and homogenized with 5 ml of perchloric acid (0.2 M). After 15 min of centrifugation at 13 000 x *g* at 4 °C, the resulting supernatant was neutralized to pH 7.5 with KOH (4 M), and then centrifuged at 1 000 x *g* for 3 min at the same temperature to remove insoluble potassium perchlorate. The obtained supernatant was immediately used for spectrophotometric determination of

H_2O_2. The reaction mixture contained 50 µl of the collected supernatant, 400 µl of 12 mM 3–dimethylaminobenzoic acid (DMAB) in 0.375 M phosphate buffer (pH 6.5), 80 µl of 1.3 mM 3–methyl–2–benzothiazolidone hydrazone (MBTH), 20 µl (0.25 units) horseradish peroxidase (Sigma; http://www.sigmaaldrich.com/) and 950 µl of deionized H_2O for a total volume of 1.5 ml. The reaction started with the addition of peroxidase. Increase in absorbance at 590 nm was monitored after 5 min at 25 °C and compared with the absorbance obtained with known amounts of H_2O_2 detected for 5 min at 590 nm. The results are expressed as µmol g^{-1} DW and correspond to the means of the values ± SD obtained with five different extracts.

5.3.3. In situ localization of reactive oxygen species

Intracellular production of ROS was measured by using 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H_2DCFHDA, Molecular Probes, USA). This non-polar compound is converted to the membrane-impermeant polar derivative H_2DCFH by esterases upon uptake by the cell. H_2DCFH is non-fluorescent but is rapidly oxidized to the highly fluorescent DCF (2',7'-dichlorofluorescein) by the H_2O_2 and other peroxides (Maxwell *et al.* 1999, Schopfer *et al.* 2001).

This procedure was adapted for the *in vivo* measurement of cytoplasmic ROS with the working solution containing 100 µM of CM-H_2DCFH-DA. Changes in fluorescence were measured from time 0 (a moment of CM-H_2DCFHDA injection) until 15th min of incubation, using a confocal laser scanning microscope (FV 500, Olympus Polska sp. z o.o., Warsaw, Poland). To analyse the results Fluoview software (Olympus Polska sp. z o.o., Warsaw, Poland) was used. The fluorescence intensity is expressed in arbitrary units.

5.3.4. Protein analysis

5.3.4.1. Preparation of protein extracts

Axes isolated from sunflower embryos (140 mg FW, corresponding approximately to 30 axes) were ground in liquid nitrogen using a mortar and

pestle. Soluble proteins were extracted from the resulting powder at 4 °C in 1.0 ml of buffer containing 10 mM HEPES, 1 mM EDTA, the protease inhibitor cocktail □complete Mini` from Roche Molecular Biochemicals, 60 U DNAse I (Roche Diagnostics, www.roche-applied-science.com), and 6 U RNAse A (Sigma, www.sigmaaldrich.com). After 10 min at 4 °C, 20 mM dithiothreitol (DTT) was added, and the protein extracts were stirred for 20 min at 4 °C and then centrifuged (20 000 x *g*, 15 min) at 4 °C. The final supernatant corresponded to the soluble protein extract. Protein concentrations in the various extracts were measured according to the method described by Bradford (1976) using a Bio-Rad assay kit (http://www.bio-rad.com/). Bovine serum albumin was used as a standard.

5.3.4.2. One- and two-dimensional electrophoresis

One-dimensional SDS-Page of seed protein extracts (5 µg protein) was performed using 12 % w/v polyacrylamide resolving gels, as described by Laemli (1970).

Proteins were also analyzed by two-dimensional gel electrophoresis as described previously (Görg *et al.* 1987, Job *et al.* 2005). Isoelectrofocusing (100 mg protein) was carried out using gel strips forming an immobilized non-linear pH gradient from 3 to 10 (Immobiline DryStrip, pH 3-10 NL, 18 cm; Amersham Pharmacia Biotech; http://www5.amershambiosciences.com/). Strips were rehydrated for 14 h at 22 °C with the thiourea/urea lysis buffer as described previously (Harder *et al.* 1999), containing 2 % v/v Triton X-100, 20 mM dithiotreitol and the protein extracts. Isoelectrofocusing was performed at 22 °C in the Multiphor II system (Amersham Pharmacia Biotech) for 1 h at 300 V and 7 h at 3500 V. Then, the gel strips were equilibrated for 2 x 20 min in 2x 100 ml of equilibration solution containing 6 M urea, 30 % v/v glycerol, 2.5 % w/v SDS, 0.15 M bis-Tris and 0.1 M HCl (Görg *et al.* 1987, Harder *et al.* 1999). DTT (50 mM) was added to the first equilibration solution, and iodoacetamide (4 % w/v) was added to the second (Harder *et al.* 1999). Separation in the second dimension was carried out in polyacrylamide gels (10 % w/v acrylamide, 0.33 % w/v piperazidine diacrylamide, 0.18 M Trizma

base, 0.166 M HCl, 0.07 % w/v ammonium persulfate and 0.035 % v/v Temed). Electrophoresis was performed at 10 °C in a buffer (pH 8.3) containing 25 mM Trizma base, 200 mM taurine and 0.1 % w/v SDS for 1 h at 35 V and for 14 h at 110 V. Ten gels (200 x 250 x 1.0 mm) were run in parallel (Isodalt system, Amersham Pharmacia Biotech). For each treatment analyzed, 2D gels were run in triplicate.

One-dimensional gels were stained with the GelCode blue stain from Pierce (www.piercenet.com). Two-dimensional gels were stained with silver nitrate according to the methods described by Blum *et al.* (1987) for densitometric analyses or Shevchenko *et al.* (1996) for mass spectrometry analyses. Stained gels were scanned with a UMAX Powerlook III scanner equipped with MagicScan version 4.5 from UMAX Data Systems (Amersham Biosciences, www.amershambiosciences.com).

5.3.4.3. Detection of carbonylated proteins and Western blotting

The appearance of carbonyl groups in proteins was analyzed by immunodetection of 2,4-dinitrophenylhydrazone (DNP)-derivatized protein as described previously (Korolainen *et al.* 2002, Job *et al.* 2005). SDS was added to the protein extract (100 μl, 10 μg μl^{-1}) to a final concentration of 0.8 %. Following dialysis, four volumes of 10 mM DNPH (Sigma)/2 M HCl were added. Samples were shaken for 30 min at room temperature, and five volumes of 20/80 ice-cold TCA/acetone containing 1 mM DTT were added to each sample. The samples were centrifuged for 15 min at 15 000 x *g* at 4 °C. The precipitated protein was then washed three times with ice-cold acetone containing 1 mM DTT, then with 1 ml of 1:1 v/v ethanol:ethyl acetate, and resolubilized in the thiourea/urea lysis buffer containing 2 % v/v Triton X-100 and 20 mM DTT. Proteins were separated by 1D or 2D SDS-PAGE as described above, and transferred to nitrocellulose sheets (Bio-Rad) using standard procedures. Carbonylated proteins were revealed by incubation with rabbit anti-DNP antibodies (Chemicon, www.chemicon.com) followed by incubation with antirabbit secondary antibodies conjugated to horseradish peroxidase (Sigma) and detection with the ECL kit (Roche Diagnostics) (Job *et al.* 2005).

Relative protein carbonyl levels were quantitated by densitometric analyses of the blots as described above.

5.3.4.4. Protein identification by mass spectrometry

Bands and spots of interest were excised from 1D and 2D SDS-PAGE gels using sterile tips and placed in 1.5 ml sterile tubes. Each polyacrylamide piece was rinsed with water, then reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and incubated overnight at 37 °C with 12.5 ng μl^{-1} trypsin (sequencing grade; Roche Diagnostics) in 25 mM NH_4HCO_3. The tryptic fragments were extracted, dried, reconstituted with 2 % v/v acetonitrile, 0.1 % formic acid and sonicated for 10 min. Analysis of tryptic peptides by tandem mass spectrometry was performed on a nanoelectrospray ionization quadrupole time-of-flight hybrid mass spectrometer (Q-TOF Ultima Global; Waters Micromass, www.waters.com) coupled with a nano-HPLC (Cap-LC; Waters Micromass). The samples were loaded and desalted on a C18 pre-column (LC-Packings Pep-Map C18, 5 μm, 100 Å, 300 μm x 5 mm; Dionex Corp., www.dionex.com) at a flow rate of 20 μl min^{-1} isocratically with 0.1 % formic acid. The peptides were separated on a C18 column (Atlantis dC18, 3 μm, 75 μm x 150 mm Nano Ease; Waters). After washing with solvent A (water/acetonitrile 98/2 v/v, 0.1 % formic acid), a linear gradient from 5 % to 60 % of solvent B (water/acetonitrile 20/80 v/v, formic acid) was developed over 80 min at a flow rate of 180 nl min^{-1}. The Q-TOF spectrometer was operated in data-dependent analysis mode using a 1 sec mass spectrometry (MS) survey scan on three different precursor ions. The peptide masses and sequences obtained were either matched automatically to proteins in a non-redundant database National Center for Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov using the Mascot MS/MS ions search algorithm (http://matrixscience.com) or manual BLAST searches were performed against the current databases. (NCBI, Swiss-Prot, http://expasy.org/sprot/)

5.3.5. Malondialdehyde measurements

Lipid peroxidation was estimated by measuring spectrophotometrically malondialdehyde (MDA) contents of axes. Determination of MDA was carried out according to Heath and Parker (1968) as described by Bailly *et al.* (1996). The tissue (25-30 axes) was homogenized in 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was boiled for 30 min at 95 °C in a water bath with 0.5 % thiobarbituric acid (TBA in 20 % TCA) and then cooled quickly on ice. After that procedure, the homogenate was centrifuged at 16 000 x *g* for 30 min. The obtained supernatant was used for malondialdehyde determination. MDA equivalent was calculated from the difference in absorbance at 532 and 600 nm using extinction coefficient 155 mM^{-1} cm^{-1}. Results are expressed as nmol g^{-1} DW and represent the mean measurements carried out with three extracts ± SD.

5.3.6. HPLC analysis of glutathione and glutathione disulfide

The GSH and GSSG extraction and determination in excised axes was determined according to the method described by Kranner and Grill (1996a).

5.3.6.1. Extraction of thiols and disulfides

Extraction was performed with 25 mg (FW) of freeze-dried material (5 axes) in the presence of 4 ml of 0.1 M HCl on ice. The low pH value (pH 1) of the extraction medium resulted in precipitating of proteins and preventing oxidation of GSH to GSSG. Extract was shaken using a vortex and centrifuged for 20 min, at 20 000 x *g*, at 4 °C. One part of the supernatant was used to determine total glutathione (i.e. the sum of GSH + GSSG), the other one for GSSH determination after blockage of GSH with N-ethylmaleimide (NEM).

5.3.6.2. Determination of total glutathione

For determination of total glutathione, a 120 µl of the supernatant was mixed with 180 µl of 200 mM 2-5N-cyclohexyl(amino)ethane-sulphonic acid (CHES) buffer (pH 9.3) and 30 µl of dithiothreitol (DTT) and left for 60 min at room temperature to allow the glutathione reduction. After that time, the aliquot was labeled with 20 µl of monobromobiamine (mBBr) for 15 min at

room temperature, in the dark. This reaction was terminated by acidifying the sample with 250 μl of 0.25 % (MSA) and centrifuged for 45 min at 20 000 x *g*, at 4 °C. Obtained supernatant was ready for reversed-phase HPLC (Jasco) analysis. Low-molecular-weight thiols were separated on a RP-18 column and detected fluorimetrically (excitation: 380nm, emission: 480nm).

5.3.6.3. Determination of GSSG

In order to determination of GSSG, a 400 μl of the supernatant was treated with 30 μl of 50 mM N-ethylmaleimide (NEM) to block free thiols and 600 μl of 200 mM CHES (pH 9.3) buffer immediately after extraction. The reaction was carried out at room temperature for 15 min. Afterwards, excess NEM was removed by extracting five times with equal volumes of toluene. Thereafter, 30 μl of 3 mM DTT were added to a 300 μl aliquot of the NEM-treated extract and left for 60 min at room temperature to allow the reduction of disulphides. These aliquots were labeled with mBBr, acidified with 0.25 % MSA, centrifuged and analyzed as previously described.

The amounts of glutathione and glutathione disulfide were calculated using the results of the standards and expressed as μmol g^{-1} DW. All results correspond to the means ± SD of the values obtained with four measurements.

5.3.7. Measurement of antioxidative enzyme activities: catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD)

5.3.7.1. Extraction of SOD, CAT and GR

Enzyme activities were determined from the extract prepared according to the method of Bailly *et al.* (1996). Axes (1 g FW) were homogenized in a cold mortar in 10 ml of 0.1 M potassium phosphate buffer (pH 7.8) containing 2 mM α–dithiothreitol (DDT), 0.1 mM EDTA, 1.25 mM polyethylene glycol 4000 (PEG 4000) and 0.2 g polivinylpyrrolidone (PVP) and mixed for 15 min. The homogenate was centrifuged at 11 000 x *g* for 15 min and the supernatant was filtered through Miracloth, desalted on PD 10 column (Amersham Biosciences). The obtained extract was immediately used for determination of enzyme activities. All steps of the extraction were carried out at 4 °C.

5.3.7.2. SOD (EC 1.15.1.1) activity

Activity of SOD was determined by inhibition of *p*–nitro blue tetrazolium chloride (NBT) photoreduction (Giannopolitis and Ries 1977). The assay was conducted at 25 °C in total volume of 3 ml of 0.1 M potassium phosphate buffer (pH 7.8) containing 1.3 μM riboflavine, 13 mM methionine, 63 μM nitro blue tetrazolium (NBT) and 50 μl of enzymatic extract as described by Bailly *et al.* (1996). Absorbance was monitored at 560 nm after 15 min illumination, using a spectrophotometer Pharma Spec UV–1700 (Shimadzu, Japan). One unit of SOD is defined as the amount of enzyme that inhibits NBT photoreduction to blue formazan by 50 % monitored at 560 nm. SOD activity of the extracts was expressed as units SOD mg^{-1} prot.

5.3.7.3. CAT (EC 1.11.1.6) activity

Catalase activity was determined according to Clairbone (1985) with some modifications (Bailly *et al.* 1996). The assay was carried out in total volume 3 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 37.5 mM H_2O_2 and 200 μl enzymatic extract. The decrease in absorbance at 240 nm due to reduction of H_2O_2 (ε = 39.4 mM^{-1} cm^{-1}) was monitored every 3 sec for 2 min, using a spectrophotometer Pharma Spec UV–1700 (Shimadzu, Japan). Catalase activity was expressed as nmol H_2O_2 decomposed mg^{-1} prot min^{-1}.

5.3.7.4. GR (EC 1.6.4.2) activity

Glutathione reductase activity was determined in 800 μl of 0.1 M potassium phosphate buffer (pH 7.8) containing 0.5 mM β–nicotinamide adenine dinucleotide 2′–phosphate, reduced form (2′–NADPH), 10 mM glutathione, oxidized form (GSSG), 3 mM $MgCl_2$ and 50 μl enzymatic extract (Bailly *et al.* 1996). The decline in absorbance due to NADPH (ε = 6.22 mM^{-1} cm^{-1}) oxidation was recorded at 340 nm every 30 sec during 6-8 min using a spectrophotometer Pharma Spec UV–1700 (Shimadzu, Japan). GR activity was expressed as nmol NADPH oxidized mg^{-1} prot min^{-1}.

All results correspond to the means ± SD of the values obtained with three measurements carried out in three independent experiences.

5.3.7.5. Protein determination

Protein content in the enzymatic extracts was determined using Bradford reagent (Bradford 1976). Bovine serum albumin (BSA, Sigma) was used as a calibration standard.

5.3.8. Cyanide detoxication – estimation of β-CAS (EC 4.4.1.9) activity

The β-cyanoalanine synthase assay was based on the reduction of methylene blue by H_2S (modified from Blumenthal *et al.* 1968). Plant material (100 mg axes) was ground with a mortar and pestle and the soluble proteins were extracted in 4 ml Tris-HCl (50 mM, pH 8.5). After centrifugation (12 000 x *g*, 15 min) the supernatant was filtered through Miracloth, desalted on a PD 10 column (Amersham Biosciences). The obtained extract was immediately used for determination of enzyme activities. All steps of the extraction were carried out at 4 °C.

The assay contained in a total volume of 1 ml: 5 mM L-cysteine, 15 mM KCN, 100 mM Tris-HCl (pH 9.0), and 300 µl protein solution. The reaction was initiated by the addition of L-cysteine. After incubation for 30 min at 30 °C the reaction was terminated by adding 500 µl stop solution containing: 3 mM $FeCl_3$ and 15 mM N,N-dimethyl-p-phenylenediamine dihydrochloride dissolved in 8.4 N HCl. The samples were then centrifuged at 5 000 x *g* for 5 min to remove precipitated proteins. The formation of methylene blue was determined at 650 nm in a spectrophotometer Pharma Spec UV–1700 (Shimadzu, Japan). Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples and were used for the quantification of enzymatically formed H_2S.

The activity of β-CAS was expressed as nmol H_2S mg $prot^{-1}$ min^{-1}. All results correspond to the means ± SD of the values obtained with four measurements.

5.3.9. Ethylene emission measurement (GC)

The conversion of ACC to ethylene in axes of naked sunflower embryos after 24 h of imbibition on water (control) or treatment by MV or HCN, was determined by gas chromatograph (Hewlett Packard 5890 series II). Twenty axes were placed into 10 ml glass flask with addition of 200 µl of deionised H_2O or ACC (1 mM) for 20 min at 20 °C. After this period, the tubes were sealed tightly and incubated during 3 h at the same temperature conditions (20 °C). Gas samples at volume 2.5 ml were fractionated on an alumina column. Quantification was done by comparison of peak areas obtained from incubated samples to those produced by standard amounts of ethylene. Ethylene production was expressed as nmol C_2H_4 g^{-1} DW h^{-1} based on the average of four repetitions of head-space concentration of ethylene in each tube.

5.4. Molecular study

5.4.1. QRT-PCR analysis of expression of genes related to ROS production (NADPHox, AO1, AO2, POX) and signaling (Ser/ThrPK, MAPK6, PTP, CaM), ethylene formation (ACS, ACO) and signal transduction induced by ethylene (ETR2, CTR1, ERF1)

5.4.1.1. Extraction of total RNA

Axes were isolated from the sunflower embryos using a sharp scalpel blade, immediately frozen in liquid nitrogen, and then stored at -80 °C until use. For each extract, 25 axes were ground to a fine powder in liquid N_2, and total RNA was extracted by a hot phenol procedure according to Verwoerd *et al.* (1989). After grinding 1 ml of hot extraction buffer (80 °C) is added [phenol □0.1 M LiCl, 100 mM Tris-HCl pH=8.0, 10 mM EDTA, 1 % SDS (1:1)]. The mixtures were homogenized using a vortex for 2 min, and 500 µl chloroform-isoamylalcohol (24:1) was added and mixed (1 min). After centrifugation (20 000 x *g*, 5 min), the aqueous phases were removed and mixed with one volume of 4 m LiCl. RNAs were allowed to precipitate overnight at 4 °C and collected after centrifugation (20 000 x *g*, 45 min, 4 °C) next day. The pellets were dissolved in 250 µl H_2O and precipitated with 25 µl NaAc 2,5 M

(pH 5.6) and 500 µl of ethanol absolute at -80 °C during 1h. After centrifugation (20 000 x *g*, 45 min, 4 °C) pellets were washed three times with 70 % ethanol, dried in speedvac and diluted in 100 µl of H_2O. RNA concentration in obtained extracts was determined spectrophotometrically at 260 nm.

5.4.1.2. Design of primers

The oligonucleotide primer sets used for real-time qPCR analysis were designed on the basis of sunflower gene or EST sequences.

ACO primers were chosen in conserved region (using multiple alignment, clustalW) between sunflower *ACO1*, *ACO2* and *ACO3* genes in order to have the whole expression of the three isoforms (gene bank accession number: U62555, U62554, L29405). Candidate *NADPHox*, *AO1*, *AO2*, *POX*, *Ser/ThrPK*, *MAPK6*, *PTP*, *CaM*, *ACS*, *ETR2*, *CTR1* and *ERF1*sequences were found in the CGP EST database of sunflower (http://cgpdb.ucdavis.edu/) using the BLAST algorithm. Names of used EST, homology percentage with other plant sequences, amplified probe length and primer sets sequences are listed in Tables 1 and 2 (supplementary data).

In addition of high homology score with *Arabidopsis thaliana* sequence, *ACS*-like sunflower EST (QHG4g06.yg.ab1) contains an aminotranferase class I and II conserved domain ACS specific. *ACS* primers were designed in conserved region of three *Arabidopsis thaliana ACS* (gene bank accession number: NM116016, NM 122719, NM 100030) and sunflower *ACS*-like EST sequences to have the total *ACS* expression. *ERF1*-like sunflower EST display 86 % amino-acid homology compared to *Arabidopsis thaliana* sequence and contains an AP2 domain which is a DNA-binding domain found in plant transcription regulators such as EREBP (ethylene responsive element binding protein).

Gene-specific primers used were designed with Primer3 Input software. The length of all PCR products ranged from 120 to 180 bp. The PCR products were cloned into pCR2.1 vectors (invitrogen) and sequence analysis confirmed the correct amplicons produced from each pair of primers.

5.4.1.3. Real time quantitative RT-PCR

Total RNA (4 μg) was treated with DNase I (Sigma) and then was reverse transcribed with Revertaid H minus M-MuLV RT (Fermentas) (incubation during 2 h at 42 °C). After enzyme inactivation (10 min, at 95 °C), the first strand cDNA obtained was checked by 1 % agarose gel electrophoresis. The amplifications were performed with real-time PCR (iCycler iQ, Bio-Rad) using 5 μl of 50 times diluted cDNA solution for *ACO*, *ACS*, *ETR2*, *ERF1* and 10 times diluted cDNA solution for *CTR1*. Gene-specific primers were designed with Primer3 Input software ensuring that the reversed primer was located in order to prevent similar cDNAs being amplificated. Used primers and the amplified sequence length are listed in Table 2 (supplementary data).

As an internal standard, a fragment of sunflower β-*tubulin* or *EF1*α gene was used. Real-time PCR reactions were performed with the Absolute qPCR Syber Green Fluorescein mix (Abgene, Epsom, UK) and 0.25 μM of each primer in a 25 μl reaction volume. They were initiated at 94 °C for 15 min followed by 40 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s. Calculations of Critical threshold (Ct) and relative expressions were performed using the iCycler iQ software (Bio-Rad). Data from QRT-PCR (mean of four replicates ± SD) are expressed in arbitrary units.

5.4.2. Cloning of genes (ACS, ACO, ETR2, ERF1)

ACS, *ACO*, *ETR2* and *ERF1* were cloned using RT-PCR with two degenerate primers (Table 2, supplementary data). Total RNA was isolated as described previously. The RNA was stored in sterile water at -80 °C prior to use. Poly(A)$^+$ RNA was isolated using the Promega polyAtract mRNA isolation system and then used as a template for RT-PCR assay. To synthesis ss-cDNA, 1 μg poly(A)$^+$ RNA was heated at 80 °C for 3 min and cooled to 4 °C, and then added to a solution containing 50 mM Tris pH 8.3, 2.5 μM homo-polymer dT primer, 10 mM $MgCl_2$, 50 mM KCl, 10 mM DTT, 1.25 mM dNTPS, 30 units of RNA guard (Pharmacia), 40 U of AMV (Pharmacia) giving a total volume of 30 μl. The sample was incubated for 10 min at 80 °C, and again cooled at 4 °C. The ss-cDNA obtained was purified on glass beads and eluted in 15 μl of H_2O.

A 2 μl portion of the sample was transferred to 50 μl of a solution containing 0.1 μM of degUP and degLOW, 0.125 mM dNTPs, 1 x PCR buffer (Pharmacia) and 2 U *Taq* polymerase (Pharmacia) and overlaid with liquid paraffin. A total of 35 cycles of PCR were performed (94 °C/1 min; 50 °C/2 min) followed by 10 min at 72 °C. The amplified fragments were cloned into pGEM-T Vector (Promega) for sequencing.

Cloning was performed from fresh PCR products with the pCR®2.1-TOPO® TA cloning kit (Invitrogen, Carlsbad, CA, USA; map of vector in supplementary data) according to the manufacturer instructions and using chemical transformation of one shot® DH5α™ competent cells using kanamycin as the selecting agent. After plasmid purification using a miniprep Plasmix kit (Talent, Trieste, Italy), the insert size was checked by PCR amplification using M13 primers and the insert sequenced by Genomexpress (Meylan, France). Similarity studies were performed with BLAST program (Altschul *et al.* 1997) using the NCBI website (www.ncbi.nlm.nih.gov/BLAST/) and the http://cgpdb.ucdavis.edu/sitemap.html database.

6. Results

6.1. Main characteristics of sunflower embryo germination

6.1.1. Effect of temperature on germination of sunflower embryos

Figure 5A shows as an example, the germination of embryos isolated from seeds freshly harvested in 2004, placed on water and at temperatures between 10 and 25 °C.

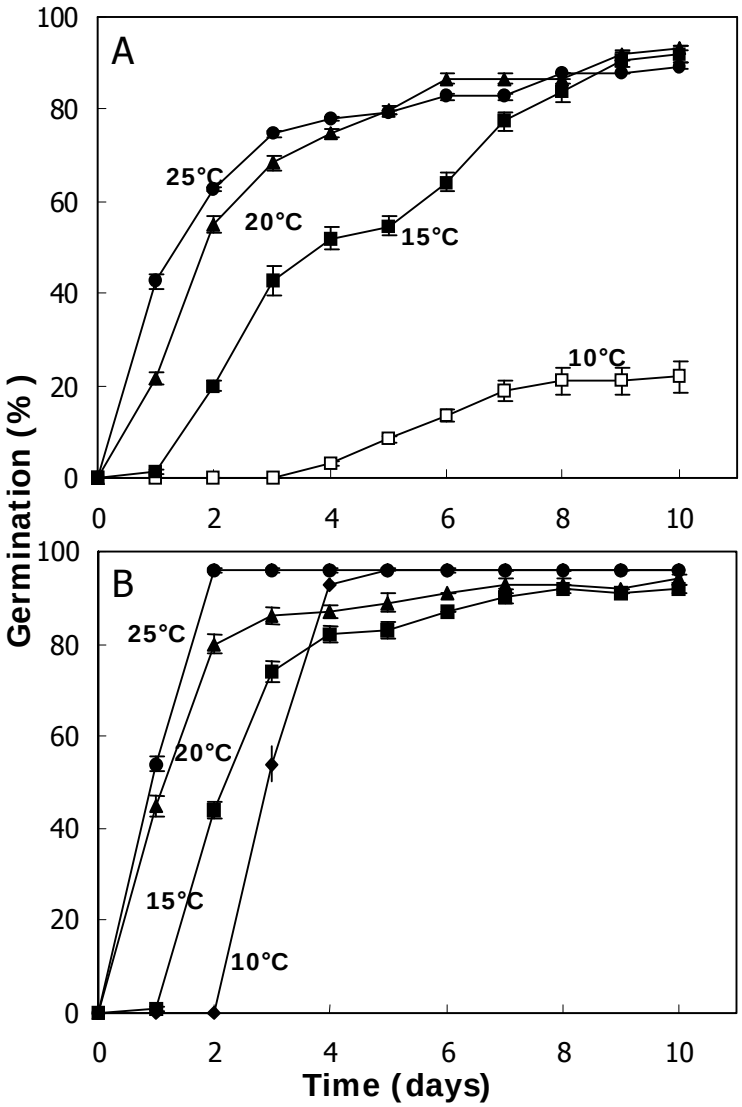


Figure 5. Effect of temperature on germination of sunflower embryos isolated from seeds harvested in 2004. Embryos isolated from freshly harvested seeds (A); Embryos isolated from seeds stored dry at 20 °C for 2 months (B). Means of four measurements ± SD. (from Oracz *et al.* 2007)

Almost 90 % of the population germinated within 8-10 days at temperatures ranging from 15 to 25 °C, the thermal optimum being 25 °C (Figure 5A). At this optimum, the lag time to onset of germination was less than 1 day, and the time to obtain 50 % germination (T_{50}) was about 1.4 day.

At 20 and 25 °C, freshly harvested (dormant) embryos germinated slowly and reached 70-75 % and 90 % germination within 3 and 6-8 days, respectively (Figure 5A). Germination became more difficult with decreasing temperature. At 15 °C, about 90 % of the population germinated within 8-10 days, but the time to obtain 50 % germination was around 4 days against 1.3-1.7 days for the embryos placed at 20-25 °C. Only about 20 % of the embryos were able to germinate at 10 °C within 10 days (Figure 5A), and no germination occurred at temperatures lower than 10 °C (data not shown).

The sensitivity of the embryos to temperature depended on the year of harvest. Table 2 regroups the germination percentages obtained at 10, 15, 20 and 25 °C, with embryos isolated from seeds harvested in 2004, 2005 and 2006.

Table 2. Germination percentages obtained at 10 (2004) or 12 (2005 and 2006), 15, 20 and 25 °C, with embryos isolated from sunflower seeds harvested in 2004, 2005 and 2006. Means of four measurements ± SD. (d, day)

Date of harvest	Germination (%)							
	10 or 12 °C		15 °C		20 °C		25 °C	
	3 d	10 d	3 d	10 d	3 d	10 d	3 d	10 d
2004	4 ± 2	18 ± 4	20 ± 2	74 ± 2	88 ± 6	98 ± 2	96 ± 4	96 ± 4
2005	1 ± 1	2 ± 2	10 ± 2	48 ± 4	90 ± 2	94 ± 2	96 ± 4	96 ± 4
2006	2 ± 1	4 ± 2	10 ± 4	52 ± 2	86 ± 4	94 ± 2	96 ± 4	96 ± 4

After dry storage, embryos become able to germinate at low temperature and germinated faster at 20-25 °C (Figure 5B). 95-99 % of the population germinated within 2 days at 25 °C, the T_{50} being about 1 day. Decrease in temperature resulted in slower germination. However, 95 % of the population germinated within 4 days at 10 °C (Figure 5B).

6.1.2. Effects of ethylene and ABA on germination of sunflower embryos

Dormant embryos placed at 20 °C germinated slowly and reached around 80 % of germination within 6-8 days (Figure 6).

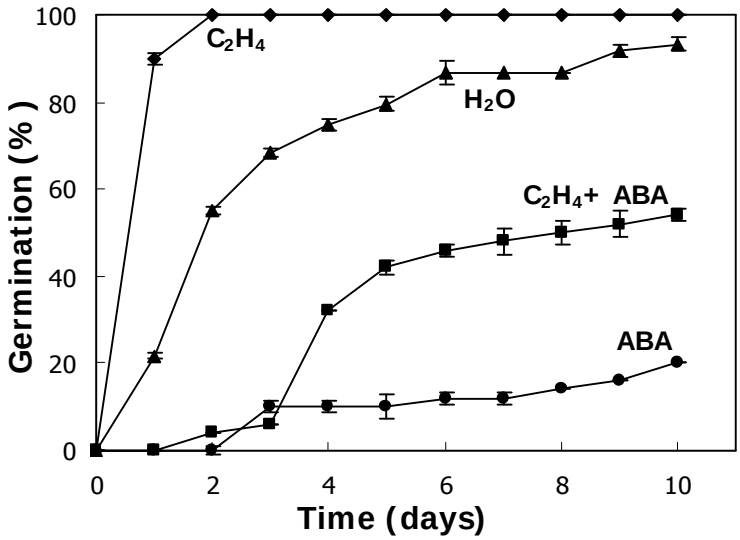


Figure 6. Effect of ethylene (50 μM) and ABA (0.1 mM) applied continuously on germination of dormant sunflower embryos placed at 20 °C, in darkness. Means of four measurements ± SD.

Incubation with ethylene improved the germination of the dormant embryos isolated from seeds harvested in 2004. 90 % of the population germinated within one day, against 22 % for the control embryos incubated in the air conditions. In contrast, 0.1 mM ABA inhibited the germination and only 20 % of embryos germinated within 10 days. When embryos were treated by ethylene (50 μM) in the presence of ABA (0.1 mM), the inhibitory effect of abscisic acid was reduced as about 40 % of embryos germinating within 10 days (Figure 6).

6.1.3. Effects of HCN on germination of sunflower embryos

6.1.3.1. Effects of HCN concentration and of the duration of treatment

Figure 7 shows the effects of 3 h treatment by HCN at various concentrations on the subsequent germination of dormant embryos placed at 10 °C, a temperature at which dormancy was expressed. The lowest used concentration (10^{-6} M) of HCN had slight stimulatory effect and only 35 % of

embryos germinated within 10 days. Using of higher concentrations of HCN strengthened the stimulatory effect on germination. In the presence of 10^{-3} M cyanide, around 80 and 98 % of the embryos germinated within 3 and 10 days, respectively (Figure 7A).

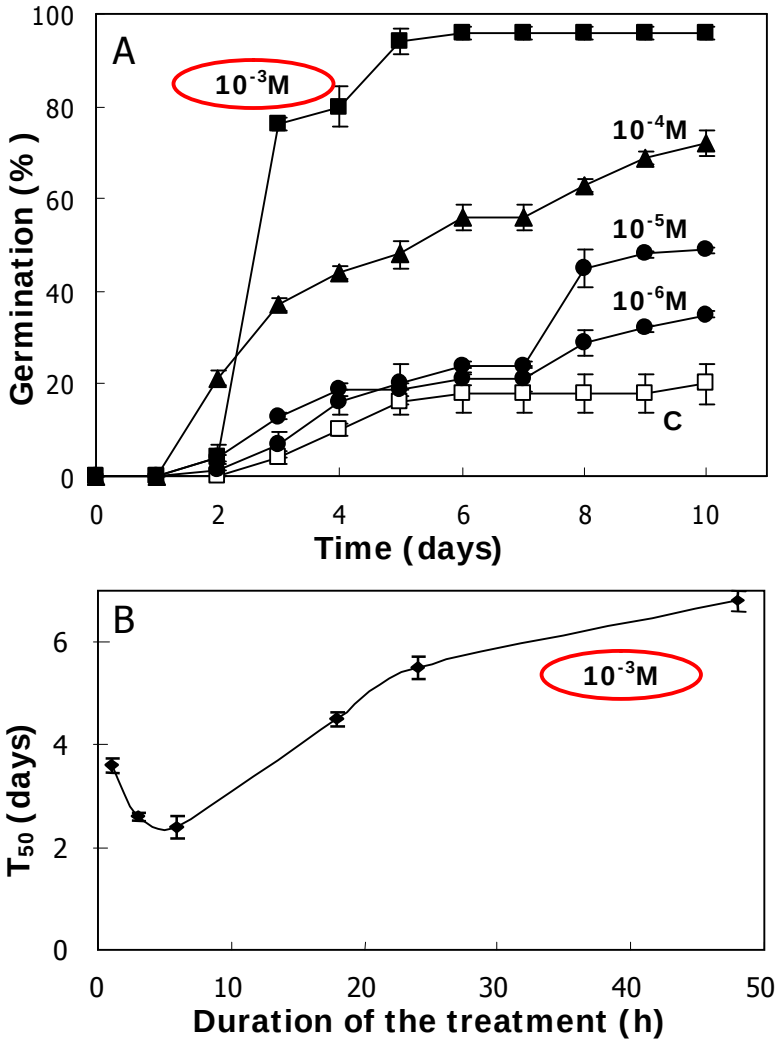


Figure 7. Effect of HCN on germination of dormant sunflower embryos. Effect of HCN concentration applied for 3 h on the germination at 10 °C, in the dark (A); Effect of the duration of treatment in the presence of 1mM HCN on the time to obtain 50 % germination of embryos transferred on water (B). (C, control embryos placed on water and non-treated by HCN). Means of three replicates ± SD.

Figure 7B shows the effect of the duration of treatment by HCN at 10^{-3} M on the subsequent germination of the embryo placed on water and in air

conditions, evaluated by the time to obtain 50 % germination (T_{50}). The optimal stimulatory effect was obtained after 1-6 h of treatment. In such condition T_{50} was the lowest (around 2.5-3.5 days). During prolonged treatment with HCN, embryos were not able to germinate until their transfer on to water and air conditions. Lengthen time of HCN treatment (more than 6 h) resulted in increase in T_{50}. After 48 h of HCN treatment, it reached more than 6.5 days (Figure 7B).

6.1.3.2. Sensitivity to HCN of sunflower embryos

6.1.3.2.1. Changes of HCN sensitivity during embryo imbibition

In order to determine whether sensitivity to HCN was affected during their imbibition, embryos were incubated on water at 10 °C, for various durations before being treated by 1 mM HCN for 3 h at the same temperature.

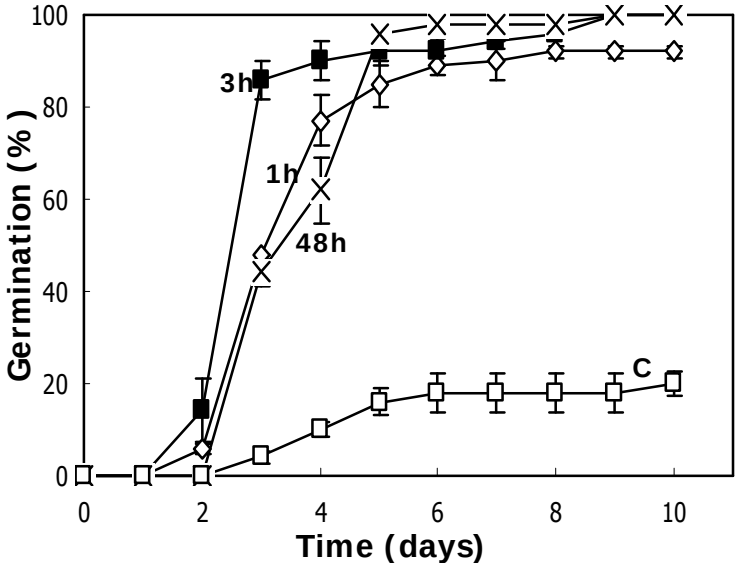


Figure 8. Effect of 3 h treatment with 1 mM HCN, applied after various duration (1, 3 and 48 h) of preimbibition. Time 0 correspond to the beginning of imbibition on water. (C, control embryos placed on water and non-treated by HCN). Means of three replicates ±SD.

Figure 8 shows that the duration of pre-imbibition of the embryos had no effect on their responsiveness to HCN. After 3 h of treatment with HCN, 80-95 % of the population germinated within 4-5 days, the T_{50} ranging around 2-3 days.

6.1.3.2.2. Changes of HCN sensitivity as function of sunflower embryo water content

To estimate the influence of embryo water content on their sensitivity to HCN, embryo water content was modulated from 5 % DW (dry embryos) to 25 % DW before the HCN treatment. Figure 9 shows the time course of germination at 10 °C of the embryo placed at 10 °C after the HCN application.

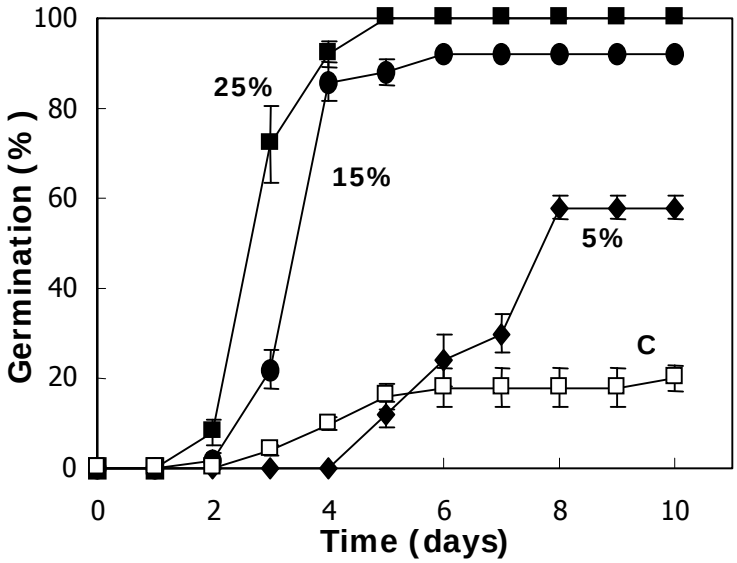


Figure 9. Effect of 3 h treatment by HCN (1 mM) on germination of dormant sunflower embryos at 10 °C, in darkness. HCN was applied on embryos at different water content (5 %, 15 % and 25 %). Time 0 correspond to the beginning of germination of control embryos or embryos at different water content and after treatment by HCN. (C, control embryos placed on water and non-treated by HCN). Means of three replicates ± SD.

Dry embryos, the moisture content of which was about 5 % DW, incubated in the presence of 1 mM for 3 h, germinated slowly at 10 °C and the subsequent germination percentage reached 60 % after 10 days. The stimulatory action of HCN increased with increasing water content of the embryo at the moment of the treatment. At 25 % DW, HCN treatment allowed the germination of the whole population within 4 days and 50 % of it germinated within around 2 days (Figure 9).

6.1.4. Effect of H_2O_2 and aminotriazol on germination of sunflower embryos

Table 3 shows the effect of H_2O_2 and aminotriazol on the germination of dormant embryos at 10 °C, in darkness.

Table 3. Effect of H_2O_2 (0.5 mM) and aminotriazol (1 mM) on germination of dormant sunflower embryos at 10 °C, in darkness. Embryos were treated for 6 h before being transferred on water or placed continuously in the presence of these compounds. Values are means of three replicates ± SD. (d, days).

Treatment		**Germination (%)**	
Compounds	**Duration**	**7 d**	**14 d**
H_2O_2 (0.5 mM)	6h	26 ± 4	72 ± 5
	continuous	50 ± 4	81 ± 3
Aminotriazol (1 mM)	6h	28 ± 3	40 ± 2
	continuous	56 ± 5	80 ± 4
H_2O	continuous	18 ± 3	32 ± 3

H_2O_2 strongly stimulated the germination of the embryos placed at 10 °C. Continuous treatment with H_2O_2 (0.5 mM) resulted in 71 % of germinated embryos within 10 days (Table 3). A short treatment (6 h) was enough to improve the germination.

Application of aminotriazol, an inhibitor of catalase activity, also stimulated germination of dormant sunflower embryos. Continuous application of this compound allowed 70 % of germination within 10 days. Six hours treatment by aminotriazol (1 mM) had the same effect as 6 h treatment by H_2O_2 (0.5 mM) (Table 3).

6.1.5. Towards elucidation of HCN effect on germination of sunflower embryos

6.1.5.1. Effect of respiratory inhibitors

To analyze whether cyanide mode of action could be associated with modification of respiratory pathways, dormant sunflower embryos were imbibed in the presence of various respiratory inhibitors at 1 mM for 3 h and then transferred on water. Table 4 shows the germination percentages of dormant

sunflower embryos obtained after 3, 7 and 10 days of incubation on water (control) or in the presence of those compounds.

Table 4. Germination of sunflower dormant embryos incubated on water or after 3 h treatment by various solutions (1 mM), at 10 °C, in darkness. Means of three replicates ± SD. (d, days).

Incubation medium	Germination (%)		
	3 d	7d	10 d
H_2O	4 ± 3	18 ± 3	20 ± 4
HCN	76 ± 3	96 ± 3	98 ± 1
NaN_3	8 ± 3	40 ± 3	50 ± 3
HCN + NaN_3	38 ± 4	98 ± 2	98 ± 2
SHAM	2 ± 3	20 ± 3	36 ± 4
HCN + SHAM	64 ± 3	78 ± 2	82 ± 2

Application of other respiratory inhibitor than HCN ☐ sodium azide (NaN_3), stimulated germination of sunflower embryos but at lower percentages than in the case of HCN. Within 10 days only 50 % of the population germinated against 98 % in the case of HCN treated ones. NaN_3 in addition with HCN did not affect the stimulatory effect of cyanide on germination of dormant sunflower embryos and resulted in similar percentages of germinated embryos (Table 4). Furthermore, incubation of embryos in the presence of an inhibitor of cyanide insensitive pathway ☐salicylhydroxamic acid (SHAM), did not change the stimulatory effect of HCN on germination. After 3, 7 and 10 days of HCN + SHAM treatment 64, 78 and 82 % of embryos germinated, whereas the presence of SHAM had no positive effect on the germination and after the same days 2, 20 and 36 % of germinated embryos were observed, respectively (Table 4).

6.1.5.2. Effect of methylviologen and menadione

In order to assess a putative casual association between HCN, ROS and dormancy alleviation, sunflower embryos were imbibed in the presence of hydrogen cyanide or in the presence of methylviologen (MV) and menadione

(MD), a ROS-generating compounds. The time courses of germination of sunflower dormant embryos treated by those compounds are presented in Figure 10.

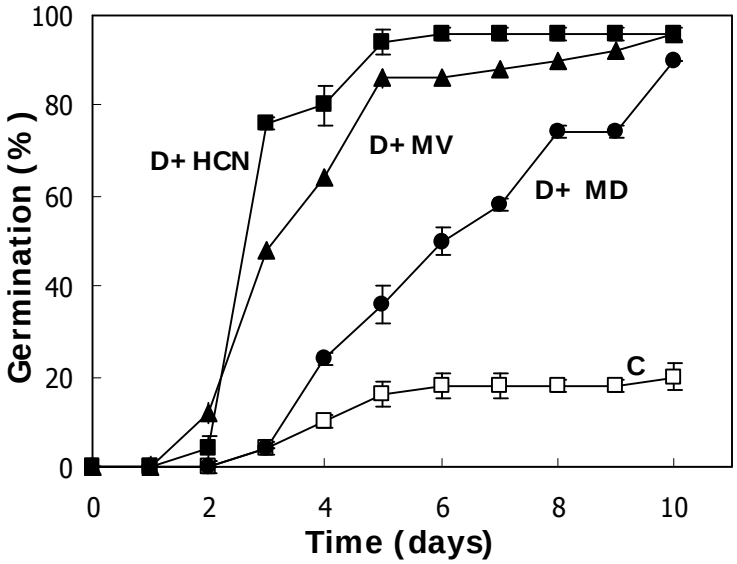


Figure 10. Effect of MV (0.1 mM), MD (1 mM) and HCN (1 mM) on the germination of dormant sunflower embryos at 10 °C, in the dark; Embryos were treated for 3 h before being transferred on water. (C, control embryos placed on water and non-treated by HCN). Means of three replicates ± SD. (from Oracz *et al.* 2007)

As HCN, a short treatment (3 h) by methylviologen (0.1 mM), as well as menadione (1 mM), stimulated the germination of dormant sunflower embryos and similar results as in the case of cyanide treatment was observed. After 7 days around 88 % and 58 % of embryos germinated respectively, against 18 % for embryos placed on water (Figure 10). For the reason of higher efficiency of MV on the germination percentages in the next part of experiments as a source of ROS, just 0.1 mM methylviologen treatment by 3 h was used.

6.1.5.3. Effect of DPI

By HCN treatment of dormant sunflower embryos in the presence of diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, it was shown that cyanide beneficial effect on germination might be associated with regulation of this enzyme activity (Figure 11).

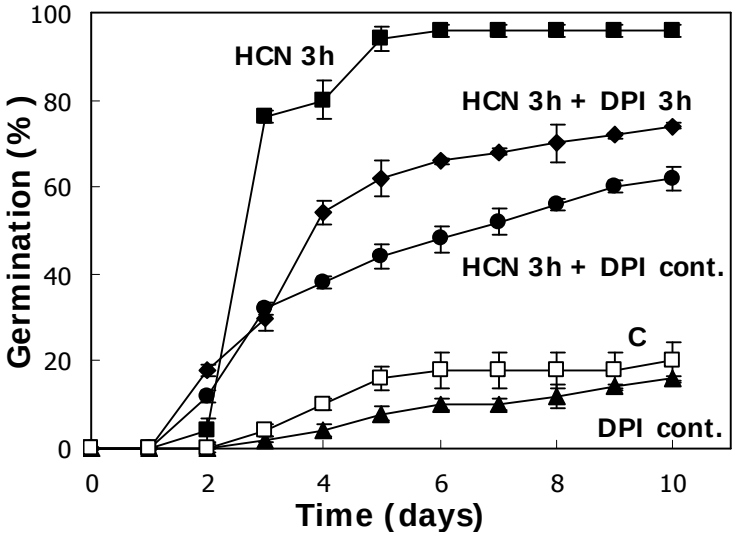


Figure 11. Effect of DPI (0.1 mM) and HCN (1 mM) on the germination of dormant sunflower embryos at 10 °C, in the dark; Embryos were treated for 3 h before being transferred on water or placed continuously in the presence of DPI. Control embryos placed on water and non-treated by HCN and/or DPI (C). Means of three replicates ± SD.

Application of both chemicals together resulted in lower % of germination than that induced by HCN treatment, and at 10th day around 50-70 % of germinated seeds was observed (about 30-50 % less than in the case of HCN treatment) (Figure 11). In addition, the continuous presence of DPI slightly slowed down and decreased germination percentages of dormant sunflower embryos.

6.1.5.4. HCN metabolism

Knowing that sunflower embryos do not contain cyanogenic compounds it was a particular interest to investigate whether imbibition process cause increase in free cyanide content released during ethylene synthesis and if there exist any relationship with stimulation of amino acids synthesis by stimulation of activity of *β*-cyanoalanine synthase (*β*-CAS) by HCN. In dry dormant and non-dormant sunflower embryos free HCN was not detected (data not shown). Imbibition process resulted in slight increase in HCN content but active detoxify

enzyme (*β*-CAS) maintained cyanide level at safe level (less than 5 μM) (not shown).

β-CAS activity in dry axes isolated from dormant and non-dormant sunflower embryos had similar values in both samples (Figure 12).

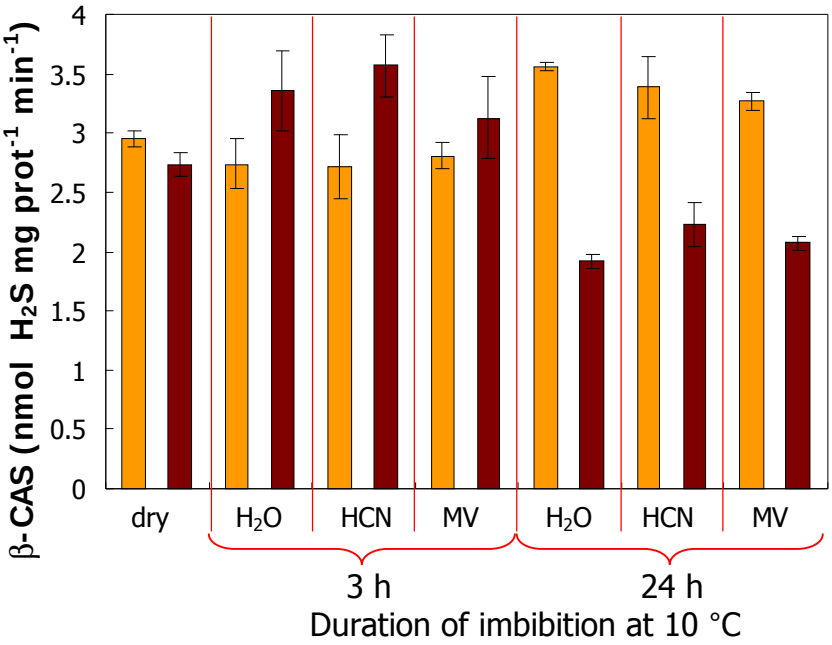


Figure 12. β-CAS activity in axes isolated from dormant (yellow bars) and non-dormant (brown bars) sunflower embryos: dry or imbibed on water, or treated for 3 h with 1 mM HCN or 0.1 mM MV, at 10 °C, in darkness. Values are mean of four replicates ± SD.

During the imbibition process, the activity of this enzyme did not change significantly. Moreover, application of methylviologen or cyanide also had no effect on *β*-CAS activity and remained stable during germination neither in the case of axes isolated from treated dormant or non-dormant embryos (Figure 12).

6.2. Mechanism of action of HCN in relation to ROS metabolism

6.2.1. Effect of HCN on ROS production and lipid peroxidation

6.2.1.1. H_2O_2, $O_2^{\cdot -}$ and MDA content

To characterize ROS production during release of dormancy by HCN, hydrogen peroxide and superoxide anion contents were determined in axes excised from dry dormant and non-dormant sunflower embryos, after 3 and 24 h of incubation at 10 °C, on water (control) and embryos treated by HCN or methylviologen (MV) solution.

Table 5. H_2O_2 content in axes isolated from dry dormant and non-dormant sunflower embryos or, after 3 and 24 h of incubation at 10 °C on water (control). Embryos were treated by HCN (1 mM) or by MV (0.1 mM) for 3 h at 10 °C. Values are means of five replicates ± SD.

Type of embryos	Treatment	H_2O_2 (μM g^{-1} DW)		
		0 h (dry)	3 h	24 h
Dormant		1.5 ± 0.21		
	H_2O		2.1 ± 0.13	2.5 ± 0.19
	HCN		2.8 ± 0.12	4.2 ± 0.35
	MV		2.7 ± 0.11	4.5 ± 0.17
Non-dormant		2.1 ± 0.14		
	H_2O		2.9 ± 0.09	4.2 ± 0.20
	HCN		3.2 ± 0.17	5.0 ± 0.11
	MV		3.1 ± 0.15	5.4 ± 0.22

In axes isolated from dry sunflower embryos H_2O_2 content was higher (2.1 μM g^{-1} DW) in non-dormant axes than in dormant ones (1.5 μM g^{-1} DW) (Table 5). Release of dormancy during after-ripening seemed then to be connected with accumulation of H_2O_2. Dormancy release was also associated with a marked enhancement of H_2O_2 content during the first hours of imbibition, particularly in axes excised from embryos treated by cyanide or methylviologen (Table 5).

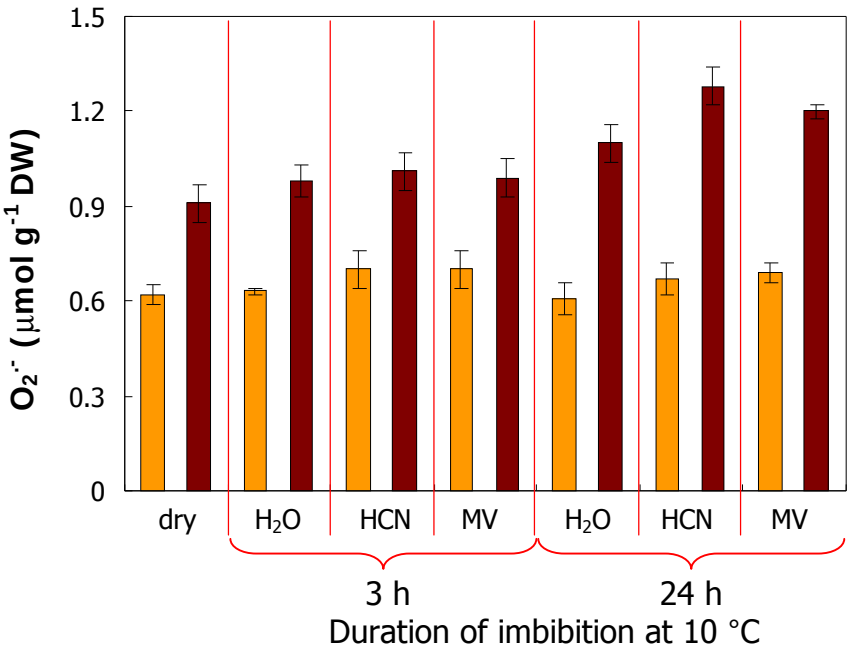


Figure 13. Superoxide radical content in axes isolated from dormant (yellow bars) and non-dormant (brown bars) sunflower embryos dry, or imbibed on water, or treated by HCN or methylviologen, at 10 °C, in darkness. Values are mean of five replicates ± SD.

Spectrophotometric determination of superoxide radical shows that there was around 30 % more $O_2^{\cdot-}$ content in axes isolated from non-dormant embryos than in those excised from dormant ones (Figure 13). When H_2O_2 content increased progressively during imbibition whatever their treatment (Table 5), the level of $O_2^{\cdot-}$ seemed to be stable during germination at 10 °C on water, and was not significantly affected by HCN or MV treatment (Figure 13).

Dormancy release during after-ripening was associated with changes in MDA content, indicating the occurrence of lipid peroxidation, and around 20 % more MDA was detected in axes isolated from non-dormant embryos (Figure 14). The first hours of imbibition resulted in a slight decrease in MDA content in axes from non-dormant embryos, but in an increase in this compound in dormant ones (Figure 14). After 3 h of imbibition in the presence of HCN or MV, MDA content of axes isolated from dormant embryos was similar to that measured in non-dormant ones. MDA content, after 24 h was not significantly different in both types of axes isolated from control or HCN or MV treated embryos (Figure 14).

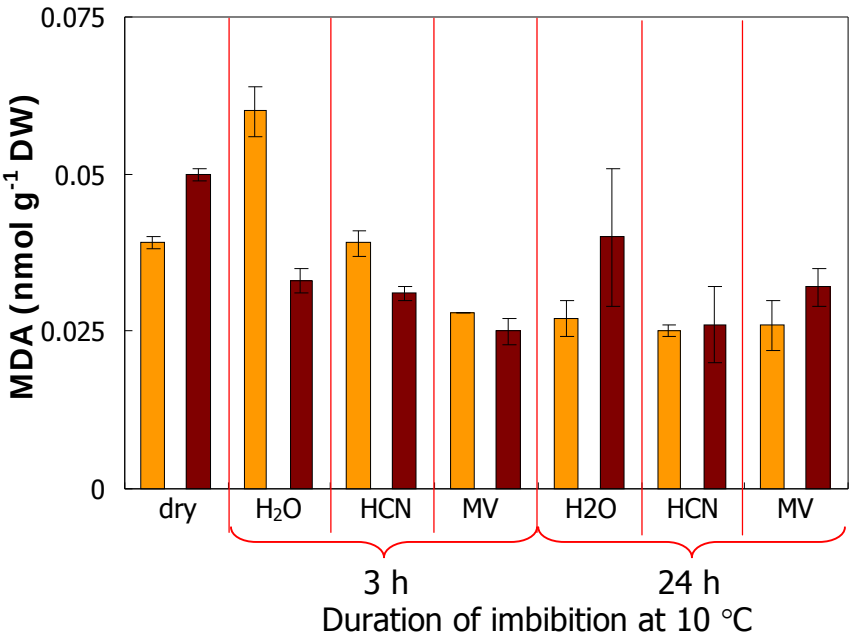


Figure 14. MDA content in axes isolated from dormant (yellow bars) and non-dormant (brown bars) sunflower embryos dry or imbibed on water, or treated by HCN or methylviologen, at 10 °C, in darkness. Values are mean of three replicates ± SD.

6.2.1.2. In situ determination of ROS

The detection of ROS was carried out using 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H_2DCFHDA), the fluorescence of which was detected by conflocal microscopy.

Changes in fluorescence were measured from the injection of CM-H_2DCFHDA (time 0) until 7th min of incubation. Figure 15 presents the microscopy images obtained after 7th min of incubation. Analysis showed that in axes isolated from dormant sunflower embryos after 3 and 24 h of imbibition in the presence of water the fluorescence intensity increased slower and was less intensive (weak green fluorescence in cells) than in axes isolated from dormant treated embryos and non-dormant (treated and non-treated by cyanide) (Figures 15 and 16).

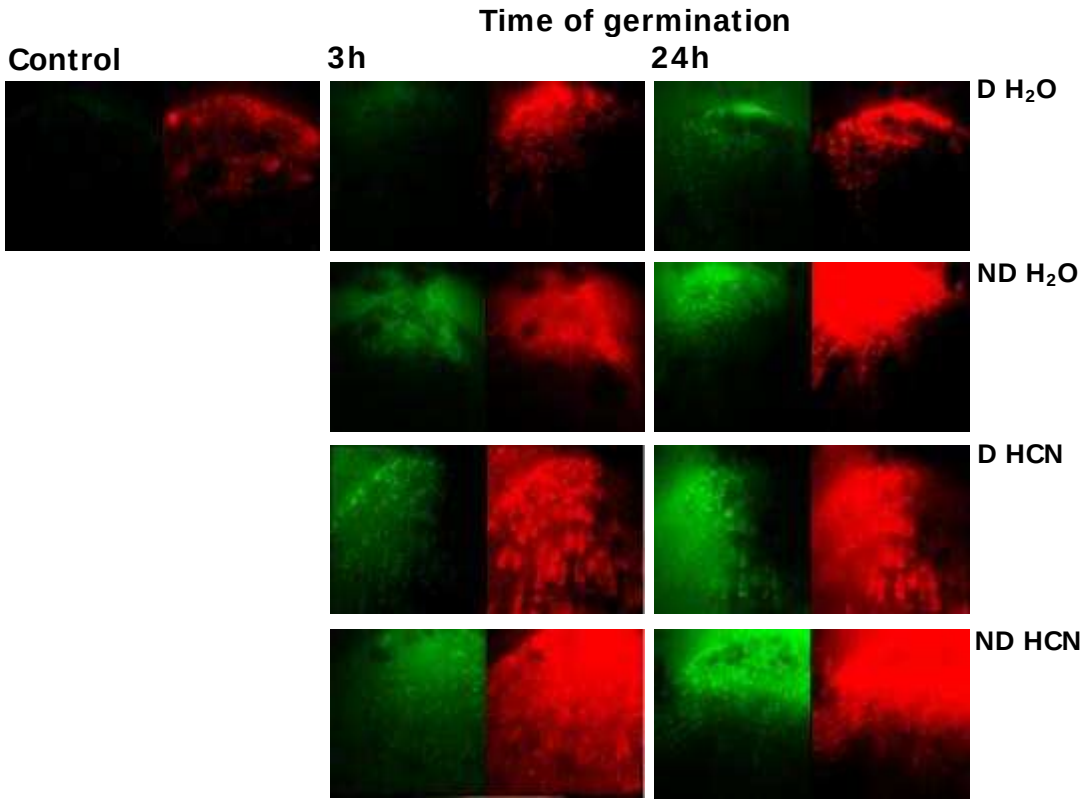


Figure 15. *In situ* ROS localization in axes isolated from dormant and non-dormant sunflower embryos dry or imbibed on water, or treated by HCN, at 10 °C, in darkness. The representative images from a confocal microscop, after 7th min of incubation with CM-H_2DCFHDA are presented. (green image – fluorescence of DCF (dichlorofluorescein), red image – autofluorescence).

Obtained results show that dormancy alleviation is associated with an accumulation of ROS content manifested by fast increase in green fluorescence intensity in the cells of axes excised from embryos which were able to germinate.

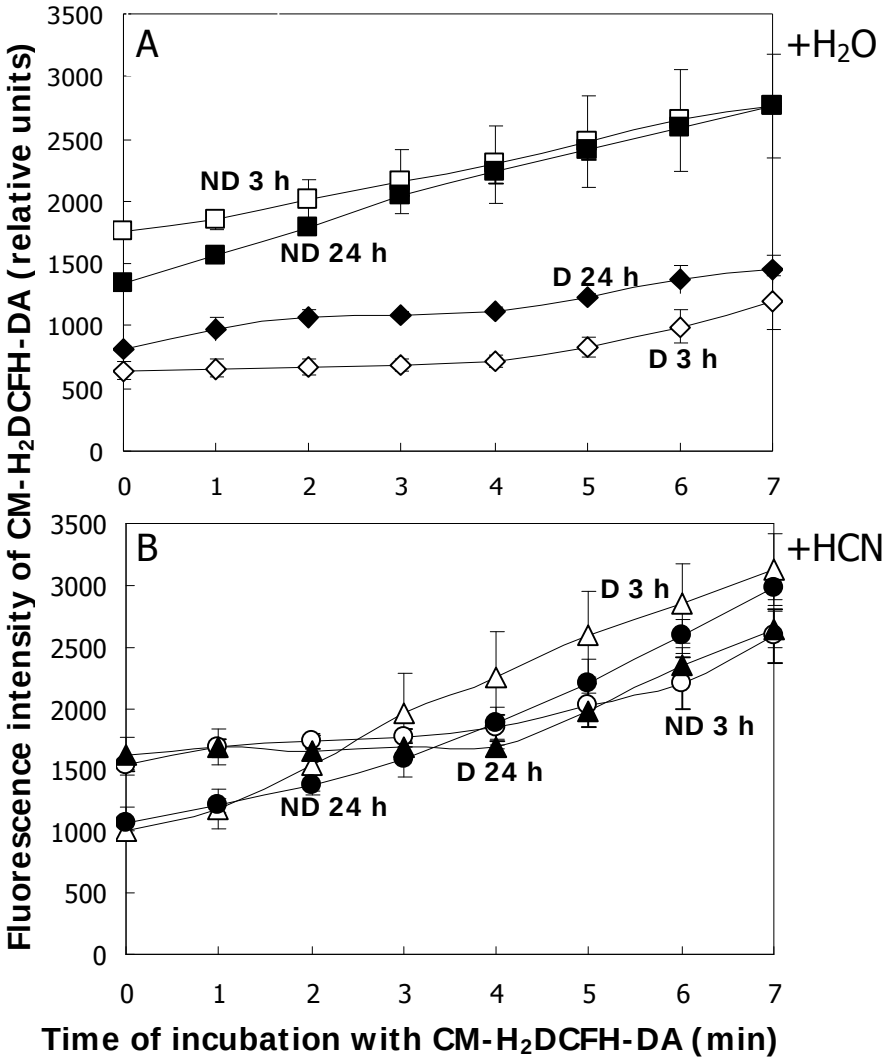


Figure 16. ROS formation in axes of dormant (D) and (ND) non-dormant sunflower embryos during incubation at 10 °C, on water. Embryos were not-pretreated by HCN (A) or pretreated during 3 h with HCN (B). Axes isolated from dormant (◇,△) or non-dormant (□,○) after 3 h of imbibition or dormant (◆,▲) and non-dormant (■,●) after 24 h of imbibition. Time course of ROS production after addition of CM-H_2DCFH-DA (time 0 to 7 min). Results represent the means (± SD) of three separate experiments.

6.2.2. Effects of HCN on the antioxidant system

6.2.2.1. Glutathione content

To determine whether ROS production during embryo dormancy alleviation by HCN might be associated with changes in non enzymatic antioxidant system, GSH and GSSG contents were measured. In dry axes excised from dormant and non-dormant GSSG+GSH, GSSG and GSH contents were about 0.92, 0.17 and 0.74 μmol g^{-1} DW, respectively (Figure 17).

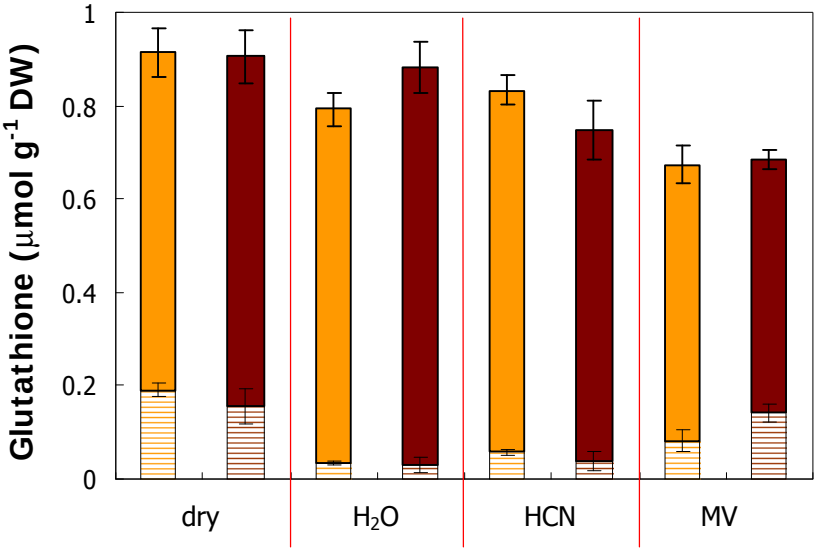


Figure 17. GSSG (striped blocks) and GSH (non-striped blocks) contents in axes isolated from dormant (yellow bars) and non-dormant (brown bars) sunflower embryos. Measurements were carried out in axes isolated from dry embryos or embryos incubated for 24 h on water, not-pretreated or pretreated by HCN (1 mM) or by MV (0.1 mM) for 3 h, at 10 °C, in darkness. Values are mean of four replicates ± SD.

Imbibition of both types of embryos, resulted in a slight decrease in GSSG+GSH content due to a reduction in GSSG content (Figure 17). Similar results were observed in axes isolated from dormant and non-dormant embryos treated by cyanide, but not in the samples treated by MV in which comparatively high GSSG content was detected (Figure 17).

Table 6 presents GSH/GSSG ratios obtained in axes isolated from sunflower embryos. Relatively low GSH/GSSG ratios observed in dry axes of dormant and non-dormant of embryos (~ 3.79 and 4.85, respectively) strongly

increased during imbibition on water (~ 22.32 and 29.41, respectively), with tendency that lower values were detected in samples obtained from dormant ones. However, HCN and MV treatments resulted in decrease of GSH/GSSG ratios (Table 6).

Table 6. GSH/GSSG ratios in axes isolated from dormant and non-dormant sunflower embryos. Measurements were carried out in axes isolated from dry embryos or embryos incubated for 24 h on water, not-pretreated or pretreated by HCN (1 mM) or by MV (0.1 mM) for 3 h, at 10 °C, in darkness. Values are mean of four replicates ± SD.

Combination	GSH/ GSSG	
	D	ND
	24 h of imbibition	
Dry	3.79 ± 0.51	4.85 ± 0.61
H_2O	22.32 ± 6.13	29.41 ± 4.13
HCN	13.63 ± 6.07	18.68 ± 2.99
MV	7.32 ± 2.15	3.85 ± 0.90

6.2.2.2. Enzymatic system

In order to investigate whether alleviation of embryo dormancy by HCN involved the antioxidant system which regulate ROS accumulation, the activities of CAT, GR and SOD were measured in axes isolated from dormant and non-dormant sunflower embryos before imbibition (dry) and after 3 and 24 h of imbibition in the presence of water at 10 °C. The embryos were placed directly on water or pretreated by HCN or MV during 3 h.

CAT specific activity in axes excised from dry dormant and non-dormant embryos was 5.96 and 6.4 nmol H_2O_2 mg^{-1} prot min^{-1}, respectively. The first 3 h of imbibition was associated with an increase in CAT activity, up to 150 % of the initial activity. The enzyme activity was then stable until 24 h (Figure 18A). Application of gaseous cyanide or methylviologen did not change CAT activity which remained constant during performed experiments (Figure 18A).

GR activity fluctuated from 0.03 to 0.057 nmol NADPH mg $protein^{-1}$ min^{-1} (Figure 18B). HCN and MV treatment had no effect on GR activity in axes

of dormant embryos. Curiously, they resulted in an increase in GR activity in axes of non-dormant embryos after 3 h of imbibition, but this improving effect was no more visible at 24 h (Figure 18B).

Relatively high SOD specific activity was detected in axes isolated from dry dormant and non-dormant sunflower embryos (7.36 and 7.16 Units mg^{-1} prot, respectively) (Figure 18C). In axes of control embryos it slightly decreased during imbibition reaching 4.94 and 5.54 Units mg^{-1} prot, respectively after 24 h. Similar results were observed in axes isolated from dormant and non-dormant embryos treated by cyanide and methylviologen in which comparatively to control, lower SOD activity was detected (Figure 18C).

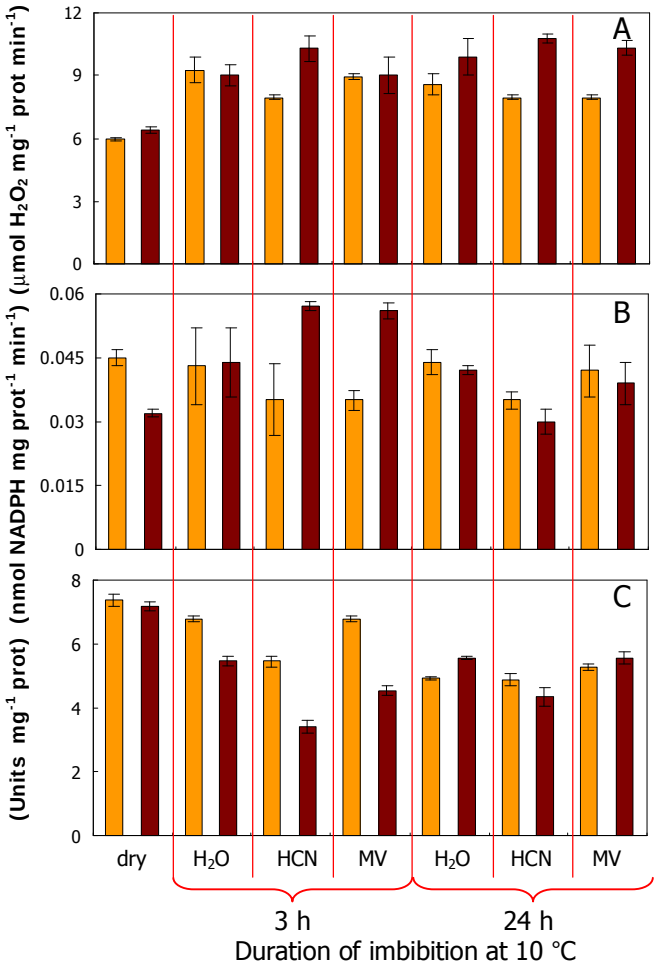


Figure 18. Activities of antioxidant enzymes CAT (A), GR (B) and SOD (C), in dry dormant (yellow bars) and non-dormant (brown bars) sunflower embryos and imbibed on water or treated by HCN or MV, at 10 °C, in darkness. Values are mean of three replicates ± SD.

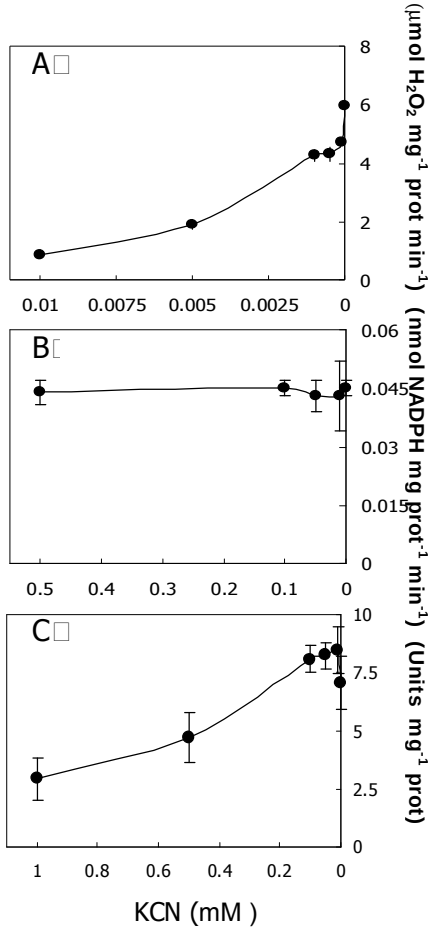


Figure 19. Inhibitory effect of KCN solutions on activities of antioxidant enzymes: CAT (A), GR (B) and SOD (C), in dry axes isolated from dormant sunflower embryos. Values are mean of three replicates ± SD.

Inhibitory effect of KCN solutions on *in vitro* activities of enzymes of antioxidant system (CAT, GR, SOD) in dry axes isolated from dormant (Figure 19) sunflower embryos was also analyzed. From all analyzed enzymes the most resistant to KCN was GR, because even relatively high concentration of used inhibitor (0.5 mM) did not affect its activity (Figure 19B`). The most sensitive enzyme was CAT, which activity was inhibited by KCN concentrations lower than 1-2 μM (Figure 19A`). In the case of SOD activity, the used KCN concentrations higher than 100 μM strongly inhibited SOD activity (Figure 19C`).

6.2.3. Protein carbonylation

This part of the study was realized within the frame of a joint project between University Pierre et Marie Curie (Paris, France) and Centre National de la Recherche Scientifique/ Bayer CropScience Joint Laboratory (Lyon, France) under the guidance of Dr D. Job and Dr C. Job.

To determine whether ROS production during dormancy alleviation by HCN in sunflower embryos could be associated with protein oxidation (carbonylation), one-dimensional (1D) and two-dimensional (2D) PAGE of axe protein extracts were performed, and the presence of carbonyl groups was detected by Western blotting using the 2,4-dinitrophenylhydrazine (DNPH) immunoassay.

The protein patterns obtained by 1D gel electrophoresis of soluble proteins from dormant and non-dormant embryonic axes are shown in Figure 20 (and Figure 4 in Oracz *et al.* 2007). Coomassie blue staining showed that the soluble protein patterns were similar for all the treatments. Soluble proteins from dry dormant axes exhibited three faint carbonylated bands of about 75, 60 and 55 kDa (Figure 20, carbonyls). In contrast, the extent of protein carbonylation was much higher in soluble proteins from dry non-dormant axes than in dormant ones, and a number of new carbonylated bands were detected (Figure 20A, B). During imbibition of non-dormant embryos on water (Figure 20D), like for dormant embryos treated by methylviologen (Figure 20E) and hydrogen cyanide (Figure 20F), a strong increase in carbonylated protein level

was observed. However, in the case of axes isolated from dormant embryos decrease in the carbonylation intensity was observed (Figure 20C).

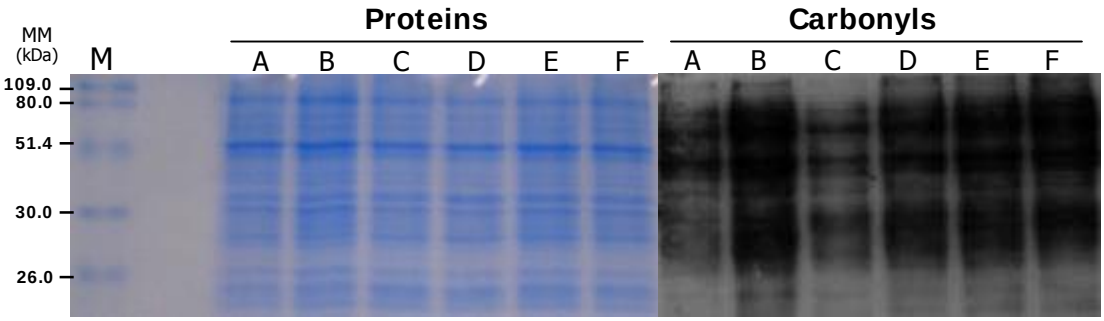


Figure 20. One-dimensional PAGE of oxidatively modified proteins from axes of dry dormant (A) and non-dormant (B) embryos, axes of non-dormant (D) and dormant (C) embryos imbibed on water during 3 h, axes of dormant embryos treated by MV (E) or HCN (F) for 3 h at 10 °C, in darkness. Protein stain (proteins) and anti-DNP immunoassay (carbonyls) are shown. (from Oracz *et al.* 2007)
M – markers; MM – molecular mass.

The protein targets of carbonylation during sunflower embryo dormancy alleviation were characterized by 2D gel electrophoresis (Figure 21 and Figure 7(a)-(d) in Oracz *et al.* 2007). Oxyblots revealed that during imbibition on water the level of protein oxidation was higher in non-dormant axes, which displayed many new oxidized proteins ranging from approximately 15-40 kDa (Figure 21B). Conversely, around 20 proteins that were found to be carbonylated during imbibition (on water) in dormant axes were not detected in non-dormant axes (Figure 21A, orange circles).

To further document a link between protein oxidation and release of seed dormancy, dormant embryos were imbibed in the presence of hydrogen cyanide. Three hours of imbibition of dormant embryos in the presence of 1 mM hydrogen cyanide triggered the carbonylation of specific proteins in dormant axes (e.g. blue circles in Figure 21C). This specific carbonylation pattern was not seen by incubating dormant embryos in water only (Figure 21A), although this latter incubation also resulted in an increase in protein carbonylation compared with the dry embryos (Figure 5(b) in Oracz *et al.* 2007). It is also interesting that the pattern of protein carbonylation is similar in all of the non-dormant axes (Figure 21B, D), whatever the treatment.

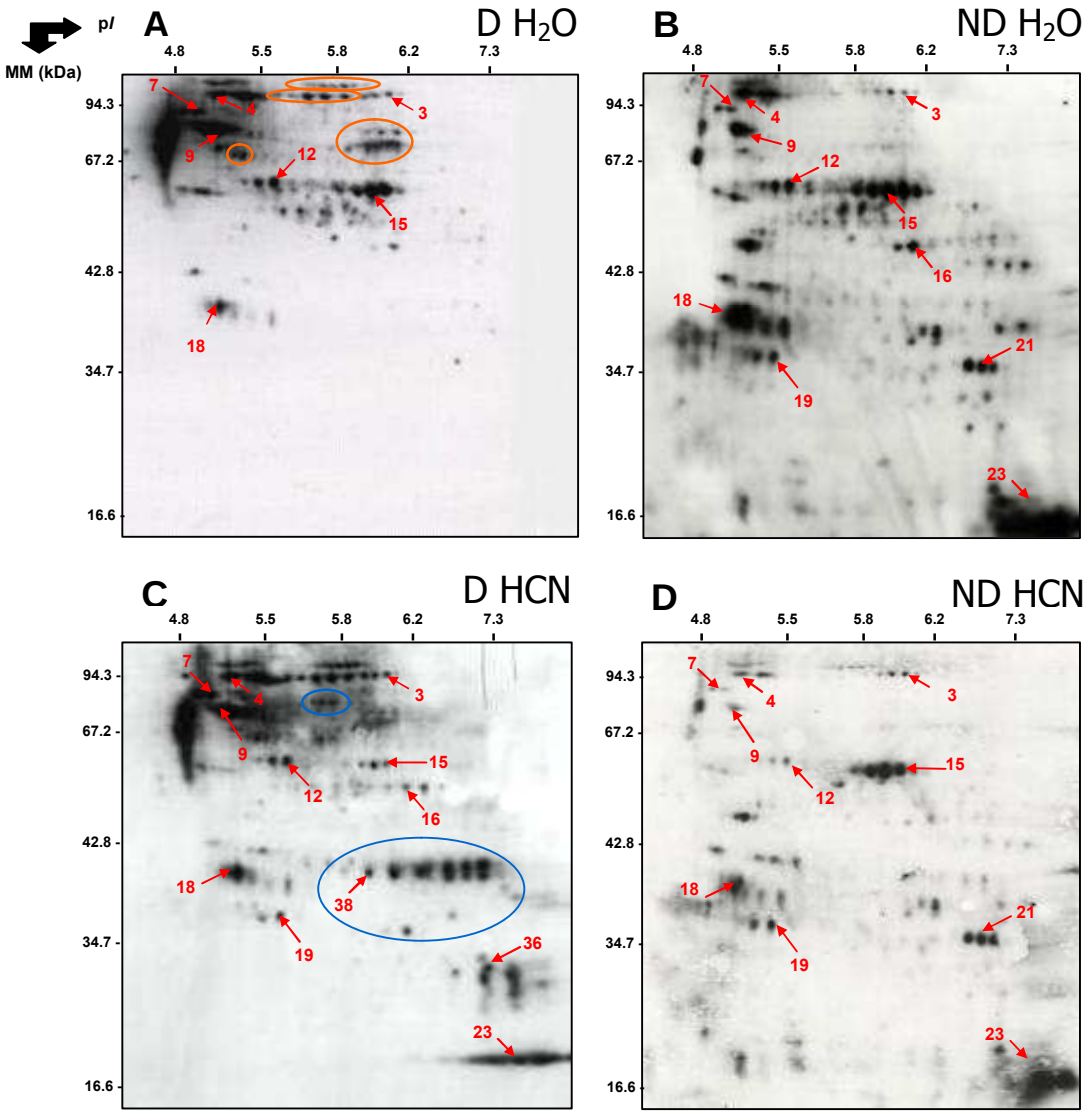


Figure 21. Two-dimensional profiles of protein oxidation in axes of (A) dormant and (B) non-dormant sunflower embryos after 3 h imbibition in the presence of water; in axes of dormant (C) and non-dormant (D) embryos after 3 h imbibitoin in the presence of 1 mM HCN at 10 °C, in darkness. Orange circles indicates proteins specifically carbonylated in dormant but not in non-dormant during imbibition on water. Blue circles indicate proteins specifically carbonylated in the presence of hydrogen cyanide. Red arrows indicated spots analyzed by MS. (from Oracz *et al*. 2007)

6.2.4. Identification of protein by MS

This part of the study was realized within the frame of a joint project between University Pierre et Marie Curie (Paris, France) and INRA (Nouzilly, France) under the guidance of Dr M. Belghazi.

Carbonylated proteins identified by mass spectroscopy are listed in Table 7.

Table 7. Identification of carbonylated proteins in embryo axes of dormant and non-dormant sunflower seeds imbibed for 3 h on water or in the presence of 1 mM HCN, at 10 °C. (from Oracz *et al.* 2007)

Spot No	Accession	Protein name	MW (kDa)	pI	Carbonylation			
					D H_2O	D HCN	ND H_2O	ND HCN
3	gil6056373	Elongation factor EF-2 (*Arabidopsis thaliana*)	93.9	6.1	+	+	+	+
4	gil3024423	Pyruwate, phosphate dikinase, chloroplast precursor (*Flaveria brownie*)	93.9	5.1	+	+	+	+
7	gil217855	81 kDa heat shock protein (*Arabidopsis thaliana*)	84.0	4.9	+	+	+	+
9	gil2827002	HSP70 (*Triticum aestivum*)	73.4	5.0	+	+	+	+
12	gil22273	Enolase (*Zea mays*)	60.0	5.5	+	+	+	+
15	gil58735933	DH0AQA1ZD02RM1 HaDevS1, Helianthus annuus 7S globulin	60.0	6.9	+	+	+	+
16	gil71793966	Alcohol dehydrogenase (*Alnus glutinosa*)	42.8	6.2	-	+	+	+
18	gil22460345	QHF6E13.yg.ab1 QH_EFGHJ sunflower RHA280, Helianthus annuus cDNA clone (≥gil1458098) globulin-like protein (*Daucus carota*)	38.0	5.2	+	+	++	+
19	gil22459294	QHF12M17.yg.ab1 OH_EFGHJ sunflower RHA280 *Helianthus annuus* cDNA clone QHF12M17, mRNA sequence ≥gil81238594lgblABB60055.1l 11S globulin precursor isoform 4 (*Sesamum indicum*)	34.0	5.5	-	+	+	+
21	gil22462073	QHG17D11.yg.ab1 QH_EFGHJ sunflower RHA280 *Helianthus annuus* cDNA clone QHG17D11, mRNA sequence ≥gil27526460lemblCAC80712.1l putative dehydrin (*Helianthus petiolaris*)	36.0	6.5	-	-	+	+
23	gil27526481	Basic 2S albumin (*Helianthus annuus*)	16.6	7.0	-	+	+	+
36	gil22310301	QHA17G24.yg.ab1 QH_ABCDI sunflower RHA801 Helianthus annuus cDNA clone QHA17G24 mRNA sequence ≥gil4127629iemblCAA76572.1l11Sl storage protein (*Coffea arabica*)	21.0	7.3	-	+	-	-
38	gil22393902	QHl21H20.yg.ab1 QH_ABCDl sunflower RHA801 *Helianthus annuus* cDNA clone QHl21lH20, mRNA sequence ≥gil5302785lemblCAB46034.1l putative epoxide hydrolase (*Arabidopsis thaliana*)	40.0	5.8	-	+	-	-

EF2 (spot number 3), PPDK (spot number 4), 81 and 70 kDa HSP (spot numbers 7 and 9), enolase (spot number 12) and 7S globulin (spot number 15) were carbonylated in the axes of all imbibed embryos (Figure 21, Table 7 and Oracz *et al.* 2007). The globulin-like protein (spot number 18) was also always carbonylated in imbibed axes, but the level of oxidation appeared to be

stronger in non-dormant axes isolated from embryos imbibed on water (Figure 21B). Treatment of imbibed dormant embryos with cyanide resulted in the carbonylation of proteins not detected in non-dormant ones. For example, proteins those show homology with 11S storage protein (spot number 36 in Figure 21) and epoxide hydrolase (spot number 38 in Figure 21) were detected in axes isolated from dormant embryos but did not appear in the axes of non-dormant embryos treated by HCN. Other, proteins sharing homology with an 11S globulin precursor, an alcohol dehydrogenase and basic 2S albumin (spot numbers 19, 16 and 23, respectively, in Figure 21) were oxidized in axes of all embryos able to germinate. Spot number 21 was detected only in imbibed non-dormant embryos (Figure 21B, D) and this protein was found to be similar to dehydrin (Table 7).

6.2.5. Effects of HCN on expression of genes involved in ROS production and signal transduction

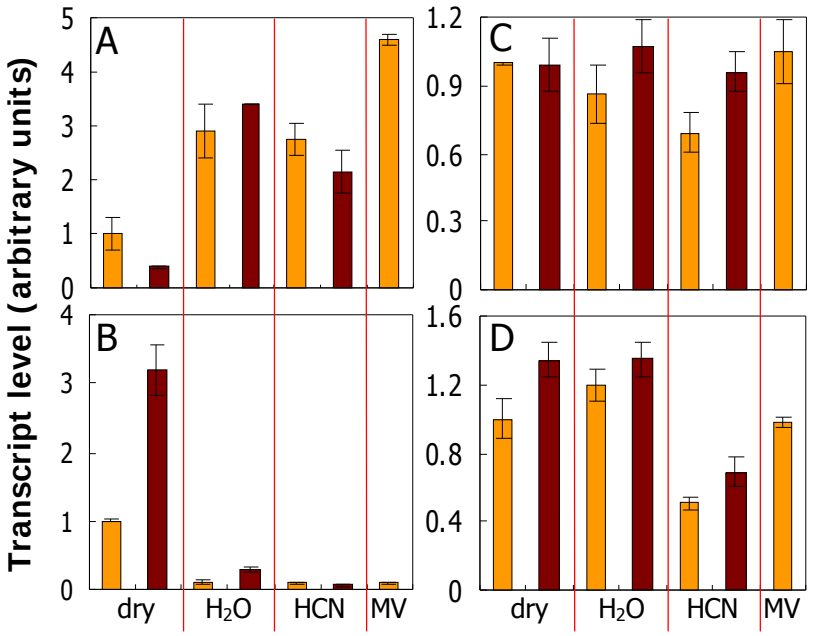


Figure 22. *NADPHox* (A), *POX* (B), *AO1* (C), *AO2* (D) transcript expression in axes isolated from dormant (yellow bars) and non-dormant (brown bars) sunflower embryos dry or after 24 h of incubation at 10 °C, on water (control), in darkness. Embryos were treated by HCN (1 mM) for 3 h or, by MV (0.1 mM) for 3 h, at 10 °C. Data from QRT-PCR (mean of four replicates ± SD) are expressed in arbitrary units.

Figure 22 presents the levels of transcripts of *NADPHox* (Figure 22A), *POX* (Figure 22B), *AO1* (Figure 22C) and *AO2* (Figure 22D) genes in axes isolated from dormant and non-dormant embryos before imbibition (dry) and after 3 h of incubation on water at 10 °C, without (control) or with a pre-treatment with HCN or methylviologen. The transcripts of the 4 genes studied are present in the dry axes. *NADPHox* expression increased after embryo imbibition, but was similar whatever the treatment (Figure 22A). In contrast, imbibition was associated with a decrease in the amount of *POX* gene transcripts (Figure 22B), but HCN and MV treatments did not affect *POX* expression. It is interesting to notice that *POX* transcripts level was significantly higher in axes isolated from dry non-dormant embryos than from dormant ones. The level of *AO1* and *AO2* transcripts were similar in dry dormant and

non-dormant axes (Figure 22C, D). They did not change during imbibition of control embryos on water. Application of cyanide or methylviologen resulted in decrease in *AO2* expression (Figure 22D), but did not affect that of *AO1* (Figure 22C).

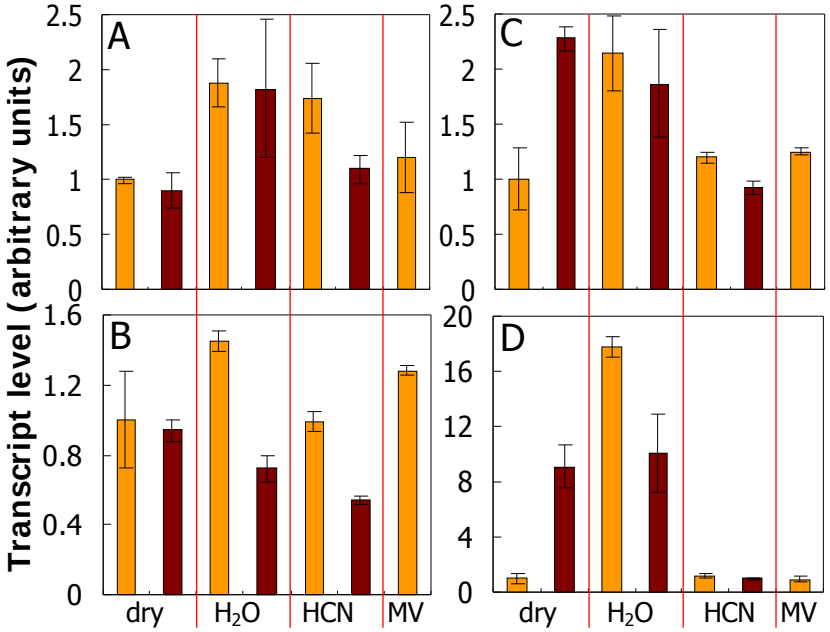


Figure 23. *MAPK6* (A), *SerThrPK* (B), *PTP* (C), *CaM* (D) transcript expression in axes isolated from dormant (yellow bars) and non-dormant (brown bars) sunflower embryos dry or after 24 h of incubation at 10 °C, on water (control), in darkness. Embryos were treated by HCN (1 mM) for 3 h or, by MV (0.1 mM) for 3 h, at 10 °C. Data from QRT-PCR (mean of four replicates ± SD) are expressed in arbitrary units.

To investigate the putative relationships between HCN and ROS signaling pathway in sunflower embryo dormancy alleviation, the transcript level of *MAPK6*, *SerThrPK*, *PTP* and *CaM* genes were also determined (Figure 23A, B, C, D). There was no significant difference in transcript level of *MAPK6* (Figure 23A) and *SerThrPK* (Figure 23B) in dry axes isolated from both types of embryos, while *PTP* (Figure 23C) and *CaM* (Figure 23D) expression seemed stronger in non-dormant embryos than in dormant ones. Imbibition at 10 °C on water of control not-treated embryos did not result in a significant change in *MAPK6*, *SerThrPK* and *PTP* expression, but it was associated in an increase in *CaM* one, particularly for the axes isolated from dormant embryos (Figure 23D).

6.3. Mechanism of action of HCN in relation to ethylene biosynthesis and action

6.3.1. Effect of inhibitors of ethylene biosynthesis and action

Table 8 presents the effects of HCN applied in the presence of inhibitors of ethylene synthesis or ethylene perception on embryos germination at 10 °C.

Table 8. Effect of $CoCl_2$, AOA, AIB and 2,5-NBD, at 1 mM on germination of dormant sunflower embryos at 10 °C, in darkness. Embryos were treated with HCN for 3 h before being transferred on water or placed continuously in the presence of these compounds. Values are means of four experiments ± SD. (d, day).

Treatment with		Germination (%)	
Ethylene biosynthesis and * receptor inhibitors	HCN (3 h)	7 d	10 d
$CoCl_2$ (1 mM)	–	22 ± 2	42 ± 2
	+	58 ± 4	74 ± 3
AOA (1 mM)	–	14 ± 2	22 ± 2
	+	78 ± 4	80 ± 2
AIB (1 mM)	–	24 ± 2	30 ± 1
	+	94 ± 3	98 ± 1
***2,5-NBD** (1 mM)	–	18 ± 1	18 ± 1
	+	22 ± 1	22 ± 1
H_2O (control)	–	18 ± 2	20 ± 4
	+	76 ± 2	96 ± 2

Inhibitors of ACC synthase activity (AOA), of ACC oxidase activity (AIB, $CoCl_2$) or of ethylene action (2,5-NBD) had no effect on germination of dormant embryos. After 10 days, 22-42 % of embryos germinated at 10 °C, against 20 % of the control embryos placed on water (Table 8). Application of HCN in the presence of ethylene biosynthesis inhibitors (AOA, AIB, $CoCl_2$) did not affect the stimulatory action of cyanide on the germination of dormant embryos whatever inhibitor used, and about 74-98 % of the embryos germinated after being treated by HCN. Moreover, the beneficial effect of HCN treatment was

suppressed in the presence of 2,5-NBD. After 10 days of HCN + 2,5-NBD application just 22 % of embryos germinated (Table 8).

6.3.2. Effect of HCN on C_2H_4 production

After 24 h of embryos incubation at 10 °C, isolated dormant axes produced very small amount of ethylene (0.33 nl h^{-1} g^{-1} FW) in comparison to the non-dormant ones (0.8 nl h^{-1} g^{-1} FW) (Figure 24).

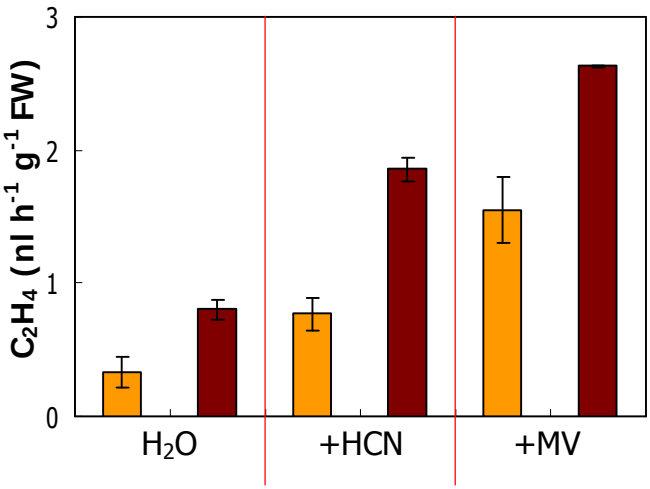


Figure 24. Ethylene emission by axes isolated from sunflower embryos. Embryos were treated by HCN (1 mM) or MV (0.1 mM) for 3 h before being transferred on water. The measurement of C_2H_4 was performed after incubation (at 20 °C, for 5 h) of axes excised from dormant (yellow bars) and non-dormant (brown bars) sunflower embryos, germinated during 24 h, at 10 °C, in darkness. Values are mean of four replicates ± SD.

In addition, 3 h treatment of dormant embryos by HCN resulted in increase in C_2H_4 production by isolated axes. This improving affect of HCN was also observed in non-dormant embryos. For both types of embryos, C_2H_4 production doubled after HCN treatment in comparison with the control. Similar tendency occurred in the case of embryos treated by methylviologen (Figure 24).

6.3.3. Effect of HCN on expression of gene involved in ethylene production and signal transduction

Transcription of genes involved in ethylene production (*ACC synthase*: *ACS* and *ACC oxidase*: *ACO* genes) or ethylene transduction signal (*ETR2*, *CTR1* and *ERF1*) was studied by qRT-PCR in axes of dry embryos, of embryos incubated for 24 h at 10 °C on water (control), or treated by HCN, C_2H_4 or MV, in order to investigate whether HCN might act at molecular level on ethylene synthesis or action, and whether ROS might be involved in this regulation. In dry dormant and non-dormant axes *ACS* and *ACO* transcripts were present in low amounts (Figure 25). In both types of embryos, imbibition resulted in a strong increase in *ACS* and *ACO* expression.

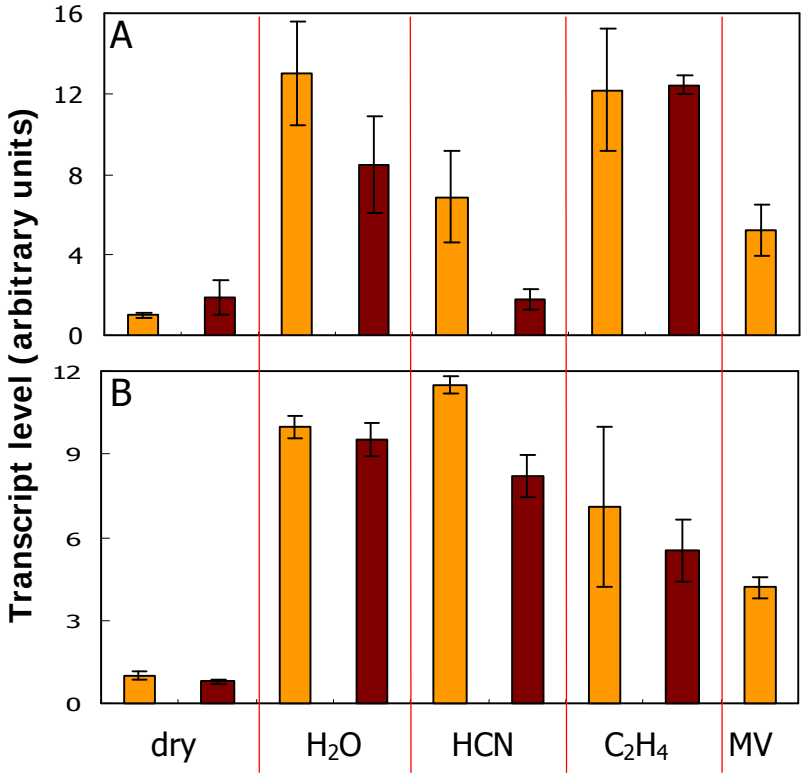


Figure 25. *ACS* and *ACO* transcript expression in axes isolated from dormant (yellow bars) and non-dormant (brown bars) sunflower embryos dry or after 24 h of incubation at 10 °C, on water (control), in darkness. Embryos were treated by HCN (1 mM) for 3 h, C_2H_4 (50 ppm) continuously, or by MV (0.1 mM) for 3 h, at 10 °C. Data from QRT-PCR (mean of four replicates ± SD) are expressed in arbitrary units.

Treatment of embryos by HCN, ethylene or methylviologen did not improve the transcription of *ACS* and *ACO* genes. In contrast, HCN and MV treatments were associated with a decrease in *ACS* transcripts, and in a lower intensity in *ACO* ones (Figure 25).

Transcription of genes involved in transduction of ethylene signal, namely *ETR2*, *CTR1* and *ERF1* (Figure 2 in the introduction), was also investigated and the results obtained are presented in Figure 26.

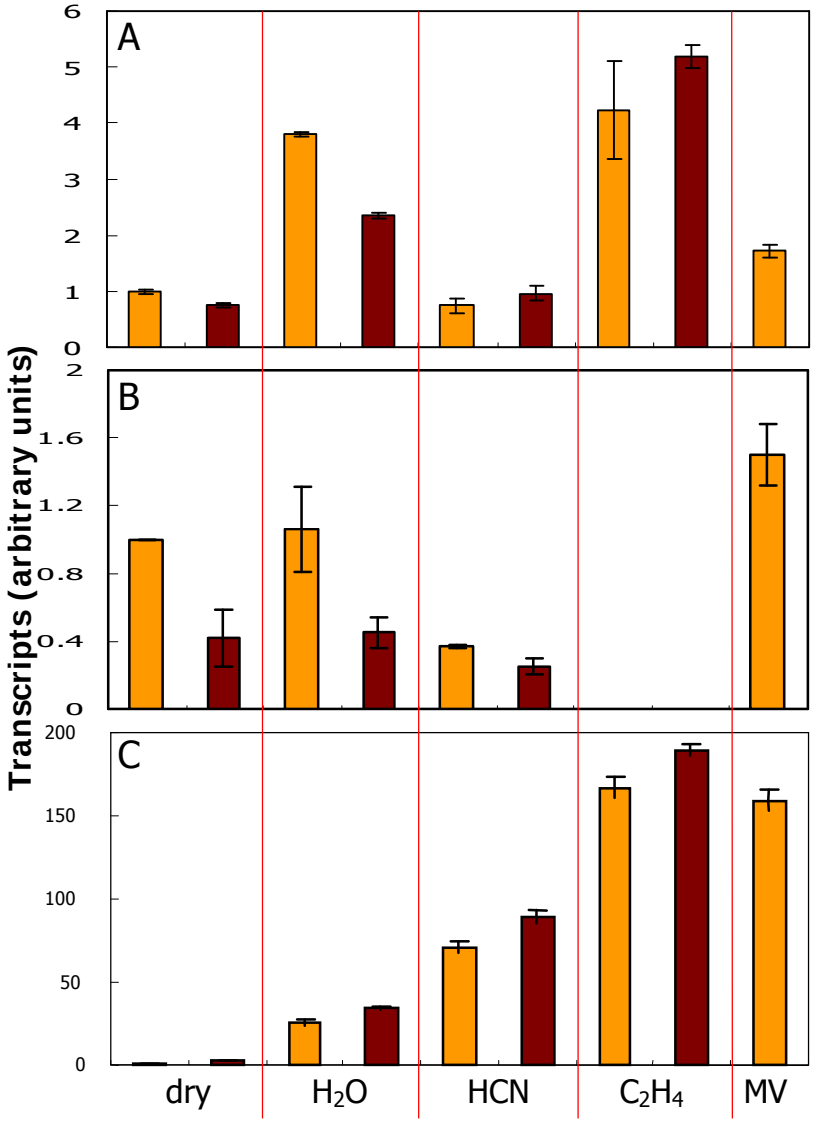


Figure 26. *ETR2*, *CTR1*, *ERF1* transcript expression in axes isolated from dormant (yellow bars) and non-dormant (brown bars) sunflower embryos dry or after 24 h of incubation at 10 °C, on water (control), in darkness. Embryos were treated by HCN (1 mM) for 3 h, C_2H_4 (50 ppm) continuously, or by MV (0.1 mM) for 3 h, at 10 °C. Data from QRT-PCR (mean of four replicates ± SD) are expressed in arbitrary units.

In axes isolated from dry embryos *ETR2*, *CTR1* and *ERF1* transcripts were present (Figure 26, dry). In both cases, the level of expression increased during imbibition on water (Figure 26, H_2O). HCN and MV treatments reduced *ETR2* expression (Figure 26A).

There was no significant difference in *CTR1* transcript level in axes isolated from dormant and non-dormant embryos, after imbibition on water, in the presence or not of HCN ethylene or MV (Figure 26B).

Figure 26C shows that level of *ERF1* transcripts in axes increased during imbibition of both types of embryos. HCN treatment like ethylene and MV strongly stimulated transcription of this factor.

7. Discussion

7.1. Main characteristics of sunflower embryo dormancy: a physiological approach

In the present work, major characteristic of the complexity of sunflower embryo dormancy is demonstrated. Obtained data confirm that at harvest naked sunflower seeds germinate poorly at 10 °C (Figure 5A and Oracz *et al.* 2007) and that after-ripening in dry conditions (20 °C, 2 months) results in a widening of the temperature range in which germination is possible (Figure 5B and Oracz *et al.* 2007). From literature it is known that ethylene strongly stimulates the germination of dormant sunflower embryos, when ABA reinforces and decrease strongly germination percentages (Corbineau *et al.* 1988, Corbineau *et al.* 1990, Corbineau and Côme 2003). Results of the present work show that ethylene decreases seed sensitivity to abscisic acid and the inhibitory effect of ABA was for a part suppressed in the presence of C_2H_4 (Figure 6). It is in agreement with results of Beaudoin *et al.* (2000) who demonstrated that in *ein2-45 abi3-4* double *Arabidopsis* mutant, ethylene suppresses seed dormancy by inhibition of ABA action. These results indicate a strong interaction between C_2H_4 and ABA signal transduction pathways, and a key conclusion is that ethylene can promote seed germination by directly interfering with ABA signaling (Beaudoin *et al.* 2000).

Interesting results concerning dormancy removal of sunflower embryos (which do not contain cyanogenic compounds) have been obtained in experiments with HCN-mediated embryos germination. Results of this work demonstrate that HCN allows the germination of dormant sunflower embryos at 10 °C, a temperature at which dormancy is expressed. This improving effect progressively increases with increasing cyanide concentrations, ranged from 10^{-6} M to 10^{-3} M, the maximal effect being obtained with 10^{-3} M HCN (Figure 7A). It is important to note that the stimulatory effect of cyanide is only observed when this compound is subsequently eliminated from the germination medium and the radicle growth is completely blocked in the continuous presence of cyanide (Figure 7B). It is also shown that the duration of pre-

imbibition of the embryos on water has no effect on their responsiveness to HCN (Figures 8 and 9). Earlier experiments performed on apple seeds (containing cyanogenic compounds) show that the stimulating effect of gaseous HCN on germination was correlated with suppressing all symptoms of dormancy such as inhibition of hypocotyl and internode elongation, as well as asymmetric growth and greening of cotyledons (Bogatek and Lewak 1991, Lewak *et al.* 2000).

It has been proposed that the amino acid pool (e.g. alanine, serine, asparagine) may increase through the activation of β-CAS by ethylene and/or HCN during germination, and consequently decreases the water potential in the embryonic axes (Hasegawa *et al.* 1994). Decrease in water potential through β-CAS activation may be one of the prerequisites for active growth of imbibed seed tissues. In the present study, no significant differences in the activity of β-CAS in axes during germination of embryos whatever their treatment were observed (Figure 12). Moreover, in axes isolated from dry dormant and non-dormant sunflower embryos, free HCN is not detectable (data not shown). Only a slight increase in HCN content was measured in axes excised from imbibed embryos, thus suggesting that β-CAS (Figure 12) maintains cyanide content at a low and not toxic level (not shown). These results demonstrate that the beneficial effect of HCN on sunflower embryo germination is not related to an increase in amino acids synthesis, by stimulation of β-CAS activity.

Esashi *et al.* (1991) postulated that the beneficial effect of cyanide on germination might result from a partial blocking of the cytochrome system. Cyanide liberated from endogenous pool of cyanogenic compounds, might act on germination through the regulation of oxidative electron transport, i.e. partial inhibition of the cytochrome pathway and/or induction of alternative, cyanide-resistant one (Hasegawa *et al.* 1994). In this work, in order to analyze whether cyanide mode of action might be associated with modification of respiratory pathways, dormant sunflower embryos were imbibed in the presence of various respiratory inhibitors (Table 4). Application of other respiratory inhibitor than HCN □sodium azide (NaN_3), stimulates germination of

sunflower embryos but at lower percentages than in the case of HCN (Table 4). The alternative cyanide-resistant respiration operates in many plant seeds during imbibition, in addition to cyanide-sensitive one (Morohashi and Matsushima 1983). The presence of an inhibitor of cyanide-insensitive pathway □ salicylhydroxamic acid (SHAM) had no effect on the germination. Furthermore, incubation of embryos in the presence of NaN_3 or SHAM did not change the stimulatory effect of HCN on germination. These observations suggest that the primary action of HCN in stimulation of dormant sunflower embryos germination is not related to its effect on respiratory metabolism (alternative pathway) neither to an activation of the pentose phosphate pathway (Table 4). This theory is highly probable since stimulatory effect of HCN on germination of sunflower embryos, is efficient even at low moisture content (0.05 g H_2O g^{-1} DM), i.e. when the respiratory pathways almost does not operate. This assumption is also supported by data obtained by Bogatek and Rychter (1984) who presented that in mitochondria isolated from apple embryos, an alternative oxidase pathway does not operate during cold stratification. It seems that cyanide-resistant respiration is not involved in the dormancy removal and germination of seeds, and therefore it is assumed that the effect of HCN pretreatment, described in the present work, results from its involvement in other metabolic processes.

7.2. Mechanisms of action of HCN in relation to ROS metabolism

It is still an intriguing question, how cyanide can be both recognized as a signal and transduced in target cells, although it induces different responses. HCN, similar to ROS, being a simple, small and diffusible molecule, it is then highly improbable that its transduction involves specific receptors (Neill *et al.* 2003). It would be difficult to imagine that cyanide acts as the only signal responsible for the various responses described above. Many molecules such as NO and ROS appear to be produced in some processes regulated by cyanide (Smith and Arteca 2000, Bogatek *et al*. 2004, Gniazdowska *et al.* 2007). Numerous recent works (Bailly 2004 and references here in) have nevertheless brought new lines of evidence showing that the role of ROS in seeds is not as

unfavorable as it was considered previously. At the opposite, it now appears more and more clearly that ROS would play a key signaling role in the achievement of major events of seed life, such as germination or dormancy release (Bailly 2004). Many reports have shown that the transition from a quiescent seed to a metabolically active organism is associated with ROS generation, suggesting that it is an ubiquitous phenomenon (El-Maarouf-Bouteau and Bailly 2008). The ROS signaling transduction pathway in plants, and therefore in seeds, from sensing to changes in gene expression, is not yet fully understood. As suggested by Moller *et al.* (2007), short-lived ROS, such as $OH^{\cdot}$, would react not far from their production site, whereas long-lived ROS, such H_2O_2, could reach targets far from their production site. A signaling role for ROS is easy to envisage since the most stable, hydrogen peroxide, carries all of the hallmarks necessary for fulfilling this role: it accumulates rapidly in response to a specific stimuli, it is soluble and readily diffuses, and the amplitude and duration of its synthesis will be proportional to the amplitude and duration of the stimulus (May *et al.* 1998). Production of hydrogen peroxide has been demonstrated during dry storage of beech (*Fagus sylvatica*) seeds (Pukacka and Ratajczak 2005), as also in the early imbibition period of seeds of sunflower (Bailly *et al*. 2002), tomato (Morohashi 2002), apple (Bogatek *et al.* 2003) and pea (Wojtyla *et al*. 2006). Results presented in this work show that dormancy removal by HCN is associated with an accumulation of hydrogen peroxide in the embryonic axes of sunflower embryos (Table 5), suggesting that it might be an essential regulatory factor of breaking of embryo dormancy. Confocal localization shows that the concentration of ROS increases faster and is much higher in axes of germinated embryos (non-dormant or HCN-treated embryos) than in not germinating ones (dormant in the presence of water) (Figures 15 and 16). This demonstrates that in sunflower embryos ROS production is initiated after cyanide application, and is in agreement with previous proposals that ROS can act as cell messengers (reviewed in Bailly 2004). Thus HCN and ROS can therefore act as a signal to allow dormancy release and improve subsequent seed germination. However, while the level of H_2O_2 (Table 5) increased progressively in axes of germinated sunflower

embryos (dormant and non-dormant treated by HCN or MV, and non-dormant in the presence of water) the level of $O_2^{\cdot -}$ seems to be constant during germination in embryos whatever their treatment. There was also observed a tendency, that in axes of non-dormant embryos (Figure 13, yellow blocks), around 20-30 % higher superoxide radical was detected in comparison with dormant ones (Figure 13, brown blocks). Potential alternative source of $O_2^{\cdot -}$ in plant cells is a reduction of O_2, catalyzed by an NAD(P)H-dependent oxidase that is localized in the plasma membrane (Vera-Estrella *et al.* 1992). However, the putative role of NADPH oxidase during germination is not yet known but might require attention regarding the various roles of this enzyme in various developmental processes. For example, Sarath *et al.* (2007) demonstrated recently that NADPH oxidase inhibition delayed germination of warm season grasses. By HCN treatment of dormant sunflower embryos in the presence of diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, it was shown that cyanide beneficial effect on germination might be associated with regulation of activity of this enzyme (Figure 11). DPI binds to the flavoprotein moiety as well as to the cytochrome b_{558} moiety of the reduced NADPH oxidase complex, inhibiting electron transfer from NADPH to O_2. The observation that DPI (in the range of 1-100 μM) inhibits the elicitor-induced production ROS in soybean (Mithofer 1997), chick-pea (Otte and Barz 1996) and rose (Auh and Murphy 1995) has been used in the present study as hypothesis that regulation of NADPH oxidase by cyanide might be responsible for its stimulating effect on germination of dormant sunflower embryos (Figure 11). Frahry and Schopfer (1998) demonstrated that DPI can interfere with a step that is specifically involved in the reduction of O_2 by electron donors such as NADH, whereas the steps involved in the binding of H_2O_2 and the subsequent steps leading to electron transfer from a phenolic substrate to H_2O_2 are unaffected. Baker *et al.* (1998) suggested that DPI may be able to block H_2O_2 generation probably by inhibiting $O_2^{\cdot -}$production that results from $NAD^{\cdot}$ oxidation or the dismutation of $O_2^{\cdot -}$ into H_2O_2. It supports the results presented in this work (Figure 11), where application of cyanide in the presence of DPI resulted in decreasing % of

germinated embryos at around 30 % less in comparison to the effect by HCN treatment without DPI.

At low moisture content (dry orthodox seeds) while respiration is prevented and enzymes are presumably not active, the non-enzymatic sources of ROS, such as lipid peroxidation (McDonald 1999) may occur. As the MDA content is higher in axes isolated from dry non-dormant embryos than in dormant ones (Figure 14), it can indicate that dormancy alleviation in dry embryos is associated with lipid peroxidation and may, in turn, be involved in free radical formation. However, the reactivation of metabolism following seed imbibition results in slight decrease in MDA level in axes isolated from sunflower embryos whatever their treatment (Figure 14), suggesting the other mechanisms for ROS production, since increase in H_2O_2 concentration during imbibition simultaneously was observed (Table 5). The lower MDA content detected in axes excised from imbibed embryos might be explained by the hypothesis of McDonald (1999), who postulated that lipid peroxidation can indeed be favoured at very low moisture content and be prevented when it is higher than 0.06 g H_2O g^{-1} DM.

Methylviologen, in light conditions generates the oxidative stress in plant tissue through the photosynthetic electron transport chain (Xiong *et al.* 2007), as well as in the dark, but by an unknown mechanism (Slooten *et al.* 1995). The improving effect of methylviologen on the germination of dormant embryos (Figure 10 and Oracz *et al.* 2007) supplies evidence that ROS play a fundamental role in dormancy alleviation. Moreover, the present data show that an application of H_2O_2 as well as of aminotriazol, an inhibitor of catalase, improves germination of dormant sunflower embryos (Table 3). These results are in agreement with other works which report that exogenous H_2O_2 stimulates seed germination of barley (*Hordeum vulgare*) (Fontaine *et al.* 1994), zinnia (*Zinnia elegans*) (Ogawa and Iwabuchi 2001).

For ROS to act as cellular messengers, seeds must be able to regulate their concentration. Various non-enzymatic and enzymatic mechanisms play these roles in plants (see Figure 4 in *Introduction*). Pukacka and Ratajczak

(2006) show that the redox status is strongly positively correlated with germination capacity of silver maple (*Acer saccharinum*). It has been reported that redox perturbations especially in GSH levels act as a signal in stimulating defense genes of the antioxidant system (May *et al.* 1998). Content of ascorbate and reduced glutathione, two related antioxidants, increase during early seed imbibition (Kranner and Grill 1993). Both compounds might then play a role in ROS scavenging through control of the cellular redox balance (Tommasi *et al.* 2001) or protein synthesis (Kranner and Grill 1996b). Axes excised from dry dormant and non-dormant sunflower embryos contain more GSSG (low GSH/GSSG ratios ~ 3.79 and 4.85, respectively) than axes isolated from embryos imbibed on water, indicating that the conversion of GSSG to GSH is slower in the dry state (Figure 17). During imbibition of dormant and non-dormant embryos on water, increase in GSH/GSSG ratios indicated faster reduction of GSSG to GSH, in axes excised from non-dormant embryos (GSH/GSSG ratios ~ 22.32 and 29.41, respectively) (Table 6). In contrary to those results, comparatively high GSSG and low GSH content (lower GSH/GSSG ratios) was detected in axes isolated from embryos treated by HCN or MV (Figure 17, Table 6). Recently, it was postulated by Prabhakaran *et al.* (2006), that selective depletion of mitochondrial GSH (mtGSH) increased oxidative stress and enhanced cell death, whereas the cytoplasmic pool was not critical to cell survival. Therefore to understand better the possible relationships which might exist between HCN, ROS and glutathione metabolism during alleviation of sunflower embryos more analysis are necessary.

In germinating sunflower seeds, activities of ROS scavenging enzymes such as GR and CAT increase prior to radicle protrusion, the latter being concomitant with the elimination of H_2O_2 and the reduction of lipid peroxidation (Bailly *et al.* 2000). Analogous stimulation of CAT activity and/or expression during germination has also been reported in other species as *Arabidopsis* (Gallardo 2001), maize (Scandalios *et al.* 1997) and soybean (Gidrol *et al.* 1994). In the present work, relatively high *in vitro* activities of CAT, GR and SOD (Figure 19A, B, C) in axes isolated from sunflower embryos are detected, whatever the treatment. Nkang (2001) demonstrates that cyanide

improves germination of *Guilfoylia monostylis* seeds by enhancing the activity of enzymes (polyphenoloxidase and peroxidase) capable of degrading hydrogen peroxide. Glutathione reductase, may be also involved in ROS alleviation in apple (Bogatek *et al.* 2003) embryos, like in other seeds such as pine (*Pinus pinea* L.) (Tommasi *et al.* 2001). Measurement of an inhibitory effect of KCN on *in vitro* activities of enzymes of antioxidant system showed that the most resistant to KCN was GR, because even relatively high concentration of this inhibitor (~ 500 μM) does not affect its activity (Figure 19B`). In the opposite, the most sensitive enzyme seemed to be CAT, which activity was inhibited by KCN concentrations lower than 1 μM (Figure 19A`). Decrease in SOD *in vitro* activity was observed after application of KCN at concentrations higher than 100 μM (Figure 19C`). These results are in agreement with data of Grossmann (2003), who demonstrated that ones of the most vulnerable for cyanide action enzymes are metalloproteins, such as Cu/Zn superoxide dismutase and catalase.

Another attempt for explaining the possible role of ROS accumulation during dormancy alleviation by cyanide was to study protein carbonylation, an irreversible oxidation process leading to a change of function of the carbonylated proteins. Protein carbonylation results from an oxidative attack on arginine, lysine, proline or threonine residues of proteins, which can affect enzyme activities or alter susceptibility of the modified proteins to proteolysis (Berlett and Stadtman 1997). Although protein carbonylation is often associated with aging in animals, it was demonstrated to be related with vigor in germinating *Arabidopsis* seeds (Job *et al*. 2005). Carbonylation of reserve proteins would help in their mobilization during germination by increasing their susceptibility to proteolytic cleavage (Job *et al*. 2005). Proteins involved in glycolysis/gluconeogenesis pathways also became carbonylated during germination which could provide reducing power through the pentose phosphate pathway (Job *et al.* 2005). The protein patterns obtained by 1D gel electrophoresis of soluble proteins from dormant and non-dormant embryonic axes show that the level of carbonylated proteins during imbibition is higher in non-dormant axes than in dormant ones (Figure 20 and Oracz *et al.* 2007).

In addition, 2D gel electrophoresis (Figure 21 and Oracz *et al.* 2007) shows that a pool of proteins became specifically carbonylated during after-ripening, as well as in HCN treated dormant embryos (proteins shown in the pink circles in Figure 21C, Table 7 and Oracz *et al.* 2007). This specific carbonylation pattern was not observed in the case of control embryos imbibed on water. Among this pool, some storage proteins became oxidized suggesting that breaking of dormancy in the dry state may be associated with a preparation toward storage protein mobilization (Oracz *et al.* 2007). Conversely, ROS generated by methylviologen (MV) also triggered specific protein oxidation (Figure 21, Table 7 and Oracz *et al.* 2007). In the group of carbonylated proteins specifically associated with breaking of dormancy by HCN and MV, some are identified as epoxide hydrolase and alcohol dehydrogenase (Table 7 and Oracz *et al*. 2007). Epoxide hydrolases catalyze the conversion of epoxides into diols. The known functions of this enzyme include detoxification of xenobiotics, drug metabolism, synthesis of signaling compounds and responses to oxidative stress (Newman *et al*. 2005). The possible role played by alcohol dehydrogenase (ADH) in breaking of seed dormancy is also well documented (Cohn *et al.* 1989, Corbineau *et al.* 1991). Taylorson and Hendricks (1979) have shown that dormancy of numerous grass seeds could be broken by treatment with low concentration of ethanol. Moreover, Corbineau *et al.* (1991) demonstrated also that ADH was involved in breaking of dormancy in oat seeds. Therefore, it is possible that inactivation of ADH by ROS might allow cellular accumulation of this compound within the embryonic axes and in turn favor their germination. However, the effect of ethanol on sunflower seed germination is not documented yet but our results suggest that it should be properly addressed. Two identified proteins, 11S globulin precursor isoform 4 (Figure 21, Table 7 and Oracz *et al.* 2007) and a basic 2S albumin (Figure 21, Table 7 and Oracz *et al.* 2007) exhibited the same pattern of oxidation as ADH, supporting the finding that germination requires the oxidation of specific reserve proteins.

Taking into account the similarities between effects of HCN and ROS, the possibility that they could act by modification of gene expression during dormancy alleviation may be also proposed. Modification of the genetic program

associated with the transition from a dormant to a non-dormant state in dry condition has been described in few reports. Apparent gene expression was demonstrated during after-ripening in *Nicotiana tabacum* (Leubner-Metzger 2005), *Arabidopsis* (Cadman *et al.* 2006) and barley (Leymarie *et al.* 2007). Many of the genes up-regulated in non-dormant seeds are associated with protein synthesis, potentially controlling the completion of germination (Cadman *et al.* 2006). Although it is known that transcriptional and translational events could be a component of dormancy breaking, there is still a little evidence concerning direct or indirect action of HCN on gene expression during dormancy alleviation. In this study, analyzes of expression of genes involved in ROS production in axes of germinating sunflower embryos (Figure 22) were performed. The results show an increase in transcription of *NADPHox* gene during seed imbibition. However, no change in transcript level of this gene occurs after treatment of the embryos with cyanide or methylviologen, suggesting that the induction of *NADPHox* transcription is related to imbibition process and is not involved in the mode of action of both chemicals. Therefore, it is highly probable that the mode of action of HCN and MV in dormancy alleviation is associated with their effect on NADPHox activity, because stimulatory effect of HCN was for a part suppressed in the presence of DPI. In plants, the production of H_2O_2 may derive from polyamine oxidation in reaction carried out by cell wall amine oxidase (AO). The level of *AO1* and *AO2* gene transcripts in dry dormant and non-dormant axes do not seem to change during imbibition in the presence of water (Figure 22C, D). On the other hand, application of HCN or MV causes a slight decrease in the transcript level of both genes. In the case of *POX* gene (Figure 22B), the transcript level observed in dry dormant and non-dormant axes decreased during imbibition in control embryos and whatever the treatment and no significant difference in transcript level were observed, suggesting that *POX* is not associated with ROS production during germination. Cyanide mode of action is then not associated with the regulation of transcription of genes involved in ROS production.

There are no data concerning specific receptor(s) of HCN in cells, and it is still known relatively little about the signal transduction pathway involved in HCN regulation of many processes within the plant cell. In literature, it is

possible to find information that in animal cells, cyanide mobilizes Ca^{2+} from intracellular compartments, which alters plasma membrane function *via* the activation of Ca^{2+}-sensitive K^+ channels (Latha *et al.* 1994) but the existence of similar mechanisms in plants remains debatable. Genetic analysis in addition to physiological studies will be required to support the existence of other elements, already detected in mammalian cells (Latha *et al.* 1994). The results presented above indicate that cyanide acts as a signal molecule leading to alleviation of seed dormancy and stimulation of germination. Moreover, ROS induced by HCN seem to play a role as a second messenger in cellular signaling which suggests that these compounds could facilitate alleviation of seed dormancy. These pathways probably do not operate independently, but rather are linked together in a complex web of interactions. A better knowledge of these events (some elements of cross-talk signal transductions) is needed for a full understanding of cyanide action in these crucial processes.

7.3. Mechanism of action of HCN in relation to ethylene biosynthesis and action

Intracellular ACO is an exclusively cytosolic enzyme which is not associated with membranes (Reinhardt *et al.* 1994). Therefore, its reaction products, ethylene and HCN, are initially released in the cytoplasm and can react directly on cytosolic enzymes. Studies of Ververidis and Dilley (1995) on *in vitro* ACC oxidase reaction showed a direct role of HCN in catalytic activation and inactivation of this enzyme. In this work is demonstrated that an application of HCN in the presence of ethylene biosynthesis inhibitors such as AOA, AIB, $CoCl_2$, does not suppress the stimulating effect of cyanide on germination of dormant sunflower embryos. However, the promoting effect of HCN is blocked in the presence of an inhibitor of the ethylene action (2,5-NBD) (Table 8). In conclusion, is proposed that the regulatory role of cyanide in dormancy alleviation of sunflower embryos does not operate by regulation of ACS and/or ACO activity, but it requires functional ethylene receptors (Table 8).

Although ethylene has been implicated in breaking of dormancy and germination of many seeds (Corbineau *et al.* 1990, Kępczyński and Kępczyńska

1997), its role in regulation of these processes is still poorly understood (Calvo *et al.* 2004). There are some hypotheses suggesting that certain components of ethylene signaling pathway are involved in the regulation of germination by gibberellins (GA) (Koornneef and Karssen 1994). Ethylene can fully rescue the germination of *A. thaliana* GA-deficient mutant *ga-1* (Koornneef and Karssen 1994). Calvo *et al.* (2004) showed that ethylene and gibberellins (GA) are involved in breaking of dormancy in beech tree (*Fagus silvatica*) seeds, by modulating of expression of *ACC oxidase* gene (*ACO*). Despite that axes from non-dormant sunflower embryos produce more ethylene in comparison to the dormant axes (Figure 24), and treatment by HCN or MV double the amount of C_2H_4, it seems to be highly probable that the mode of action of HCN is not associating with regulation of production of this hormone. Indeed the amount of detected ethylene is relatively small (~1 nl h^{-1} g^{-1} FW) and unable to affect germination process. Moreover, transcription analyze of genes down- or up-regulating ethylene production shows no significant changes in transcript level of *ACS* and *ACO* genes, indicating that regulation of ethylene synthesis is not required for sunflower embryo dormancy removal by cyanide and/or ROS (Figure 25). However, in apple embryo HCN markedly enhanced ethylene emission by stimulating of *ACS6* expression (Bogatek *et al.* 2004).

Signal molecules such as ROS might be good candidates for the interaction between cyanide and hormone signal transduction pathways. Ethylene and ROS signaling pathways seem also to share some common mechanisms. Stimulation of ethylene synthesis by environmental stresses, such as ozone, UV irradiation, and wounding, involves generation of reactive oxygen species (Surplus *et al.* 1998). Interactions among salicylic acid (SA), jasmonic acid (JA) and ethylene have been also reported and found to modulate responses to ROS (for review Wang *et al.* 2002). The studies on seeds of several species suggest that there exists a relationship between ROS metabolism, hormone regulation and seed germination (El Maarouf-Bouteau and Bailly 2008). Several components of the ethylene signal transduction have been identified and their signaling pathway has been characterized (Kucera *et al.* 2005). Ethylene receptors are normally blocked to repress ethylene response

(Bleecker and Kende 2000). Upon ethylene binding, the receptors become active which alleviates the repression on ethylene signal transduction and allows ethylene responses (Gamble *et al.* 2002). Desikan *et al.* (2005) presented that the ethylene receptor ETR1 mediate H_2O_2 signal in stomatal guard cells. This raises the question, whether the stimulatory effect of HCN on germination could be related to the ethylene signaling pathway. The observation that some mutants of *A. thaliana*, which exhibit reduced response to ethylene, also show a lowered response to cyanide treatments (Smith and Arteca 2000), may support this assumption. However, there is no experimental data indicating that HCN shares the same receptors as ethylene and the next downstream component identified in the ethylene signaling pathway as CTR1-Ser/Thr kinase. Analyze of transcription of genes involved in transduction of ethylene signal presented in this work, demonstrates that cyanide markedly increased the transcription of *ERF1* but not that of the *ETR2* and *CTR1* (Figure 26). That might suggest the involvement of ethylene receptors as also MAPK signaling cascade, seems not to operate at transcription level, during HCN dormancy alleviation of sunflower embryos. A recent study of Huang *et al.* (2003) showed that ethylene receptor and CTR1 protein formed a complex and the function of CTR1 was regulated via association/dissociation with the ethylene receptor proteins. Based on this view, it is considered that ethylene receptors could be more important modulators in the regulation of ethylene response than CTR1 (Huang *et al.* 2003). Therefore, *ERF1* might be a target gene of HCN action. Moreover, presented data assign a new mechanism of HCN and ROS (MV) action, initiating the transcription factor *ERF1* but through different mechanism of that involved by ethylene knowing for increase of mRNA level of *ETR2* (Hua *et al.* 1998, Hua and Meyerowitz 1998).

7.4. General remarks and future developments

Seed dormancy and germination are very complex phenomena which involve tightly controlled signaling pathways as well as cellular and molecular regulations. In addition to hormones, there are many signaling molecules such as HCN, ROS and NO which seem to play important roles in seed dormancy, but

whether their mechanism of action relies on a unique dominant signaling pathway or on the overlap of many is still under investigation.

The question about HCN toxic or signaling role has been stated previously (Siegień and Bogatek 2006). In addition, the regulatory (signaling) function in plant metabolism is now underlined. The present data document the effectiveness of cyanide in breaking of sunflower embryo dormancy. It seems that cyanide possesses some properties characteristic for signaling molecules: a) it is produced quickly and efficiently on demand; b) it induces specific responses within the cell at low concentrations, sometimes as low as 1 μM; c) it can be metabolized (removed) rapidly subsequently to signaling events. It is worth to note that cyanide as a gaseous molecule may easily diffuse not only through cyanogenic plant tissues, but also may be released into the surrounding atmosphere, similarly to ethylene, methyl jasmonate and fungal or bacterial substances. Therefore, cyanide seems to be involved in regulation of many processes (e.g. germination), which are also controlled by ROS and ethylene. Presented data allow us to propose the protein oxidation as a new mechanism of action for HCN and suggest that changes in gene expression are not the only mechanism leading to seed dormancy release. Moreover, in the present work it is demonstrated for the first time, that HCN did not change the gene transcription of enzymes involved in ROS production or signaling pathway but can induce the physiological response by ROS, using some elements of other signal transduction pathway (e.g. *ERF1* in ethylene signaling pathway).

The possible role of HCN in dormancy alleviation seems to be a complex phenomenon, that why additionally experiments in this area, are still necessary. Knowing that germination is associated with ROS transient accumulation which may interact with ABA and GA transduction pathways (El-Maarouf Bouteau and Bailly 2008), future work should pay more attention to the possible HCN involvement with this mechanism, since this compound is also known to stimulate ROS production and affect ABA content (Bogatek *et al.* 2003). Moreover, in last decade was observed an increase in interest about role of NO in regulation of germination (Beligni and Lamattina 2000, Kopyra and Gwóźdź 2003, Bethke *et al*. 2007, Gniazdowska *et al.* 2007). It was suggested

that, HCN- and NO- mediated germination might be related to variation of ethylene biosynthesis as well as ABA/ethylene sensitivity (Bogatek and Sykała 2005, Dobrzyńska *et al.* 2005, Bogatek and Gniazdowska 2006). Further experiments in this area, at transcript and protein levels, would be highly informative and help to better understand the possible role of cyanide as also the other signaling molecules in dormancy alleviation.

8. Conclusions

1. A short (3 h) pre-treatment of dormant sunflower embryos (*Helianthus annuus* L.) by gaseous HCN (1 mM) releases their dormancy and allows their subsequent germination at low temperature (10-15 °C).

2. Regulatory role of HCN in alleviation of sunflower embryo dormancy is associated with:

a) an increase in ROS accumulation (H_2O_2);

b) an specific oxidation of a set of proteins among which epoxide hydrolase and alcohol dehydrogenase have been identified;

c) an increase in the expression of the ethylene transcription factor – *ERF1.*

3. Mode of action of HCN during sunflower dormancy breaking does not result from:

a) an activation of the pentose phosphate or the cyanide-insensitive pathways;

b) an increase in amino acids synthesis (as alanine, serine, asparagine), by stimulation of activity of β-CAS;

c) a regulation of transcription of genes involved in ethylene production (*ACS* and *ACO*) and a stimulation of ethylene production;

d) a modification of the transcription of genes coding for enzymes involved in ROS production (*NADPHox*, *AO1*, *AO2*, *POX*) and ROS signaling pathway (*MAPK6*, *SerThrPK*, *PTP*, *CaM*).

9. References

Aalen R.B. 1999. Peroxiredoxin antioxidants in seed physiology. *Seed Sci. Res.* **9**: 285-295.

Abeles F.B. and Lonski J. 1969. Stimulation of lettuce seed germination by ethylene. *Plant Physiol.* **44**: 277-280.

Abeles F.B., Morgan P.W., Saltveit J.M.E. 1992. Ethylene in plant biology. 2nd ed. Abeles F.B., Morgan P.W., Saltveit J.M.E. (eds), Academic Press, New York.

Achard P., Wriezen W.H., Van Der Straeten D., Harberd N.P. 2003. Ethylene regulates *Arabidopsis* development *via* the modulation of DELLA protein growth repressor function. *Plant Cell.* **15**: 2816-2825.

Akopyan T., Braunstein A., Goryachenkova E. 1975. β-Cyanoalanine synthase: purification and characterization. *Proc. Natl. Acad. Sci. USA* **72**: 1617-1621.

Ali-Rachedi S., Bouinot D., Wagner M.H., Bonnet M., Sotta B., Grappin P., Jullien M. 2004. Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta* **219**: 479-488.

Alonso J.M., Hirayama T., Roman G., Nourizadeh S., Ecker J.R. 1999. EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science* **284**: 2148-2152.

Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucl. Acids Res.* **25**: 3389-3402.

Apel K. and Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **55**: 373-399.

Asano M., Suzuki S., Kawai M., Miwa T., Shibai H. 1999. Characterization of novel cysteine proteinases from germinating cotyledons of soybean (*Glycine max* L. Merrill). *J. Biochem.* **126**: 296-301.

Auh C.K. and Murphy T.M. 1995. Plasma membrane redox enzyme is involved in the synthesis of O_2^- and H_2O_2 by *Phytophthora* elicitor-stimulated rose cells. *Plant Physiol.* **107**: 1241-1427.

Bagniol S. 1987. Mise en évidence de l□intervention de éthylène dans la germination et la dormance des semences de tournesol (*Helianthus annuus* L.). Diplôme d□Études Approfondies. Université Pierre et Marie Curie, Paris.

Bailly C. 2004. Active oxygen species and antioxidants in seed biology. *Seed Sci. Res.* **14**: 93-107.

Bailly C., Benamar A., Corbineau F., Côme D. 1996. Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related to deterioration during accelerated aging. *Physiol. Plant.* **97**: 104-110.

Bailly C., Benamar A., Corbineau F., Côme D. 2000. Antioxidant systems in sunflower (*Helianthus annuus* L.) seeds as affected by priming. *Seed Sci. Res.* **10**: 35-42.

Bailly C., Bogatek-Leszczyńska R., Côme D., Corbineau F. 2002. Changes in activities of antioxidant enzymes and lipoxygenase during growth of sunflower seedlings from seeds of different vigour. *Seed Sci. Res.* **12**: 47-55.

Baker C.J., Deahl K., Domek J., Orlandi E.W. 1998. Oxygen metabolism in plant/bacteria interactions: Effect of DPI on the pseudo-NAD(P)H oxidase activity of peroxidase. *Biochem. Biophys. Res. Commun.* **252**: 461-464.

Baskin J.M. and Baskin C.C. 2004. A classification system for seed dormancy. *Seed Sci. Res.* **14**: 1-16.

Batlla D. and Benech-Arnold R.L. 2005. Predicting changes in dormancy level in weed seed soil banks: Implications for weed management. *Crop Prot.* **26**: 189-197.

Beaudoin N., Serizet C., Gosti F., Giraudat J. 2000. Interactions between abscisic acid and ethylene signaling cascades. *Plant Cell* **12**: 1103-1115.

Beligni M.V., Fath A., Bethke P.C., Lamattina L., Jones R.L. 2002. Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiol.* **129**: 1642-1650.

Beligni M.V. and Lamattina L. 2000. Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyls elongation, three light-inducible responses in plants. *Planta* **210**: 215-221.

Benech-Arnold R.L., Sánchez R.A., Forcella F., Kruk B.C., Ghersa C.M. 2000. Environmental control of dormancy in weed seed banks in soil. *Field Crops Res.* **67**: 105-122.

Berlett B.S. and Stadtman E.R. 1997. Protein oxidation in aging, disease and oxidative stress. *J. Biol. Chem.* **272**: 20313-20316.

Bethke P.C., Libourel I.G.L., Aoyama N., Chung Y.Y., Still D.W., Jones R.L. 2007. The *Arabidopsis* aleurone layer Responds to nitric oxide, gibberellin, and abscisic acid and is sufficient and necessary for seed dormancy. *Plant Physiol.* **143**: 1173-1188.

Bethke P.C., Libourel I.G.L., Reinöhl V., Jones R.L. 2006. Sodium nitroprusside, cyanide, nitrite, and nitrate break *Arabidopsis* seed dormancy in a nitric oxide-dependent manner. *Planta* **223**: 805-812.

Bewley J.D. 1997. Seed germination and dormancy. *Plant Cell* **9**: 1055-1066.

Bewley J.D. and Black M. 1994. Seeds-physiology of development and germination. Bewley J.D., Black M. (eds), Plenum Press, New York.

Bleecker A.B. 1999. Ethylene perception and signaling: an evolutionary perspective. *Trends Plant Sci.* **4**: 269-274.

Bleecker A.B. and Kende H. 2000. Ethylene: a gaseous signal molecule in plants. *Annu. Rev. Cell Dev. Biol.* **16**: 1-18.

Blum H., Beier H., Gross H.J. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**: 93-99.

Blumenthal S., Hendrickson H., Abrol Y., Conn E. 1968. Cyanide metabolism in higher plants: III. The biosynthesis of β-cyanoalanine. *J. Biol. Chem.* **243**: 5302-5307.

Bogatek R., Côme D., Corbineau F., Picard M.A., Żarska-Maciejewska B., Lewak S. 1999. Sugar metabolism as related to the cyanide-mediated elimination of dormancy in apple embryos. *Plant Physiol. Biochem.* **37**: 577-585.

Bogatek R., Gawrońska H., Oracz K. 2003. Involvement of oxidative stress and ABA in CN-mediated elimination of embryonic dormancy in apple. In: Nicolas G., Bradford K.J., Côme D. and Pritchard H.W. (eds), The Biology of seeds: Recent Res. Adv. pp. 211-216.

Bogatek R. and Gniazdowska A. 2006. Nitric oxide and HCN reduce deep dormancy of apple seeds. *Acta Physiol. Plant.* **28**: 281-287.

Bogatek R. and Lewak S. 1988. Effect of cyanide and cold treatment on sugar catabolism in apple seeds during dormancy removal. *Physiol. Plant.* **73**: 406-411.

Bogatek R. and Lewak S. 1991. Cyanide controls enzymes involved in lipid and sugar catabolism in dormant apple embryos during culture. *Physiol. Plant.* **83**: 422-426.

Bogatek R. and Rychter A. 1984. Respiratory activity of apple seed during dormancy removal and germination. *Physiol. Veg.* **22**: 181-191.

Bogatek R. and Sykała A. 2005. Signaling action of cyanide on ethylene biosynthesis in dormant apple embryos. In: Proceedings of the 8th International Workshop on Seeds: Germinating New Ideas. Brisbane, Australia, p. 72.

Bogatek R., Sykała A., Krysiak C. 2004. Cyanide-induced ethylene biosynthesis in dormant apple embryos. *Acta Physiol. Plant.* **26** (suppl.): 16.

Bogatek R., Żarska-Maciejewska B., Sińska I., Lewak S. 1989. The embryonic axis controls lipid catabolism in cotyledons of apple seeds during germination. *Physiol. Plant.* **76**: 557-562.

Borghetti F., Noda F.N, de Sa C.M. 2002. Possible involvement of proteasome activity in ethylene-induced germination of dormant sunflower embryos. *Braz. J. Plant Physiol.* **14**: 125-131.

Bowler C., Van Montagu M., Inzé D. 1992. Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**: 83-116.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

Cadman C.S.C., Toorop P.E., Hilhorst H.W.M., Finch-Savage W.E. 2006. Gene expression profiles of *Arabidopsis* Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant J.* **46**: 805-822.

Calvo A.P., Nicolas C., Lorenzo O., Nicolas G., Rodriguez D. 2004. Evidence for positive regulation by gibberellins and ethylene of ACC oxidase expression and activity during transition from dormancy to germination in *Fagus sylvatica* L. seeds. *J. Plant Growth Regul.* **23**: 44-53.

Castric P., Farnden K., Conn E. 1972. Cyanide metabolism in higher plants: V. The formation of asparagines from β-cyanoalanine. *Arch. Biochem. Biophys.* **152**: 62-69.

Chao Q., Rothenberg M., Solano R., Roman G., Terzaghi W., Ecker J.R. 1997. Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* **89**: 1133-1144.

Chen Y.F., Etheridge N., Schaller G.E. 2005. Ethylene signal transduction. *Ann. Bot.* **95**: 901-915.

Chivasa S. and Carr J.P. 1998. Cyanide restores *N* gene-mediated resistance to tobacco mosaic virus in transgenic tobacco expressing salicylic acid hydroxylase. *Plant Cell* **10**: 1489-1498.

Clairbone A. 1985. Catalase activity. In: Greenwald R.A. (ed.), Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, FL., pp. 283-284.

Cohn M.A., Jones K.L., Chiles L.A., Church D.F. 1989. Seed dormancy in red rice. 7. Structure-activity studies of germination stimulants. *Plant Physiol.* **89**: 879-882.

Cona A., Rea G., Angelini R., Federico R., Tavladoraki P. 2006. Functions of amine oxidases in plant development and defence. *Trends Plant Sci.* **11**: 80-88.

Corbineau F., Bagniol S., Côme D. 1990. Sunflower (*Helianthus annuus* L.) seed and its regulation by ethylene. *Isr. J. Bot.* **39**: 313-325.

Corbineau F. and Côme D. 1987. Regulation de la germination des semences de tournesol par l'éthylène. In: 2ème colloque sur les substances de croissance et leurs utilisations en agriculture. Paris: ANPP, pp. 271□282.

Corbineau F. and Côme D. 2003. Germination of sunflower seeds as related to ethylene synthesis and sensitivity - an overview. In: Vendrell M., Klee H., Pech J.C., Romojaro F. (eds), Biology and Biotechnology of the Plant Hormone Ethylene III, IOS Press, NATO Sciences Series, Amsterdam, pp.: 216-221.

Corbineau F., Gouble B., Lecat S., Côme D. 1991. Stimulation of germination of dormant oat (*Avena sativa* L.) seeds by ethanol and other alcohols. *Seed Sci. Res.* **1**: 21-28.

Corbineau F., Rudnicki R.M., Côme D. 1988. Induction of secondary dormancy in sunflower seeds by high temperature. Possible involvement of ethylene biosynthesis. *Physiol. Plant.* **73**: 368-373.

Desikan R., Hancock J.T., Bright J., Harrison J., Weir I., Hooley R., Neill S.J. 2005. A role for ETR1 in hydrogen peroxide signaling in stomatal guard cells. *Plant Physiol.* **137**: 831-834.

Dobrzyńska U., Gniazdowska A., Babańczyk T., Bogatek R. 2005. Cyanide and nitric oxide as a regulatory factor of seed germination. *Biol. Lett.* **42**: 151.

Droux M., Martins J., Sajus P., Douce R. 1992. Purification and characterization of *O*-acetylserine(thiol) lyase from spinach chloroplasts. *Arch. Biochem. Biophys.* **295**: 379-390.

Dziewanowska K., Niedźwiedź I., Chodelska I., Lewak S. 1979a. Hydrogen cyanide and cyanogenic compounds in seeds. I. Influence of hydrogen cyanide on germination of apple embryos. *Physiol. Veg.* **17**: 279-303.

Dziewanowska K., Niedźwiedź I., Lewak S. 1979b. Hydrogen cyanide and cyanogenic compounds in seeds. II. Changes in free HCN level in apple seeds during stratification. *Physiol. Veg.* **17**: 681-686.

Ebbs S. 2004. Biological degradation of cyanide compounds. *Curr. Opin. Biotechnol.* **15**: 231-236.

Elstner E.F. and Heupel A. 1976. Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. *Anal. Biochem.* **70**: 616-620.

El-Maarouf-Bouteau H. and Bailly C. 2008. Oxidative signaling in seed germination and dormancy. *Plant Signal. Behav.* □in press.

Esashi Y., Isuzugawa K., Matsuyama S., Ashino H., Hasegawa R. 1991. Endogenous evolution of HCN during pre-germination periods in many seed species. *Physiol. Plant.* **83**: 27-33.

Esashi Y., Maruyama A., Sasaki S., Tani A., Yoshiyama M. 1996. Involvement of cyanogens in the promotion of germination of cocklebur seeds in response to various nitrogenous compounds, inhibitors of respiratory and ethylene. *Plant Cell Physiol.* **37**: 545-549.

Finch-Savage W. and Leubner-Metzger G. 2006. Seed dormancy and the control of germination. *New Phytol.* **171**: 501-523.

Fontaine O., Huault C., Pavis N., Billard J.P. 1994. Dormancy breakage of *Hordeum vulgare* seeds: effects of hydrogen peroxide and scarification on glutathione level and glutathione reductase activity. *Plant Physiol. Bioch.* **32**: 677-683.

Frachry G. and Schopfer P. 1998. Inhibition of O_2-reducing activity of horseradish peroxidase by diphenyleneiodonium. *Phytochemistry* **48**: 223-227.

Fujimoto S.Y., Ohta M., Usui A., Shinshi H., Ohme-Takagi M. 2000. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* **12**: 393-404.

Gallardo K., Job C., Groot S.P.C., Puype M., Demol H., Vandekerckhove J., Job D. 2001. Proteomics analysis of *Arabidopsis* seed germination and priming. *Plant Physiol.* **126**: 835-848.

Gamble R.L., Qu X., Schaller G.E. 2002. Mutational analysis of the ethylene receptor ETR1. Role of histidine kinase domain in dominant ethylene insensitivity. *Plant Physiol.* **128**: 1428-1438.

Giannopolitis C.N. and Ries S.K. 1977. Superoxide dismutases I. Occurrence in higher plants. *Plant Physiol.* **59**: 309-314.

Gidrol X., Lin W.S., Degousee N., Yip S.F., Kush A. 1994. Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. *Eur. J. Biochem.* **224**: 21-28.

Gleadow R.M. and Woodrow I.E. 2002. Constraints on effectiveness of cyanogenic glycosides in herbivore defense. *J. Chem. Ecol.* **28**: 1301-1313.

Gniazdowska A., Dobrzyńska U., Babańczyk T., Bogatek R. 2007. Breaking the apple embryo dormancy by nitric oxide involves the stimulation of ethylene production. *Planta* **225**: 1051-1057.

Görg A., Postel W., Weser J., Günther S., Strahler J.R., Hanash S.M., Somerlot L. 1987. Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. *Electrophoresis* **8**: 122-124.

Grossmann K. 1996. A role for cyanide, derived from ethylene biosynthesis, in the development of stress symptoms. *Physiol. Plant.* **97**: 772-775.

Grossmann K. 2003. Mediation of herbicide effects by hormone interactions. *J. Plant Growth Reg.* **22**: 109-122.

Gunasekar P.G., Borowitz J.L., Isom G.E. 1998. Cyanide-induced generation of oxidative species: involvement of nitric oxides synthase and cyclooxygenase-2. *J. Pharmacol. Exp. Ther.* **285**: 236-241.

Gubler F., Millar A.A., Jacobsen J. 2005. Dormancy release, ABA and pre-harvest sprouting. *Curr. Opin. Plant Biol.* **8**: 183-187.

Gurbuz F., Ciftci H., Akcil A., Karahan A.G. 2004. Microbial detoxification of cyanide solutions: a new biotechnological approach using algae. *Hydrometallurgy* **72**: 167-176.

Harder A., Wildgruber R., Nawrocki A., Fey S.J., Larsen P.M., Görg A. 1999. Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. *Electrophoresis* **20**: 826-829.

Hasegawa R., Tada T., Torii Y., Esashi Y. 1994. Presence of β-cyanoalanine synthase in unimbibed dry seeds and its activation by ethylene during pre-germination. *Physiol. Plant.* **1**: 141-146.

Heath R.L. and Parker L. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Bioch. Bioph.* **125**: 189-198.

Hu X., Jiang M., Zhang J., Zhang A., Lin F., Tan M. 2007. Calcium-calmodulin is required for abscisic acid-induced antioxidant defense and functions both upstream and downstream of H_2O_2 production in leaves of maize (*Zea mays*) plants. *New Phytol.* **173**: 27-38.

Hua J. and Meyerowitz E.M. 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* **94**: 261-271.

Hua J., Sakai H., Nourzadeh S., Chen Q.G., Bleecker A.B., Ecker J.R., Meyerowitz E.M. 1998. EIN4 and ERS2 are members of the putative ethylene receptor family in *Arabidopsis*. *Plant Cell* **10**: 1321-1332.

Huang Y., Li H., Hutchison C.E., Laskey J., Kieber J.J. 2003. Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in *Arabidopsis*. *Plant J.* **33**: 221-233.

Hucklesby D.P., Dowling M.J., Hewitt E.J. 1982. Cyanide formation from glyoxylate and hydroxylamine catalyzed by extracts of higher-plant leaves. *Planta* **156**: 487-491.

Ikegami F., Takayama K., Murakoshi I. 1988. Purification and properties of β-cyano-L-alanine synthase from *Lathyrus latifolius*. *Phytochemistry* **27**: 3385-3389.

Jensen M.S., Ahlemeyer B., Ravati A., Thakur P., Mennel H.D., Krieglstein J. 2002. Preconditioning-induced protection against cyanide-induced neurotoxicity is mediated by preserving mitochondrial function. *Neurochem. Int.* **40**: 285-293.

Job C., Rajjou L., Lovigny Y., Belghazi M., Job D. 2005. Patterns of protein oxidation in *Arabidopsis* seeds and during germination. *Plant Physiol.* **138**: 790-802.

Jonak C., Ökrész L., Bögre L., Hirt H. 2002. Complexity, cross talk and integration of plant MAP kinase signalling. *Curr. Opin. Plant Biol.* **5**: 415-424.

Jones P.R., Andersen M.D., Nielsen J.S., Hoj P.B., Moller B.L. 2000. The biosynthesis, degradation, transport and possible function of cyanogenic glycosides. In: Romero J.T., Ibrahim R., Varin L., De Luca V. (eds). Evolution of Metabolic Pathways. Elsevier Science, New York, pp.: 191-247.

Karssen C.M., Brinkhorst-Van der Swan D.L.C., Breekland A.D., Koornneef M. 1983. Induction of seed dormancy during seed development by endogenous abscisic acid: studies on abscisic acid-deficient genotypes of *Arabidopsis thaliana* L. Heynh. *Planta* **157**: 158-165.

Karssen C.M., Haigh A., van der Toorn P., Weges R. 1989. Physiological mechanisms involved in seed priming. In: Taylorson R.B. (ed.), Recent Advances in the Development and Germination of Seeds. Plenum Press, New York, pp.: 269-280.

Kępczyński J. and Karssen C.M. 1985. Requirement for the action of endogenous ethylene during germination of non-dormant seeds pf *Amaranthus caudatus*. *Physiol. Plant.* **63**: 49-52.

Kępczyński J. and Kępczyńska E. 1997. Ethylene in seed dormancy and germination. *Physiol. Plant.* **101**: 720-726.

Kieber J.J. 1997. The ethylene response pathway in *Arabidopsis*. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**: 277-296.

Kopyra M. and Gwóźdź E.A. 2003. Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. *Plant Physiol. Biochem.* **41**: 1011-1017.

Koornneef M., Bentsink L., Hilhorst H. 2002. Seed dormancy and germination. *Curr. Opin. Plant Biol.* **5**: 33-36.

Koornneef M. and Karssen C.M. 1994. Seed dormancy and germination. In: Somerville C.R., Meyerowitz E.M. (eds), Arabidopsis. Cold Spring Harbour laboratory Press, New York, pp.: 313-334.

Korolainen M.A., Goldsteins G., Alafuzoff N.M., Koistinaho J., Pirttilä T. 2002. Proteomic analysis of protein oxidation in Alzheimer□ disease brain. *Electrophoresis.* **23**: 3428-3433.

Kranner I. and Grill D. 1993. Content of low-molecular-weight thiols during the imbibition of pea-seeds. *Physiol. Plant.* **88**: 557-562.

Kranner I. and Grill D. 1996a. Determination of glutathione and glutathione disulphide in lichens: a comparison of frequently used methods. *Phytochem. Anal.* **7**: 24-28.

Kranner I. and Grill D. 1996b. Significance of thiol-disulphide exchange in resting stages of plant development. *Bot. Acta* **109**: 8-14.

Kremer R.J. and Souissi T. 2001. Cyanide production by rhizobacteria and potential for suppression of weed seedling growth. *Curr. Microbiol.* **43**: 182-186.

Kucera B., Cohn M.A., Leubner-Metzger G. 2005. Plant hormone interactions during seed dormancy release and germination. Invited review: *Seed Sci. Res.* **15**: 281-307.

Laemli U.K. 1970. Cleavage of structural protein during the assembly of the head of the bacteriophage T4. *Nature* **227**: 680-685.

Latha M.V., Borowitz J.L., Yim G.K.W., Kanthasamy A., Isom G.E. 1994. Plasma membrane hyperpolarization by cyanide in chromaffin cells: role of potassium channels. *Arch. Toxicol.* **68**: 370-374.

LePage-Degivry M.T. and Garello G. 1992. *In situ* abscisic acid synthesis. A requirement for induction of embryo dormancy in *Helianthus annuus*. *Plant Physiol.* **98**: 1386-1390.

Leubner-Metzger G. 2005. Beta-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *Plant J.* **41**: 133-145.

Lewak S., Bogatek R., Żarska-Maciejewska B. 2000. Sugar metabolism in apple embryos. In: Viemont J.D. and Grabbe J. (eds), Dormancy in Plants. CAB International, pp.: 47-55.

Leymarie J., Bruneaux E., Gibot-Leclerc S., Corbineau F. 2007. Identification of transcripts potentially involved in barley seed germination and dormancy using cDNA-AFLP. *J. Exp. Bot.* **58**: 425-437.

Liang W.S. 2003. Drought stress increases both cyanogenesis and *β*-cyanoalanine synthase activity in tobacco. *Plant Sci.* **165**: 1109-1115.

Liang W.S. and Li D.B. 2001. The two β-cyanoalanine synthase isozymes of tobacco showed different antioxidative abilities. *Plant Sci.* **161**: 1171-1177.

Lu C., Han M.H., Guevara-Garcia A., Fedoroff N.V. 2002. Mitogen-activated protein kinase signaling in postgermination arrest of development by abscisic acid. *Proc. Natl. Acad. Sci. USA* **99**: 15812-15817.

Lunn J., Droux M., Martin J., Douce R. 1990. Localization of ATP sulfurylase and *O*-acetylserine(thiol) lyase in spinach leaves. *Plant Physiol.* **94**: 1345-1352.

Maruyama A., Kazuki S., Ishizawa K. 2001. β-Cyanoalanine synthase and cysteine synthase from potato: molecular cloning, biochemical characterization, and spatial and hormonal regulation. *Plant Mol. Biol.* **46**: 749-760.

Maruyama A., Yoshiyama M., Adachi Y., Tani A., Hasegawa R., Esashi Y. 1996. Promotion of cocklebur seed germination by allyl, sulfur and cyanogenic compounds. *Plant Cell Physiol.* **37**: 1054-1058.

Matilla A.J. 2000. Ethylene in seed formation and germination. *Seed Sci. Res.* **10**: 111-126.

Maxwell D.P., Wang Y. McIntosh L. 1999. The alternative oxidase lowers mitochondria reactive oxygen production in plant cells. *Proc. Natl. Acad. Sci. USA* **96**: 8271-8276.

May M.J., Vernoux T., Leaver C., Van Montagu M., Inzé D. 1998. Glutathione homeostasis in plants: implications for environmental sensing and plant development. *J. Exp. Bot.* **49**: 649-667.

McAdam A. and Knowels C. 1984. Purification and properties of β-cyanoalanine synthase from the cyanide-producing bacterium *Chromobacterium violaceum. Biochem. Biophys. Acta* **786**: 123-132.

McDonald M.B. 1999. Seed deterioration: physiology, repair and assessment. *Seed Sci. Tech.* **27**: 177-237.

Meyer T., Burow M., Bauer M., Papenbrock J. 2003. *Arabidopsis* sulfurtransferases: investigation of their function during senescence and in cyanide detoxification. *Planta* **217**: 1-10.

Meyers D. and Ahmad S. 1991. Link between L-3-cyanoalanine synthase activity and differential cyanide sensitivity of insects. *Biochim. Biophys. Acta* **1075**: 195-197.

Miller J.M. and Conn E.E. 1980. Metabolism of hydrogen cyanide by higher plants. *Plant Physiol.* **65**: 1199-1202.

Mithofer A., Daxberger A., Fromhold-Treu D., Ebel J. 1997. Involvement of an NAD(P)H oxidase in the elicitor-inducible oxidative burst of soybean. *Phytochemistry* **45**: 1101-1107.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* **7**: 405-410.

Mittler R., Vanderauwera S., Gollery M., Van Breusegem F. 2004. Reactive oxygen gene networks of plants. *Trends Plant Sci.* **9**: 490-498.

Moller I.M., Jensen P.E., Hansson A. 2007. Oxidative modifications to cellular components in plants. *Ann. Rev. Plant Biol.* **58**: 459-481.

Moon H., Lee B., Choi G., Shin D., Prasad D.T., Lee O., Kwak S.S., Kim D.H., Nam J., Bahk J., Hong J.C., Lee S.Y., Cho M.J., Lim C.O., Yun D.J. 2003. NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. *Proc. Natl. Acad. Sci. USA* **100**: 358-363.

Morohashi Y. 2002. Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. *J. Exp. Bot.* **53**: 1643-1650.

Morohashi Y. and Matsushima H. 1983. Appearance and disappearance of cyanide-resistant respiration in *Vigna mungo* cotyledons during and following germination of the axis. *Plant Physiol.* **73**: 82-86.

Nahrstedt A. 1993. Cyanogenesis and foodplants. In: van Beek T.A., Breteler H. (eds), Proc. of the Phytochem. Soc. in Europe, Clarendon Press, Oxford, pp.: 107-129.

Neill S.J., Desikan R., Clarke A., Hurst R.D., Hancock J.T. 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. Exp. Bot.* **53**: 1237-1247.

Neill S.J., Desikan R., Hancock J.T. 2003. Nitric oxide signalling in plants. *New Phytol.* **159**: 11-35.

Newman J.W., Morisseau C., Hammock B.D. 2005. Epoxide hydrolases: their roles and interactions with lipid metabolism. *Prog. Lipid Res.* **44**: 1-51.

Niedźwiedź-Siegień I. 1998. Cyanogenic glucosides in *Linum usitatissimum*. *Phytochemistry* **49**: 59-63.

Niedźwiedź-Siegień I. and Gierasimiuk A. 2001. Environmental factors affecting the cyanogenic potential of flax seedlings. *Acta Physiol. Plant.* **23**: 383-390.

Nkang A. 2001. Effect of cyanide pre-treatment on activities of polyphenoloxidase and peroxidase in seeds of *Guilfoylia monostylis*. *Seed Sci. Technol.* **29**: 557-565.

Ogawa K. and Iwabuchi M. 2001. A mechanism for promoting the germination of *Zinnia elegans* seeds by hydrogen peroxide. *Plant Cell Physiol*. **42**: 286-291.

Ohme-Takagi M. and Shinshi H. 1995. Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell* **7**: 173-182.

O'Kane D., Gill V., Boyd P., Burdon R. 1996. Chilling, oxidative stress and antioxidant responses in Arabidopsis thaliana callus. *Planta* **198**: 371-377.

Oracz K., El-Maarouf Bouteau H., Farrant J.M., Cooper K., Belghazi M., Job C., Job D., Corbineau F., Bailly C. 2007. ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. *Plant J.* **50**: 452-465.

Otte O. and Barz W. 1996. The elicitor-induced oxidative burst in cultured chickpea cells drives the rapid insolublilization of two cell wall structural proteins. *Planta* **200**: 238-246.

Peiser G.D., Wang N.E., Hoffman N.E., Yang S.F., Liu H.W., Walsh C.T. 1984. Formation of cyanide from carbon 1 of 1-aminocyclopropane-1-carboxylic acid during its conversion to ethylene. *Proc. Natl. Acad. Sci. USA* **81**: 3059-3063.

Pérez-Flores L., Carrari F., Osuna-Fernández R., Rodriguez M.V., Encisco S., Stanelloni R., Sánchez R.A., Bottini R., Iusem N.D., Benech-Arnold R. 2003. Expression analysis of GA 20-oxidase in embryos from two sorghum lines with contrasting dormancy: possible participation of this gene in the hormonal control of germination. *J. Exp. Bot.* **54**: 2071-2079.

Pitzschke A. and Hirt H. 2006. Mitogen-activated protein kinases and reactive oxygen species signaling in plants. *Plant Physiol.* **141**: 351-356.

Prabhakaran K., Li L., Borowitz J.L., Isom G.E. 2006. Inducible nitric oxide synthase up-regulation and mitochondrial glutathione depletion mediate cyanide-induced necrosis in mesencephalic cells. *J. Neurosci. Res.* **84**: 1003-1011.

Pukacka S. and Ratajczak E. 2005. Production and scavenging of reactive oxygen species in *Fagus sylvatica* seeds during storage at varied temperature and humidity. *J. Plant Physiol.* **162**: 873-885.

Pukacka S. and Ratajczak E. 2006. Antioxidative response of ascorbate-glutathione pathway enzymes and metabolites to desiccation of recalcitrant *Acer saccharinum* seeds. *J. Plant Physiol.* **163**: 1259-1266.

Reinhardt D., Kende H., Boller T. 1994. Subcellular localization of 1-amino-cyclopropane-1-carboxylate oxidase in tomato cells. *Planta* **195**: 142-146.

Roberts E.H. 1969. Seed dormancy and oxidation processes. *Symp. Soc. Exp. Biol.* **23**: 161-192.

Rolland N., Droux M., Douce R. 1992. Subcellular distribution of *O*-acetylserine(thiol) lyase in cauliflower (*Brassica oleracea* L.) inflorescence. *Plant Physiol.* **98**: 927-935.

Roman G., Lubarsky B., Kieber J.J., Rothenberg M., Ecker J.R. 1995. Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: five novel mutant loci integrated into a stress response pathway. *Genetics* **139**: 1393-1409.

Samuel M.A. and Ellis B.E. 2002. Double jeopardy: both overexpression and suppression of a redox-activated plant mitogen-activated protein kinase render tobacco plants ozone sensitive. *Plant Cell* **14**: 2059-2069.

Samuilov V.D., Lagunova E.M., Beshta O.E., Kitashov A.V. 2000. CN-induced degradation of nuclei in cells of pea leaves. *Biochemistry* (Moscow) **65**: 696-702.

Sarath G., Hou G., Baird L.M., Mitchell R.B. 2007. Reactive oxygen species, ABA and nitric oxide interactions on the germination of warm-season C(4)-grasses. *Planta* **226**: 697-708.

Scandalios J.G., Guan L., Polidoros A.N. 1997. Catalases in plants: gene structure, properties, regulation and expression. In: Scandalios J.G. (ed.), Oxidative stress and the molecular biology of antioxidant defenses. New York, Cold Spring Harbor Laboratory Press, pp.: 343-406.

Schopfer P. and Liszkay A. 2006. Plasma membrane-generated reactive oxygen intermediates and their role in cell growth of plants. *Biofactors* **28**: 73-81.

Schopfer P., Plachy C., Frahry G. 2001. Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. *Plant Physiol.* **125**: 1591-1602.

Seigler D.S. 1991. Cyanide and cyanogenic glycosides. In: Rosenthal G.A. and Berenbaum M.R. (eds), Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, San Diego, pp.: 35-77.

Selmar D. 1993. Apoplastic occurrence of cyanogenic β-glucosidases and consequences for the metabolism of cyanogenic glucosides. In: Esen A. (ed.), β-Glucosidases: Biochemistry and Molecular Biology, Am. Chem. Soc. Symp. Ser. 533, American Chemical Society, Washington, DC, pp.: 191-204.

Selmar D., Lieberei R., Biehl B. 1988. Mobilization and utilization of cyanogenic glucosides. The linustatin pathway. *Plant Physiol.* **86**: 711-716.

Shevchenko A., Wilm M., Vorm O., Mann M. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **68**: 850-858.

Siefert F., Kwiatkowski J., Sarkar S., Grossmann K. 1995. Changes in endogenous cyanide and 1-aminocyclopropane-1-carboxylic acid levels during the hypersensitive response of tobacco mosaic virus-infected tobacco leaves. *Plant Growth Regul.* **17**: 109-113.

Siegień I. and Bogatek R. 2006. Cyanide action in plants ☐ from toxic to regulatory. *Acta Physiol. Plant.* **28**: 483-497.

Slooten L., Capiau K., Van Camp W., Van Montagu M., Sybesma C., Inzé D. 1995. Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide dismutase in the chloroplasts. *Plant Physiol.* **107**: 737-750.

Smith J.M. and Arteca R.N. 2000. Molecular control of ethylene production by cyanide in *Arabidopsis thaliana. Physiol. Plant.* **109**: 180-187.

Solano R., Stepanova A., Chao Q., Ecker J.R. 1998. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev.* **12**: 3703-3714.

Solomonson L.P. and Barber M.J. 1990. Assimilatory nitrate reductase: functional properties and regulation. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **41**: 225-253.

Surplus S.L., Jordan B.R., Murphy A.M., Carr J.P., Thomas B., Mackerness S.A.H. 1998. Ultraviolet-B-induced responses in *Arabidopsis thaliana*: Role of salicylic acid and reactive oxygen species in the regulation of transcripts encoding photosynthetic and acidic pathogenesis-related proteins. *Plant Cell Environ.* **21**: 685-694.

Taylorson R.B. and Hendricks S.B. 1973. Promotion of seed germination by cyanide. *Plant Physiol.* **52**: 23-27.

Taylorson R.B. and Hendricks S.B. 1979. Overcoming dormancy in seeds with ethanol and other anesthetics. *Planta* **145**: 597-510.

Tena G., Asai T., Chiu W.L., Sheen J. 2001. Plant mitogen-activated protein kinase signaling cascades. *Curr. Opin. Plant Biol.* **4**: 392-400.

Tommasi F., Paciolla C., de Pinto M.C., de Gara L. 2001. A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seeds. *J. Exp. Bot.* **52**: 1647-1654.

Vera-Estrella R., Blumwald E., Higgins V.J. 1992. Effect of specific elicitors of *Cladosporium fulvum* on tomato suspension cells. *Plant Physiol.* **99**: 1208-1215.

Ververidis P. and Dilley D.R. 1995. Catalytic-and non-catalytic inactivation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase in ethylene biosynthesis: role of cyanide product. Proc. 22nd Ann. Mtg. Plant Growth Regulator Soc. Am., pp.: 183-199.

Verwoerd T.C., Dekker B.M.M., Hoekema A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. *Nucl. Acids Res.* **17**: 2362.

Vetter J. 2000. Plant cyanogenic glycosides. *Toxicon* **38**: 11-36.

Vleeshouwers L.M., Bouwmeester H.J., Karssen C.M. 1995. Redefining seed dormancy: an attempt to integrate physiology and ecology. *J. Ecol.* **83**: 1031-1037.

Walters C., Landre P., Hill L.M., Corbineau F., Bailly C. 2005. Organization of lipid reserves in cotyledons of primed and aged sunflower seeds. *Planta* **222**: 397-407.

Wang K.L.C., Li H., Ecker J.R. 2002. Ethylene biosynthesis and signaling networks. *Plant Cell* S131-S151.

Willekens H., Inzé D., Van Montagu M., Van Camp W. 1995. Catalases in plants. *Mol. Breeding* **1**: 207-228.

Wurtele E.S., Nikolau B.J., Conn E.E. 1985. Subcellular and developmental distribution of β-cyanoalanine synthase in barley (*Hordeum vulgare*) leaves. *Plant Physiol.* **78**: 285-290.

Wojtyla L., Garnczarska M., Zalewski T., Bednarski W., Ratajczak L., Jurga S. 2006. A comparative study of water distribution, free radical production and activation of antioxidative metabolism in germinating pea seeds. *J. Plant Physiol.* **163**: 1207-1220.

Xiong Y., Contento A.L., Nguyen P.Q., Bassham D.C. 2007. Degradation of oxidized proteins by autophagy during oxidative stress in *Arabidopsis*. *Plant. Physiol.* **143**: 291-299.

Xiong L. and Yang Y. 2003. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell* **15**: 745-59.

Yip W.K. and Yang S.F. 1988. Cyanide metabolism in relation to ethylene production in plant tissues. *Plant Physiol.* **88**: 473-476.

Zagórski S. and Lewak S. 1985. Germination of lettuce seeds promoted by red light, gibberellin or cyanide is differently affected by far red illumination and temperature. *Acta Physiol. Plant.* **7**: 65-70.

Zhang S. and Klessig D.F. 2001. MAPK cascades in plant defense signaling. *Trends Plant Sci.* **6**: 520-527.

10. Supplementary data

Tables 1 and **2** presenting primers used in QRT-PCR gene analysis;

Map of vector used in cloning of genes;

Publication: **Oracz *et al.* 2007** and supplements;

Presented posters:

- Bailly C., Mamadou N., **Oracz K.**, Come D., Corbineau F. (24-28 May 2004). "Is there a role for cyanide in sunflower seed germination". 3rd International Symposium on Plant Dormancy, Wageningen, Netherlands.
- **Oracz K.**, Bailly C., Corbineau F. (8-13 May 2005). "Effect of cyanide on the germination of dormant sunflower (*Helianthus annuus* L.) seeds". 8th International Workshop on Seeds - Germinating New Ideas, Brisbane, Australia.
- **Oracz K.**, El-Maarouf-Bouteau H., Bogatek R., Corbineau F., Bailly C. (26-30 August 2007). "Possible interplay of HCN and ROS with ethylene signalling pathway in alleviation of sunflower embryo dormancy. 3rd Conference of Polish Society of Experimental Plant Biology, Warsaw, Poland - ***award in the category of the very interesting results presented on the conference.***

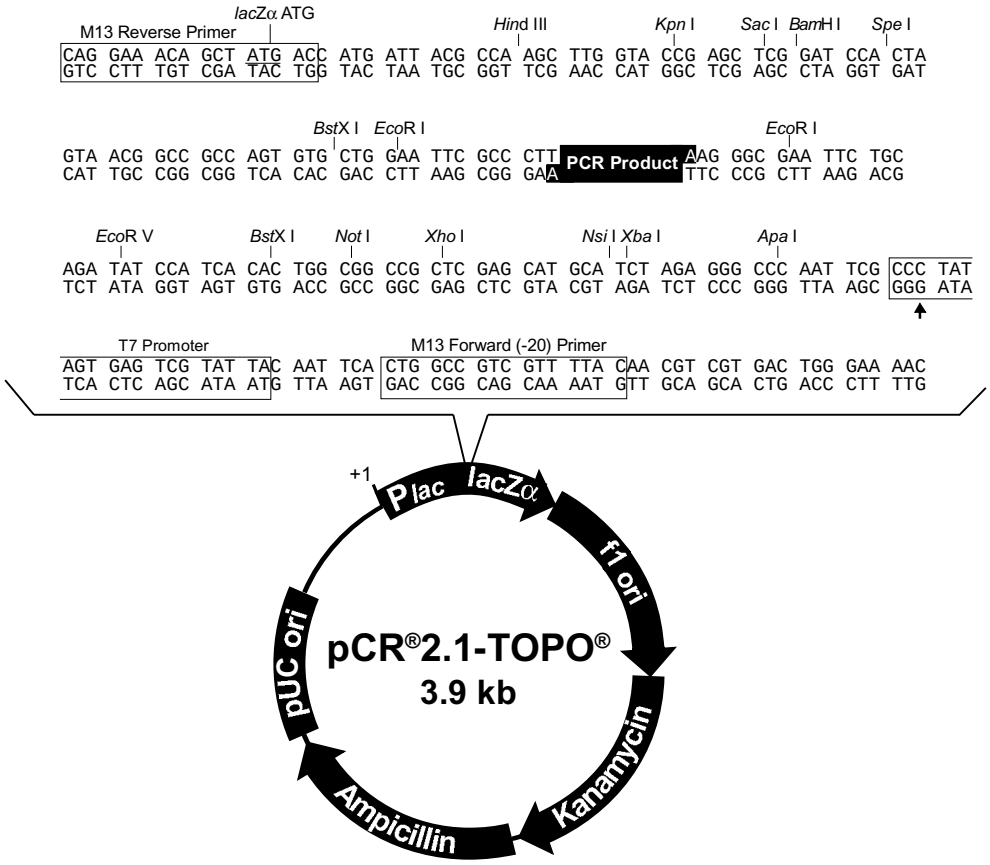
Award in category the very interesting results presented at 3rd Conference of Polish Society of Experimental Plant Biology (Warsaw, Poland), by the title: "Possible interplay of HCN and ROS with ethylene signalling pathway in alleviation of sunflower embryos dormancy".

Table 1. Characteristic of primers used in QRT-PCR in order to study the involvement of ROS production or ROS signaling pathways in germination. (aa: amino-acid, nt: nucleotide).

Name of *H.annuus* L. gene	Gene bank of CGP EST accession number	Amplification product size	Primers sequence	Homology percentage of EST sequences with other plants (plant, accession no)
involved in ROS production				
NADPHox (NADPH oxidase)	>QH_CA_Contig929	116	Left: AGGGTCGTTTGACTGGTTC Right: ACCGAGCATCACCTTCTTC	88 aa (*Nicotiana tabacum, CAC84140*)
POX (Peroxidase - cell wall)	>QH_CA_Contig2935	101	Left: CCTCCGTTATTCGCCTTC Right: ATTGGCTGCTGACTCCTTC	70 aa (*Arabidopsis*, BT008314)
AO (Amine oxidase - cell wall)	>QHL17I14.yg.ab1	108	Left: CCGACTGTATCATCATCGAGTT Right: GCGTTACAAACTGGCAAATC	68 aa (*Arabidopsis*, AAB87690.1)
involved in ROS signal transduction				
Ser/ ThrPK (Ser/ Thr protein kinase)	>QHB16I20.yg.ab1	195	Left: CAAGGGAGGTGACTTTGG Right: ATGTTGGCATACGGCTCT	87 aa (*Arabidopsis*, NP_173077)
MAPK6 (Mitogen activated protein kinase 6)	>QH_CA_Contig1463	182	Left: CAAGCAACCCTCTACTGAAC Right: GCAACCCACAGACCATAC	81 nt (*Arabidopsis*, NM_129941)
PTP (Protein tyrosine phosphatase)	>QH_CA_Contig640	149	Left: TTTCAAGTGGAGGTTGTGGT Right:GAGGGAGGATTTGGTGTTG	82 nt (Arabidopsis, AY070042)
CaM (Calmodulin)	>QH_CA_Contig1037	168	Left: GAGTTCCTTGGTGGTGATG Right: GACCTGTGTTGTCGTTTCAG	94 aa (*Lycopersicon esculentum*, CAA75056)
constitutive gene				
EF1α (Elongation factor 1α)	QH_CA_Contig2764	159	Left: TCTCCACTCCTCCAACAC Right: CTCAATCACTCGCTACACC	98 id aa (*Lycopersicon esculentum*, P17786)
β-tubulin	QH_CA_Contig4019	132	Left: GGCGTCTACCTTCATTGGT Right: TCCATCTCATCCATTCCTTC	88 nt (*Arabidopsis*, AAK96884)

Table 2. Characteristic of primers used in QRT-PCR in order to study the involvement of the ethylene production or ethylene signal transduction pathways in germination. (nt: nucleotide).

Name of *H.annuus* L. gene	Gene bank of CGP EST accession number	Amplification product size	Primers sequence	Homology percentage of EST sequences with other plants (plant, accession no)
involved in ethylene production				
ACO (ACC oxidase)	L 29405	124	Left: GAAGTGTATGGAGCAGAGGTTT Right: GTTGGAGGTAGGGCGATG	
ACS (ACC synthase)	QHG4g06.yg.ab1	126	Left: CGGTTATTAGAGGGGGTAGTG Right: TATTGTGTCGGGAGGAGGA	80 nt (*Arabidopsis thaliana*, NM_116016)
involved in ethylene signal transduction				
ETR2 (Ethylene response 2)	QHF11C05.yg.ab1	131	Left: AGTCGGAAGGCTCTGGTG Right: TCCTGTGGGATACGGAACT	77 nt (*Cucumis sativus*, AB026500)
CTR1 (Constitutive triple response 1)	QHG13O10.yg.ab1	166	Left: CCGTCCACTCTCTTGTAGGT Right: TCGTCGTCTGGCTCTTCT	84 nt (*Arabidopsis thaliana*, At5g03730.1)
ERF1 (Ethylene response factor 1)	QH_CA_Contig5791	176	Left: TCTTGACTCAATCCAACACC Right: ACTCTTGGTTTTCCACCACT	86 nt (*Arabidopsis thaliana*, AF076278)



Comments for pCR®2.1-TOPO®
3931 nucleotides

*Lac*Zα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Invitrogen™
life technologies

The Plant Journal (2007) **50**, 452–465 doi: 10.1111/j.1365-313X.2007.03063.x

ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation

Krystyna Oracz[1,2], Hayat El-Maarouf Bouteau[2], Jill M. Farrant[3], Keren Cooper[3], Maya Belghazi[4], Claudette Job[5], Dominique Job[5], Françoise Corbineau[2] and Christophe Bailly[2,*]

[1]*Department of Plant Physiology, Warsaw Agricultural University, Nowoursynowska 159, 02-776 Warsaw, Poland,*

[2]*Université Pierre et Marie Curie-Paris 6, EA 2388 Physiologie des Semences, Le Raphaël, Site d'Ivry, Boîte 152, 4 Place Jussieu, Paris F-75005 France,*

[3]*Department of Molecular and Cell Biology, University of Cape Town, Private Bag 7701 Rondebosch, South Africa,*

[4]*Institut National de la Recherche Agronomique, Unité Mixte de Recherche 6175, Service de Spectrométrie de Masse pour la Protéomique, Nouzilly, F-37380 France, and*

[5]*Centre National de la Recherche Scientifique/Bayer CropScience Joint Laboratory, Unité Mixte de Recherche 2847, Bayer CropScience, Lyon, F-69283 France*

Received 17 November 2006; revised 21 December 2006; accepted 4 January 2007
*For correspondence (fax +33 1 44 27 59 27; e-mail bailly@ccr.jussieu.fr).

Summary

At harvest, sunflower (*Helianthus annuus* L.) seeds are dormant and unable to germinate at temperatures below 15°C. Seed storage in the dry state, known as after-ripening, is associated with an alleviation of embryonic dormancy allowing subsequent germination at suboptimal temperatures. To identify the process by which dormancy is broken during after-ripening, we focused on the role of reactive oxygen species (ROS) in this phenomenon. After-ripening entailed a progressive accumulation of ROS, namely superoxide anions and hydrogen peroxide, in cells of embryonic axes. This accumulation, which was investigated at the cellular level by electron microscopy, occurred concomitantly with lipid peroxidation and oxidation (carbonylation) of specific embryo proteins. Incubation of dormant seeds for 3 h in the presence of hydrogen cyanide (a compound that breaks dormancy) or methylviologen (a ROS-generating compound) also released dormancy and caused the oxidation of a specific set of embryo proteins. From these observations, we propose a novel mechanism for seed dormancy alleviation. This mechanism involves ROS production and targeted changes in protein carbonylation patterns.

Keywords: seed dormancy, after-ripening, reactive oxygen species, proteome analysis, carbonylation, sunflower.

Introduction

Seed dormancy, defined as the failure of viable mature seeds to germinate under favorable conditions, is assumed to be an important adaptive trait in nature, enabling seeds to remain quiescent until the conditions for germination and seedling establishment become favorable (Bewley, 1997; Bewley and Black, 1994; Finch-Savage and Leubner-Metzger, 2006). This trait can have an embryo and/or a coat component, hence the terms 'embryo' and 'coat' dormancy to distinguish between these two mechanisms. Under natural conditions, release of dormancy generally occurs during after-ripening (storage in dry conditions) or during stratification (imbibition at low temperature), which result in widening of the conditions allowing seed germination (Baskin and Baskin, 1998; Bewley, 1997; Bewley and Black, 1994; Donohue *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006; Koornneef *et al.*, 2002).

After-ripening is an intriguing phenomenon as it occurs at low seed moisture contents (MC), generally <0.10 g H_2O/g dry weight (DW). Under these extreme conditions, water is generally not available for biochemical reactions, and very little is known about the cellular and molecular mechanisms involved in this process. However, changes in gene expression and/or protein synthesis during after-ripening have been shown to occur in seeds of *Nicotiana tabacum*

(Leubner-Metzger, 2005), *Nicotiana plumbaginifolia* (Bove *et al.*, 2005) and *Arabidopsis thaliana* (Cadman *et al.*, 2006; Chibani *et al.*, 2006), which presumably reflects the existence of hydrated pockets within cells or tissues of the mature seeds (Leubner-Metzger, 2005). Non-enzymatic reactions are also likely to occur during dry storage of seeds, such as lipid peroxidation (McDonald, 1999; Priestley, 1986; Wilson and McDonald, 1986) or the Amadori and Maillard reactions associated with free radical production and oxidation processes (Esashi *et al.*, 1993; Murthy and Sun, 2000; Murthy *et al.*, 2003; Sun and Leopold, 1995). Furthermore, several studies have documented the production of reactive oxygen species (ROS) during seed storage in the dry state (Bucharov and Gantcheff, 1984; Hendry, 1993; McDonald, 1999; Pukacka and Ratajczak, 2005). ROS can react with virtually all biological molecules including lipids, DNA and proteins. Because proteins have numerous biological functions, their oxidation may result in modification of their enzymatic and binding properties and lead to diverse functional changes. Oxidation of proteins can occur through a number of different mechanisms, such as the formation of disulfide cross-links and glycoxidation adducts, nitration of tyrosine residues, and carbonylation of specific amino acid residues (Davies, 2005). Recent studies have indicated that protein oxidation is not necessarily a deleterious phenomenon in plants (Job *et al.*, 2005; Johansson *et al.*, 2004). Moreover, cellular ROS may show some selectivity with respect to their targets. For example, H_2O_2, which is an oxidant, can react with specific molecules at specific sites (Davies, 2005; Halliwell and Gutteridge, 1999). Importantly, ROS have been invoked to play a role in cellular signaling (for review, see Bailly, 2004), raising the hypothesis that these compounds can facilitate the shift from a dormant to a non-dormant status in seeds.

Sunflower (*Helianthus annuus*) seeds provide an excellent system for studying dormancy because they are deeply dormant at harvest, but this dormancy is progressively lost during dry storage (Corbineau *et al.*, 1990). Their dormancy results from both seed coat- and embryo-imposed dormancy, the latter being involved in the failure to germinate at 10–15°C. However, the molecular mechanisms of embryo dormancy and of its release during after-ripening are still largely unknown.

The objective of this study was to investigate whether the production of ROS and lipid autoxidation occur after harvest, and whether this can, in turn, bring about modifications in seed protein oxidation patterns that facilitate alleviation of seed dormancy. Moreover, in order to assess a putative causal association between ROS production, protein oxidation and dormancy alleviation, dormant sunflower seeds were imbibed in the presence of hydrogen cyanide, a compound that breaks dormancy (Bethke *et al.*, 2006; Bogatek *et al.*, 1991; Esashi *et al.*, 1991; Taylorson and Hendricks, 1973), or in the presence of methylviologen (MV), a ROS-generating compound (Slooten *et al.*, 1995). Although MV has not yet been reported as a dormancy-breaking chemical, we have used it here as a tool to assess the proposition that ROS generation is involved in dormancy alleviation in this species. The general finding is that dormancy release, both in dry and imbibed states, is associated with ROS production and the carbonylation of specific embryo proteins.

Results

Seed germination

Figure 1 shows the germination behavior of dormant and non-dormant de-coated (naked) sunflower seeds at various temperatures. At 15, 20 and 25°C, dormant seeds germinated slowly and reached 80–90% germination within 6–8 days (Figure 1a). Only about 20% of dormant naked seeds were able to germinate at 10°C within 10 days (Figure 1a), but dry storage markedly enhanced their germination at all temperatures tested, and, in particular, they became able to fully germinate at 10°C within 4 days (Figure 1b).

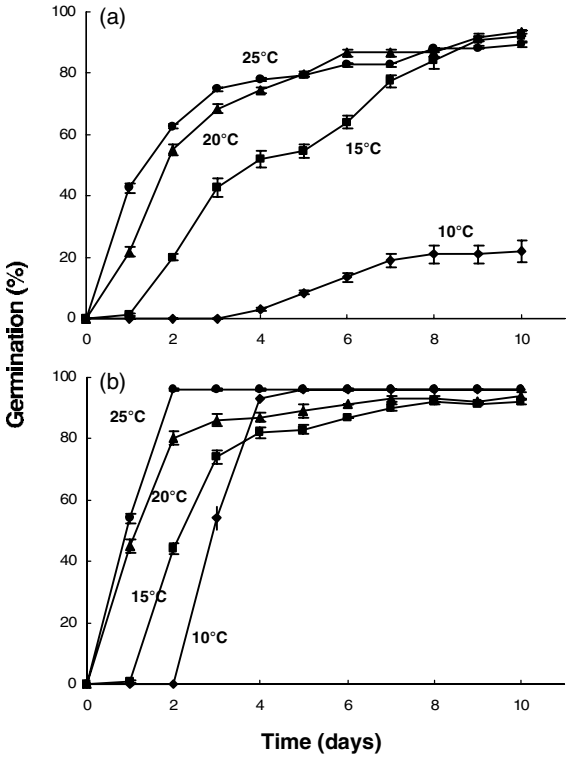


Figure 1. Germination of dormant and non-dormant sunflower embryos (i.e. naked seeds without pericarp) at various temperatures.
(a) Dormant embryos (immediately after harvest).
(b) Non-dormant embryos (after 2 months of dry storage).
Values are means of four replicates ± SD.

ROS production during dry storage

To characterize ROS production during after-ripening, hydrogen peroxide and superoxide anion contents were determined in axes (see Figure 2) excised from dormant and non-dormant seeds (Table 1). Dormancy release was associated with a marked enhancement in both compounds, as the level of H_2O_2 doubled and that of $O_2^{\cdot-}$ increased by about 50%. Dry storage was also associated with a slight but significant increase in malondialdehyde (MDA) content, indicating the occurrence of lipid peroxidation (Table 1).

Table 1 Hydrogen peroxide, superoxide and malondialdehyde (MDA) contents in dry dormant and non-dormant sunflower axes

	H_2O_2 (μmol g DW^{-1})	$O_2^{\cdot-}$ (μmol g DW^{-1})	MDA (μmol g DW^{-1})
Dormant axes	1.37 ± 0.21	5.29 ± 0.25	76.75 ± 3.65
Non-dormant axes	2.10 ± 0.14	8.66 ± 0.51	90.62 ± 0.72

Values are means of five replicates ± SD.

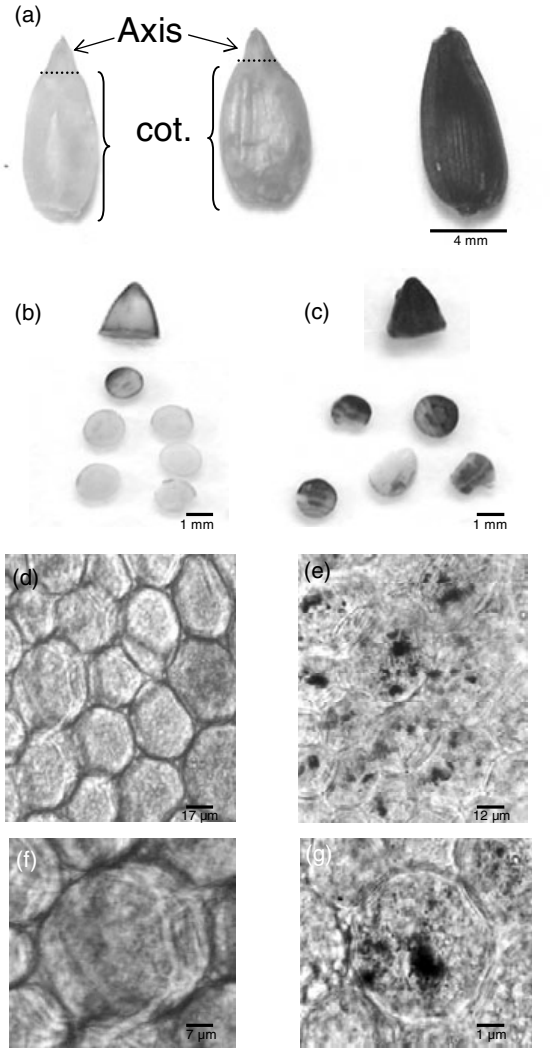


Figure 2. Localization of $O_2^{\cdot-}$ in sunflower embryonic axes.
(a) Whole seed (right), embryo (center) and half-embryo (showing only one cotyledon, left) of sunflower, showing axis and cotyledon (cot.).
(b, c) Whole axes and hand-cut sections of dormant (b) and non-dormant (c) sunflower axes. The superoxide anions were visualized with NBT staining.
(d, f) and (e, g) Sections of dormant and non-dormant axes, respectively, stained with NBT and viewed by light microscopy. The superoxide anion is detected as formazan precipitates within the cytoplasm of the cells of non-dormant axes (e, g). No marked coloration was visible in the cells of dormant axes (d, f).
Scale is indicated on bars within the figures.

In situ accumulation of $O_2^{\cdot-}$ in whole seeds (embryos) and in sections through axes during dry storage is shown in Figure 2. At the whole-tissue level, accumulation of formazans occurred almost homogeneously in axes of non-dormant seeds (Figure 2c). In contrast, staining of dormant axes by nitroblue tetrazolium (NBT) was only very slight (Figure 2b). Transverse sections through the axes confirmed the presence of formazan deposits at the cellular level. In dormant axes, no NBT precipitation was visible within the cells (Figure 2d,f), whereas numerous dark spots indicated the presence of superoxide anion within the cells of non-dormant axes (Figure 2e,g).

The detection of hydrogen peroxide was carried out by $CeCl_3$ staining and revelation by transmission electron microscopy (Bestwick *et al.*, 1997; Pellinen *et al.*, 2002). Cells from axes of both dormant and non-dormant seeds were filled with large lipid bodies and smaller protein bodies, which somewhat occluded observation of the cytoplasm (Figure 3a,b). However, in the presence of $CeCl_3$, H_2O_2 was clearly evident as electron dense (cerium perhydroxide) spots (arrowed) within the cytoplasm of the cells (Figure 3c–f).While some H_2O_2 could be detected in dormant seeds (Figure 3c,e), there was much higher staining in the cytoplasm of cells from axes of non-dormant seeds, revealing endogenous H_2O_2 production mainly in the non-dormant tissues (Figure 3d,f).

To determine whether ROS accumulation during dry storage caused seed dormancy alleviation, and was not simply a side-effect accompanying seed storage, whole dormant sunflower seeds were stored at 75% RH (relative humidity) or 5% RH at 25°C for various durations, and the H_2O_2 content in the axes was determined (Table 2). Storage of seeds at 75% RH was associated with a progressive reduction of dormancy, whereas dormancy was maintained when seeds were stored at 5% RH. After 6 weeks of storage at 25°C and 75% RH, dormancy was lost, as these seeds germinated at 10°C almost as fast as the non-dormant ones (i.e. the seeds obtained after 2 months of dry storage at 25°C and 60% RH; Table 3 and Figure 1b). Under these conditions,

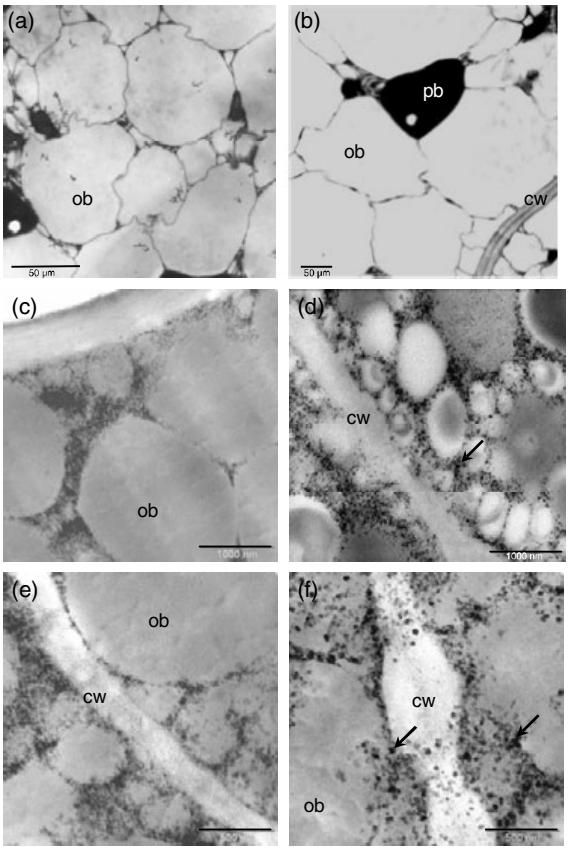


Figure 3. Subcellular localization of H_2O_2 in sunflower embryonic axes.
(a, b) Dormant and non-dormant axes, respectively, viewed by TEM without H_2O_2 staining (sections not infiltrated by $CeCl_3$).
(c, e) and (d, f) Dormant and non-dormant axes, respectively, viewed by TEM with $CeCl_3$-staining. H_2O_2 is visualized as black spots corresponding to electron-dense cerium perhydroxide precipitates (arrows). Abbreviations: cw, cell wall; ob, oil body; pb, protein body. Scale bars represent 50 µm (a,b), 1000 nm (c,d) and 500 nm (e,f).

Table 2 Germination after 7 days at 10°C and hydrogen peroxide content of axes of dormant sunflower seeds stored for 3 and 6 weeks under 75% and 5% RH at 25°C

Duration and conditions of storage	Germination (%) at 10°C after 7 days	H_2O_2 content (µmol g DW^{-1})
None (after harvest)	19 ± 2.2	1.37 ± 0.21
3 weeks, 75% RH	42 ± 1.1	2.01 ± 0.12
3 weeks, 5% RH	22 ± 1.5	1.41 ± 0.04
6 weeks, 75% RH	70 ± 2.0	2.21 ± 0.22
6 weeks, 5% RH	21 ± 0.5	1.40 ± 0.03

Values are means of four (germination) or five (H_2O_2) replicates ± SD

the hydrogen peroxide content of the embryos increased from 1.3 to 2.2 µmol g DW^{-1}, whereas this content remained unchanged when dormant seeds were stored for the same duration but at 5% RH, i.e. conditions that do not permit seed dormancy release (Table 3).

Protein carbonylation during dry after-ripening

To determine whether ROS production during after-ripening could be associated with protein oxidation (carbonylation), one-dimensional (1D) and two-dimensional (2D) PAGE of seed protein extracts were performed, and the presence of carbonyl groups was detected by Western blotting using the 2,4-dinitrophenylhydrazine (DNPH) immunoassay (Korolainen *et al.*, 2002).

The protein patterns obtained by 1D gel electrophoresis of soluble proteins from dormant and non-dormant embryonic axes are shown in Figure 4. Coomassie blue staining showed the soluble proteins from both dormant and non-dormant embryonic axes to be very similar. Soluble proteins from dormant axes exhibited three faint carbonylated bands of about 75, 60 and 55 kDa (Figure 4). In contrast, the extent of protein carbonylation was much higher in soluble proteins from non-dormant axes, and a number of new carbonylated bands were detected (Figure 4).

The protein targets of carbonylation during after-ripening were characterized by 2D gel electrophoresis and LC/MS–MS analyses. The 2D protein patterns of soluble proteins extracted from dormant and non-dormant axes were very similar (Figure 5a,c). However, in agreement with the data obtained by 1D gel electrophoresis (Figure 4), after-ripening was associated with increased carbonylation of several proteins (red arrows and red circles in Figure 5). There was also a decrease in the carbonylation level of some proteins during this process (yellow arrows in Figure 5). Finally, some carbonylated proteins remained at a constant level during after-ripening (green arrows in Figure 5). It is clear that not all seed proteins detected by silver staining were carbonylated, testifying to the specificity of protein oxidation during after-ripening.

Twenty spots representing carbonylated proteins were excised from 2D gels and analyzed by mass spectrometry. Identification failed for 12 proteins. Table 2 and Table S1 list the oxidized proteins that were identified by this approach, mainly by sequence homology with proteins from species other than sunflower. However, several proteins could be identified using the sunflower EST collections (http://cgpdb.ucdavis.edu/; http://genoplante-info.infobiogen.fr/), highlighting the usefulness of such collections as a tool for proteomics in the absence of extensive genomic sequence information. The data showed that the carbonylation level of elongation factor 2 (EF2, spot number 3 in Figure 5), pyruvate orthophosphate dikinase (PPDK, spot number 4 in Figure 5) and a protein corresponding to a sunflower EST, which has a strong homology with a 7S globulin of *Sesamum indicum* and with various vicilin-like proteins (spot number 15 in Figure 5) increased during dry after-

Table 3 Identification of carbonylated proteins in embryo axes of dry dormant and non-dormant sunflower seeds

Spot number	Accession	Protein name	MW (kDa)	pI	Change in carbonylation during after-ripening
3	gi|6056373	Elongation factor EF-2 (*Arabidopsis thaliana*)	93.9	6.1	Increase
4	gi|3024423	Pyruvate, phosphate dikinase, chloroplast precursor (*Flaveria brownie*)	93.9	5.1	Increase
7	gi|217855	81 kDa heat shock protein (*Arabidopsis thaliana*)	84.0	4.9	Constant
9	gi|2827002	HSP70 (*Triticum aestivum*)	73.4	5.0	Constant
12	gi|22273	Enolase (*Zea mays*)	60.0	5.5	Constant
15	gi|58735933	DH0AQA1ZD02RM1 HaDevS1, *Helianthus annuus* 7S globulin	60.0	6.9	Increase
18	gi|22460345	QHF6E13.yg.ab1 QH_EFGHJ sunflower RHA280, *Helianthus annuus* cDNA clone (≥gi|1458098) globulin-like protein (*Daucus carota*)	38.0	5.2	Constant
46	gi|32530040	DH0AB43ZF02RM1 HaDevR1, *Helianthus annuus* cDNA clone HaDevR143F02, mRNA sequence ≥gi|82621184|gb|ABB86280.1| proteasome-like protein alpha subunit-like (*Solanum tuberosum*)	37.0	6.6	Decrease

Peptide sequences were identified by MS-MS sequencing, see Table S1 for the peptide sequences. Experimental molecular weight (MW) and pI are indicated.

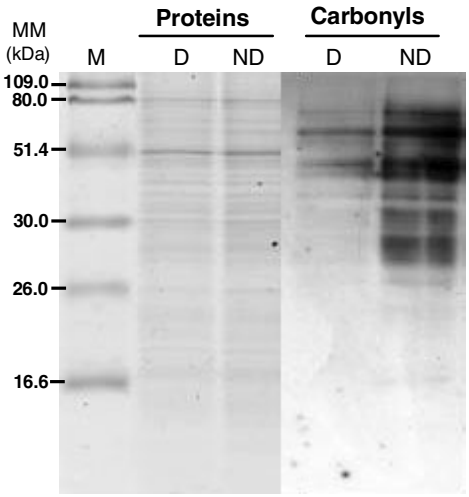


Figure 4. One-dimensional PAGE of oxidatively modified proteins from axes of dry dormant and non-dormant sunflower seeds.
Protein stain (proteins) and anti-DNP immunoassay (carbonyls) are shown. D, dormant; ND, non-dormant; M, markers; MM, molecular mass.

ripening. In contrast, seed dormancy alleviation during dry storage was associated with decreased carbonylation of several proteins including a 20S proteasome α-subunit (spot number 46 in Figure 5). Proteins whose carbonylation level remained constant during dry storage included 81 and 70 kDa heat shock proteins (HSPs, spot numbers 7 and 9 in Figure 5), enolase (spot number 12 in Figure 5) and a protein with strong homology to a globulin-like protein from carrot (*Daucus carota*) (spot number 18 in Figure 5).

Effect of cyanide and methylviologen on the protein oxidation pattern

To further document a link between protein oxidation and release of seed dormancy, dormant sunflower seeds were imbibed in the presence of hydrogen cyanide, a compound known to break dormancy in many species (Bethke *et al.*, 2006; Bogatek *et al.*, 1991; Côme *et al.*, 1988; Esashi *et al.*, 1991; Taylorson and Hendricks, 1973). A 3 h imbibition of the dormant sunflower seeds in the presence of 1 mM hydrogen cyanide efficiently released dormancy (Figure 6). Furthermore, this incubation triggered the carbonylation of specific proteins in dormant axes only (e.g. the blue circles in Figure 7). This carbonylation pattern was specific as it was not seen with non-dormant seeds incubated either in water or in the presence of hydrogen cyanide (Figure 7d). To further assess the correlation between ROS accumulation, protein carbonylation and break of dormancy, dormant and non-dormant seeds were imbibed for 3 h in the presence of methylviologen, a compound known to induce ROS production (Slooten *et al.*, 1995). After this treatment, the H_2O_2 content of dormant axes was 2.7 ± 0.11 μmol g DW^{-1}, whereas it was 2.0 ± 0.13 μmol g DW^{-1} when dormant seeds were imbibed on water (data not shown). Figure 6 shows that this compound was very effective in promoting dormancy release in sunflower seeds, lending further support to the hypothesis that dormancy release is associated with the generation of ROS. Remarkably, not only did this compound break dormancy, but this also entailed the same specific pattern of protein oxidation as seen with hydrogen cyanide (blue circle in Figure 7). Oxyblots also revealed that imbibition of dormant and non-dormant embryos on water

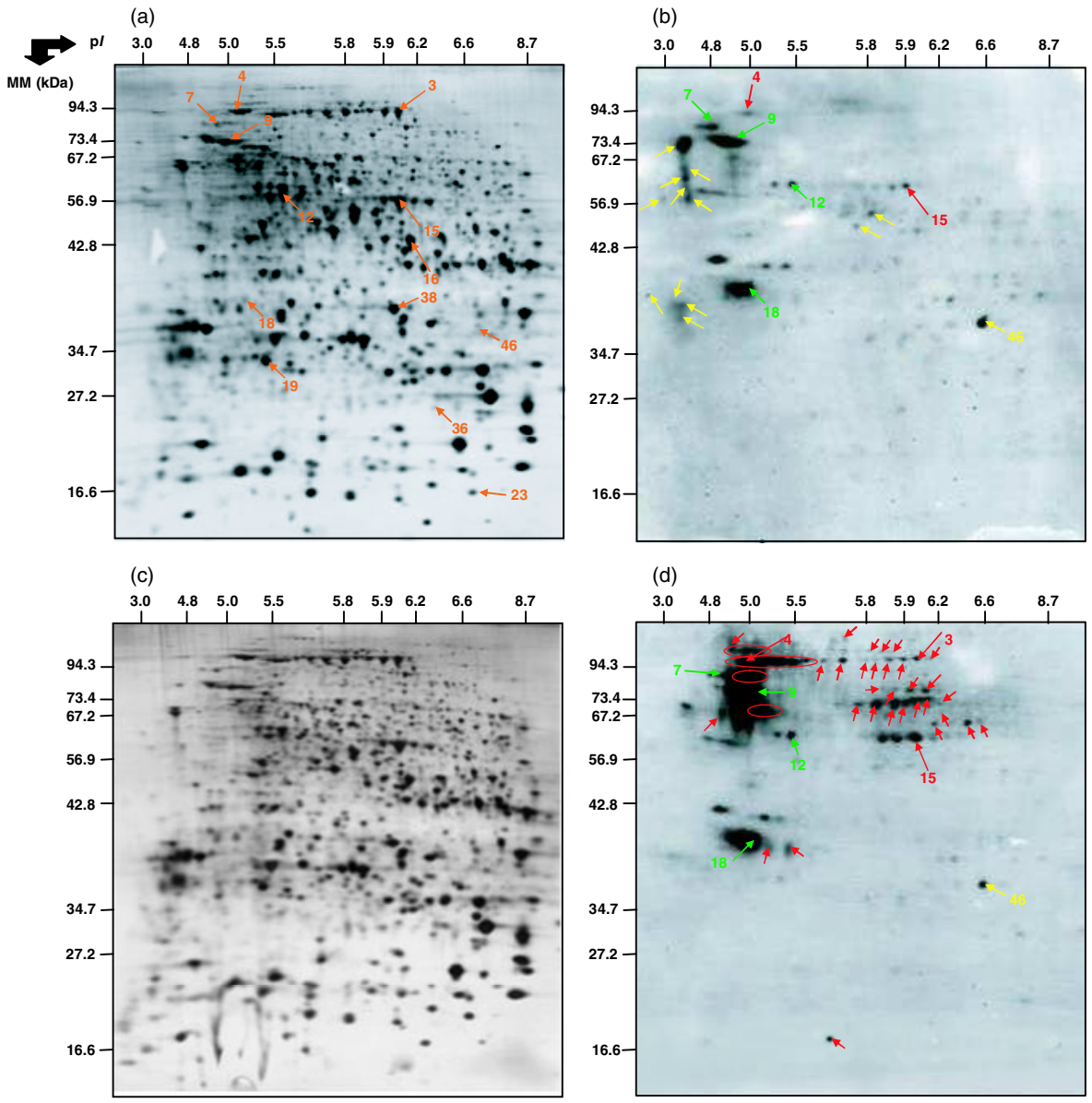


Figure 5. Two-dimensional profiles of protein abundance and oxidation in axes of dry dormant and non-dormant sunflower seeds.
(a, b) Dormant axes; (c, d) non-dormant axes.
Protein stains (a, c) and anti-DNP immunoassays (b, d) are shown. Proteins undergoing increased carbonylation during after-ripening are labeled with red arrows, those for which the carbonylation level remained constant during after-ripening are labeled with green arrows, and those for which the carbonylation level decreased during after-ripening are labeled by yellow arrows. Numbers indicated on the arrows correspond to the proteins that have been identified by mass spectrometry (listed in Table 3).

was associated with an increase in protein carbonylation (compare Figures 5 and 7). However, the level of protein oxidation was higher in non-dormant axes, which displayed many new oxidized proteins ranging from approximately 15–40 kDa (Figure 7b). Conversely, around 20 proteins that were found to be carbonylated during imbibition in dormant axes were not detected in non-dormant axes (Figure 7a, yellow circles). Carbonylated proteins identified by mass spectroscopy are listed in Table 4. EF2 (spot number 3), PPDK (spot number 4), 81 and 70 kDa HSP (spot numbers 7 and 9), enolase (spot number 12) and 7S globulin (spot number 15), previously identified in dry-after ripened seeds, were also carbonylated in the axes of all imbibed seeds (Figure 7, Tables 3 and 4). The globulin-like protein (spot

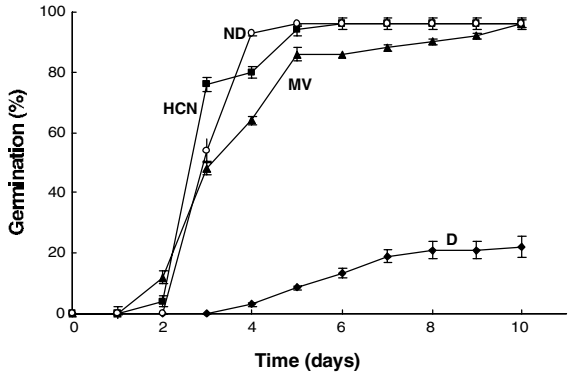


Figure 6. Effect of a 3 h treatment by 1 mM hydrogen cyanide (HCN) and 0.1 mM methylviologen (MV) on the germination of dormant sunflower embryos (D) at 10°C. ND, non-dormant embryos. Means of four replicates ± SD.

number 18) was also always carbonylated in imbibed axes, but the level of oxidation appeared to be stronger in non-dormant axes isolated from seeds imbibed on water (Figure 7b). Treatment of imbibed dormant seeds with cyanide and MV also resulted in the carbonylation of proteins not detected in dry after-ripened seeds. For example, proteins that show homology with 11S storage protein (spot number 36 in Figure 7) and epoxide hydrolase (spot number 38 in Figure 7) were detected in dormant seeds but did not appear in the axes of non-dormant seeds treated with cyanide and MV. Other proteins sharing homology with an 11S globulin precursor, an alcohol dehydrogenase and a basic 2S albumin (spot numbers 19, 16 and 23, respectively, in Figure 7) were oxidized in axes of all seeds able to germinate. Spot number 21 was detected only in imbibed non-dormant seeds (Figure 7b,d,f), and this protein was found to be similar to dehydrin (Table 4).

Discussion

Dormancy is a characteristic feature of sunflower seeds at their harvest. This dormancy exhibits both seed coat and embryo components, and progressively appears during seed development on the mother plant (Corbineau *et al.*, 1990). The seed-coat-imposed dormancy acts through the effects of phenolics on oxygen availability for the embryo, and is therefore expressed to a greater extent at high temperatures, while the embryo-imposed dormancy is likely to be involved in the restriction of germination at temperatures below 15°C (Corbineau *et al.*, 1990; Gay *et al.*, 1991). In agreement, dormant naked seeds assayed immediately after harvest only germinated very poorly at 10°C (Figure 1a), and after-ripening in dry conditions (20°C, 60% RH, 2 months) resulted in a widening of the temperatures permitting germination (Figure 1b).

Our results show that after-ripening is associated with an accumulation of superoxide anions and hydrogen peroxide in the embryonic axes (Table 1, Figures 2 and 3). $O_2^{\cdot-}$ appeared as formazan precipitates within the cells (Figure 2), and ultrastructural studies showed that hydrogen peroxide accumulated in the cytoplasm of embryonic axis cells (Figure 3). By storing the seeds at various RH, it was possible to modulate the extent of dormancy release (Table 2). This suggests that the water status within the embryo cell is likely to play a critical role in the process of after-ripening, and shows that hydrogen peroxide accumulation is tightly associated with the breaking of embryo dormancy as this compound only accumulated under conditions associated with dormancy release. Thus a causal link between ROS production, or at least H_2O_2 accumulation, and after-ripening is likely to exist, and this process is not just related to seed storage. This demonstrates that, in sunflower seeds, ROS production in the dry state is initiated after harvest, and that, in agreement with previous proposals that ROS can act as cell messengers (reviewed in Bailly, 2004), these molecules could therefore act as a signal to allow dormancy release and favor subsequent seed germination. However, it is also likely that prolonged storage of seeds in the dry state would be associated with a sustained production of ROS, which would lead to oxidative stress and to the related deteriorative events known to occur during seed aging (Bailly, 2004).

In plants, and more especially in seeds, ROS may originate from the mitochondrial respiratory chain or be produced through the action of enzymes such as NADPH oxidase (Bailly, 2004). However, in dry tissues, such as in mature orthodox seeds, respiration is prevented and enzymes are presumably not active, which suggests the occurrence of other mechanisms for ROS production. At low moisture content, 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). Enzymatic oxidation of lipids through lipoxygenase is also possible when water activity is as low as 0.4 (Drapron, 1985). Using model systems, such as those based on oil encapsulation in a glassy matrix, it has been demonstrated that glasses do not prevent oxygen diffusion, thus allowing autoxidation reactions of lipids (Andersen and Skibsted, 2002; Andersen *et al.*, 2000; Nelson and Labuza, 1992). As the MDA content increased during dormancy alleviation (Table 1), we assume that after-ripening is associated with lipid peroxidation, which could be a consequence of ROS accumulation and may, in turn, be involved in free radical formation.

To test the hypothesis that ROS accumulation during after-ripening is involved in dormancy alleviation, and to assign a possible mechanism whereby this might occur, we characterized the oxidized proteome of embryonic axes from dormant and non-dormant seeds. Protein carbonyla-

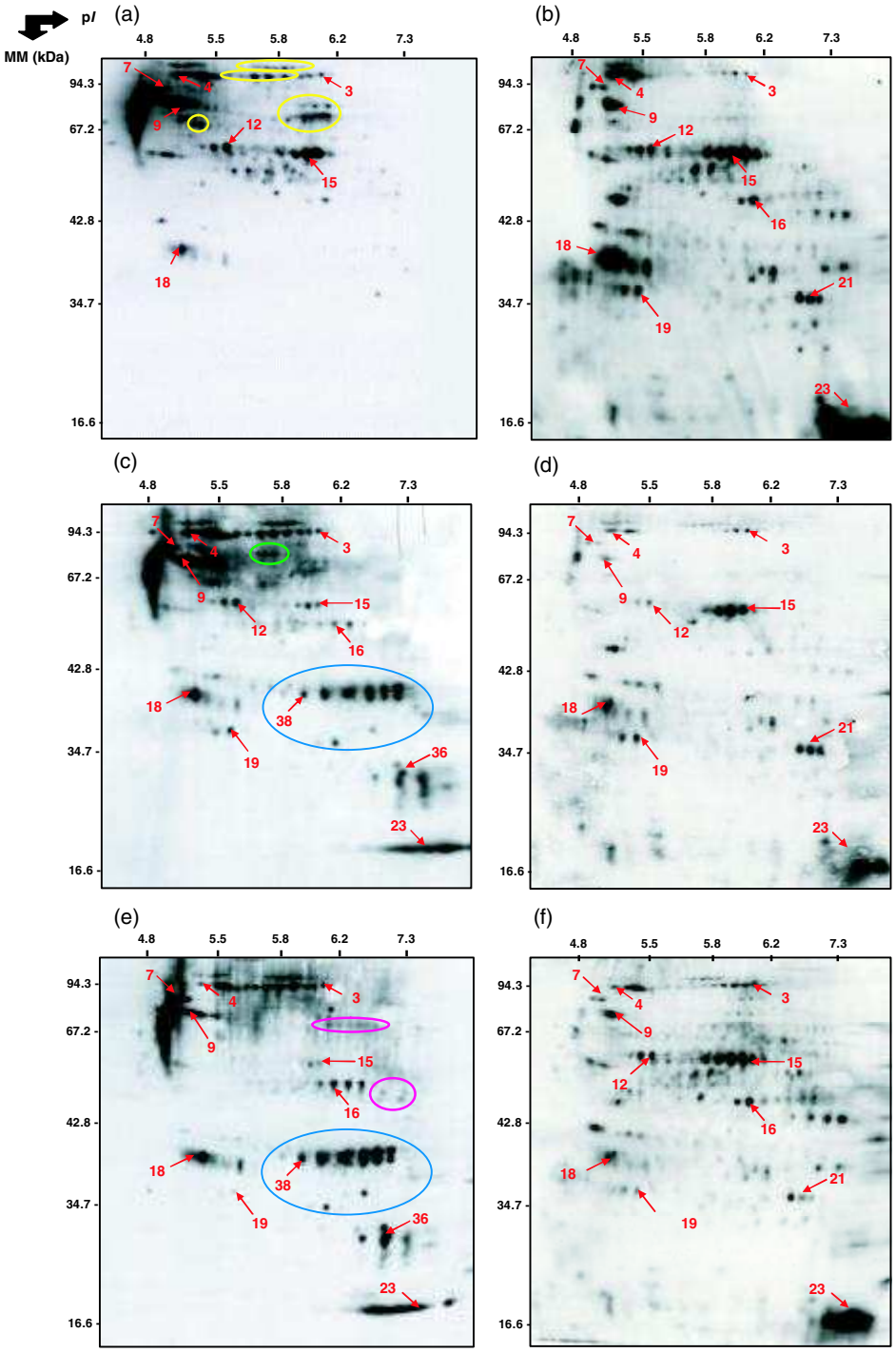


Figure 7. Two-dimensional profiles of protein oxidation in axes of dormant and non-dormant sunflower seeds after 3 h imbibition at 10°C under various conditions. (a) Dormant axes and (b) non-dormant axes imbibed on water; (c) dormant axes and (d) non-dormant axes imbibed in the presence of 1 mM HCN; (e) dormant axes and (f) non-dormant axes imbibed in the presence of 0.1 mM methylviologen.
Numbers indicated on the arrows correspond to proteins that have been identified by mass spectrometry (see Tables 3 and 4). Yellow circles, proteins carbonylated in dormant axes but not in non-dormant axes during imbibition on water. Blue circles, proteins specifically carbonylated in the presence of hydrogen cyanide or methylviologen. Green and purple circles, proteins specifically carbonylated in the presence of cyanide or methylviologen, respectively, during imbibition of dormant axes.

Table 4 Identification of carbonylated proteins in embryo axes of dormant and non-dormant sunflower seeds imbibed for 3 h on water (W) or in the presence of hydrogen cyanide (CN) or methylviologen (MV) at 10°C

					Carbonylation					
Spot number	Accession	Protein name	MW (kDa)	pI	D W	D CN	D MV	ND W	ND CN	ND MV
3	See Table 3	Elongation factor EF-2	93.9	6.1	+	+	+	+	+	+
4	See Table 3	Pyruvate, phosphate dikinase, chloroplast precursor	93.9	5.1	+	+	+	+	+	+
7	See Table 3	81 kDa heat shock protein	84.0	4.9	+	+	+	+	+	+
9	See Table 3	HSP70	73.4	5.0	+	+	+	+	+	+
12	See Table 3	Enolase	60.0	5.5	+	+	+	+	+	+
15	See Table 3	7S globulin	60.0	6.9	+	+	+	+	+	+
16	gi|71793966	alcohol dehydrogenase (*Alnus glutinosa*)	42.8	6.2	–	+	+	+	+	+
18	See Table 3	Globulin-like protein	38.0	5.2	+	+	+	++	+	+
19	gi|22459294	QHF12M17.yg.ab1 QH_EFGHJ sunflower RHA280 *Helianthus annuus* cDNA clone QHF12M17, mRNA sequence ≥gi|81238594|gb|ABB60055.1| 11S globulin precursor isoform 4 (*Sesamum indicum*)	34.0	5.5	–	+	+	+	+	+
21	gi|22462073	QHG17D11.yg.ab1 QH_EFGHJ sunflower RHA280 *Helianthus annuus* cDNA clone QHG17D11, mRNA sequence ≥gi|27526460|emb|CAC80712.1| putative dehydrin (*Helianthus petiolaris*)	36.0	6.5	–	–	–	+	+	+
23	gi|27526481	Basic 2S albumin (*Helianthus annuus*)	16.6	7.0	–	+	+	+	+	+
36	gi|22310301	QHA17G24.yg.ab1 QH_ABCDI sunflower RHA801 *Helianthus annuus* cDNA clone QHA17G24, mRNA sequence ≥gi|4127629|emb|CAA76572.1| 11S storage protein (*Coffea arabica*)	21.0	7.3	–	+	+	–	–	–
38	gi|22393902	QHI21H20.yg.ab1 QH_ABCDI sunflower RHA801 *Helianthus annuus* cDNA clone QHI21H20, mRNA sequence ≥gi|5302785|emb|CAB46034.1| putative epoxide hydrolase (*Arabidopsis thaliana*)	40.0	5.8	–	+	+	–	–	–

D, dormant seeds; ND, non-dormant seeds. Peptide sequences were identified by MS-MS sequencing; see Table 3 for characteristics of spots 3, 4, 7, 9, 12, 15 and 18, and Table S1 for the peptide sequences. Experimental molecular weight (MW) and pI are indicated.

tion results from oxidative attack on Arg, Lys, Pro or Thr residues of proteins, which can affect enzyme activities or alter susceptibility of the modified proteins to proteolysis (Berlett and Stadtman, 1997; Davies, 2005; Dunlop *et al.*, 2002). Our present data clearly indicate the occurrence of carbonylation of specific embryonic proteins during after-ripening of sunflower seeds (Figures 4 and 5). An alternative possibility to account for our results could be a redistribution of carbonylated proteins in the various protein fractions (i.e. soluble and non-soluble proteins) because of cell structural changes occurring during after-ripening that would render the carbonylated proteins more soluble from the non-dormant axes than from the corresponding dormant ones. However, the fact that no marked ultrastructural changes occurred during after-ripening (Figure 3a,b) and that the soluble proteomes revealed by silver nitrate staining were very similar for the dormant and non-dormant axes does not favor this idea (Figure 5). We suggest 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 (Table 1), which is known to react with lysine residues to form carbonyl derivatives (Burcham and Kuhan, 1996; Liu and Wang, 2005). In animals, protein carbonylation has been widely used as a measurement of oxidative damage, and it has been shown to increase in aging tissues (Dalle-Donne *et al.*, 2003; Ding *et al.*, 2006; Nyström, 2005). In marked contrast, protein carbonylation may not be an inevitable consequence of tissue aging in plants. Thus, in Arabidopsis, patterns of protein carbonylation vary widely during

progression of the life cycle, and the extent of protein carbonylation drops abruptly prior to the vegetative to reproductive transition (Johansson *et al.*, 2004). Carbonylation of numerous proteins also occurs during Arabidopsis seed germination, although the germinated seeds gave rise to vigorous plantlets (Job *et al.*, 2005).

The present data document the effectiveness of both hydrogen cyanide and methylviologen in breaking sunflower seed dormancy (Figure 6). Hydrogen cyanide has already been shown to alleviate seed dormancy in several species, including Arabidopsis (Bethke *et al.*, 2006; Bogatek *et al.*, 1991; Côme *et al.*, 1988; Esashi *et al.*, 1991; Taylorson and Hendricks, 1973). Methylviologen, on the other hand, is a compound known to generate oxidative stress in plants in the light through the photosynthetic electron transport chain (Xiong *et al.*, 2007), as well as in the dark, but by an unknown mechanism (Slooten *et al.*, 1995). However, its utilization as a compound that breaks seed dormancy has not been reported to date, and the advantage of using methylviologen in our study is that it supplies evidence that ROS probably play a fundamental role in seed dormancy release.

The oxyblots presented in Figure 7 clearly show that carbonylation of specific proteins occurred upon treating the dormant sunflower seeds with either hydrogen cyanide or methylviologen (e.g. proteins shown in the blue circles in Figure 7). This specific carbonylation pattern was not seen by incubating dormant seeds in water only (Figure 7a), although this latter incubation also resulted in an increase in protein carbonylation compared with the dry seeds (compare Figures 5b and 7a). It is also interesting that the pattern of protein carbonylation is similar in all of the non-dormant axes (Figure 7), regardless of the treatment. This has significance in the present experiments and in interpreting the data as they provide treatment controls illustrating the reproducibly of the technique and the biological behavior. In summary, seed dormancy release during dry storage and imbibition in the presence of hydrogen cyanide or methylviologen was associated with the appearance of specific carbonylation patterns, although these patterns differed when dormancy was broken in dry and in imbibed state conditions (compare Figures 5 and 7). It would nevertheless be interesting to further characterize the specific features of the carbonylated proteome during breaking of dormancy by carrying out time-course experiments during the after-ripening process. The similarity of the effects of HCN and MV on the oxidized proteome also suggests that these compounds share a common mechanism for triggering dormancy release in imbibed dormant seeds.

The role of protein oxidation in dormancy alleviation could be discussed in relation to the nature of the carbonylated proteins presently identified. EF2, PPDK and 7S globulin (spot numbers 3, 4 and 15, respectively, in Figure 5, Table 2 and Table S1) exhibited an increased carbonylation level during after-ripening. EF2 catalyzes peptidyl-tRNA translocation of the ribosome during the elongation phase of protein translation. Its level is known to decrease dramatically during wheat seed development, particularly during the desiccation phase (Gallie *et al.*, 1998). Carbonylation of EF2 during storage in the dry state after completion of seed development might therefore terminate protein synthesis that is associated strictly with developmental processes. PPDK plays a role in photosynthesis in C4 plants but its function in C3 plants is not fully elucidated. Interestingly, this enzyme appears to be absent from many dicot seeds, including Arabidopsis, with the remarkable exception of sunflower seeds (Chastain *et al.*, 2006). As PPDK is presumed to be involved in seed development rather than in seed germination (Chastain *et al.*, 2006), its carbonylation during after-ripening could provide a means to down-regulate some residual enzyme activity. In this context, it has been shown in rice that, as seed development progresses towards late maturation, the enzyme undergoes post-translational down-regulation in terms of activity and amount via regulatory phosphorylation (PPDK inactivation) and protein degradation (Chastain *et al.*, 2006). Carbonylation of storage proteins has previously been reported in dry mature Arabidopsis seeds, and it was suggested that carbonylation of these proteins facilitates their mobilization during germination (Job *et al.*, 2005). Thus, in sunflower seeds, breaking of dormancy in the dry state may be associated with preparation for storage protein mobilization.

The carbonylation level of some proteins decreased during after-ripening. For example, this was the case for a 20S proteasome α-subunit (spot number 46; Figure 5, Table 2 and Table S1). This observation is consistent with data showing a requirement of for proteasome activity in sunflower embryos for both the breaking of dormancy by ethylene and the progression of germination (Borghetti *et al.*, 2002).

Proteins that were similarly oxidized in dry and imbibed dormant and non-dormant sunflower axes were also characterized. Among them, HSP 70 and 81 (spot numbers 7 and 9, Figures 5 and 7, Tables 3 and 4 and Table S1) and enolase (spot number 12, Figures 5 and 7, Tables 3 and 4 and Table S1) were found. Molecular chaperones are known to be targets of carbonylation in yeast and bacteria challenged by oxidative stress (Cabiscol *et al.*, 2000; Tamarit *et al.*, 1998). Thus, it is possible that carbonylation of these HSPs in both dormant and non-dormant sunflower seeds reflects the occurrence of an oxidative stress during the desiccation phase of seed development. It has been suggested that the protective role these proteins play is as shields protecting other proteins against ROS damage (Cabiscol *et al.*, 2000). Enolase catalyzes the dehydration of 2-phosphoglycerate to PEP and is therefore the second enzyme involved in metabolism, after PPDK, that has been found to be oxidized in sunflower axes. This result is consistent with the observation that enolase is one of the most prominent carbonylated proteins in dry mature Arabidopsis seeds (Job *et al.*, 2005).

The use of hydrogen cyanide or methylviologen also allowed identification of carbonylated proteins that were specifically associated with breaking of dormancy in the imbibed state. This was the case for alcohol dehydrogenase (ADH, spot number 16, Figure 7, Table 4 and Table S1), which was carbonylated in all axes of seeds undergoing germination. Consistent with this, the beneficial effect of alcohol on breaking the dormancy of seeds (Cohn *et al.*, 1989; Corbineau *et al.*, 1991) or buds (Claassens *et al.*, 2005) is well documented. Two identified proteins, globulin precursor isoform 4 (spot number 19 Figure 7, Table 4 and Table S1) and a basic 2S albumin (spot number 23, Figure 7, Table 4 and Table S1) exhibited the same pattern of oxidation as ADH, supporting the finding that germination requires the oxidation of specific reserve proteins. A putative epoxide hydrolase was found to be carbonylated in dormant axes treated by hydrogen cyanide and methylviologen (spot number 38, Figure 7, Table 4 and Table S1). Epoxide hydrolases catalyze the conversion of epoxides to diols. The known functions of such enzymes include detoxification of xenobiotics, drug metabolism, synthesis of signaling compounds, intermediary metabolism and responses to oxidative stress (Newman *et al.*, 2005). In plants, epoxide hydrolases are thought to participate in general defense systems (Newman *et al.*, 2005). Therefore, the specific carbonylation of this protein is consistent with the occurrence of oxidative stress during dormancy alleviation.

In conclusion, taken together, our results allow us to propose a novel mechanism for seed dormancy release. This mechanism involves a change in proteome oxidation, resulting from an accumulation of ROS during after-ripening. Hence, ROS accumulation appears to be a key signal governing cell activity during after-ripening. Furthermore, this hypothesis may provide a more general model to account for breaking of seed dormancy in view of the similar results obtained during breaking of dormancy in the dry state and the imbibed state.

Experimental procedures

Plant material

Two batches of sunflower (*Helianthus annuus* L., cv LG5665) seeds, harvested in 2004 and 2005 near Montélimar (Drôme, France) and purchased from Limagrain (www.limagrain.com), were used in this study. Seeds were dormant at harvest and their moisture content was approximately 4% on a fresh weight (FW) basis. After-ripening was performed by placing the seeds after harvest at 25°C and 60% relative humidity (RH) for 2 months (Corbineau *et al.*, 1990). Seeds were also placed at 25°C over saturated solutions of NaCl and $ZnCl_2$ in tightly closed jars, giving RH of 75\% and 5%, respectively (Vertucci and Roos, 1993). All the results presented in this study represent means of the data obtained from seeds harvested in both 2004 and 2005.

Germination tests

Germination assays were performed with naked seeds (i.e. without pericarp) in darkness in 9 cm Petri dishes (25 seeds per dish, eight replicates) placed on a layer of cotton wool moistened with deionized water. A seed was considered as germinated when the radicle had elongated to 2–3 mm. Germination counts were made daily for 10 days.

Cyanide and methylviologen treatments

The treatment of sunflower embryos by gaseous 1 mM HCN was carried out as described by Bogatek *et al.* (1991). Naked dry seeds were placed in a tightly closed glass jar (500 ml volume) on a layer of sterile cotton wool moistened with deionized water (50 seeds per jar). A glass tube containing 5 ml of 0.1 M KCN solution placed in the jar was used as a source of gaseous HCN, which was produced by acidifying the KCN solution with 5 ml of lactic acid (10% v/v). After 3 h of treatment in darkness at 10°C, the jars were opened and gaseous cyanide released, and the seeds were rinsed carefully three times with deionized water before germination tests or biochemical analyses. Treatment by methylviologen was carried out by placing naked seeds in darkness at 10°C on a cotton wool moistened with a solution of 0.1 mM MV for 3 h. Embryos were also rinsed with deionized water after treatment.

Determination of superoxide anion content

Superoxide anion content was determined according to the method developed by Elstner and Heupel (1976). Axes (0.2 g FW) were ground in 4 ml of sodium phosphate buffer (pH 7.8, 50 mM) at 4°C. The extracts were centrifuged at 16 000 ***g*** for 15 min, and the resulting supernatants were used for $O_2^{\cdot-}$ determination. The supernatant (1 ml) was first incubated at 25°C for 30 min in the presence of 1 mM hydroxylamine hydrochloride in 50 mM sodium phosphate buffer (pH 7.8). Then, 0.5 ml of this reaction mixture was incubated with 0.5 ml of 17 mM sulfanilamide and 0.5 ml of 7 mM 2-naphtylamine at 25°C for 30 min. The absorbance was measured at 540 nm after centrifugation at 13 000 ***g*** for 10 min. A calibration curve was established using sodium nitrite. The results are expressed as $\mu mol\ g^{-1}$ DW of seeds and correspond to the means of measurements carried out on five extracts ± SD.

In situ *localization of superoxide anion*

Whole axes and hand-cut sections of axes were incubated in 6 mM nitroblue tetrazolium (NBT) in 10 mM Tris-HCl buffer (pH 7.4) at room temperature for 30 min. Superoxide anion was visualized as deposits of dark-blue insoluble formazan compounds (Beyer and Fridovich, 1987).

Determination of hydrogen peroxide content

The H_2O_2 content of excised axes was determined according to the method described by O'Kane *et al.* (1996). Axes (0.5 g FW) were ground in a mortar and homogenized with 5 ml of 0.2 M perchloric acid. After 15 min of centrifugation at 13 000 ***g*** at 4°C, the resulting supernatant was neutralized to pH 7.5 with 4 M KOH and then centrifuged at 1000 ***g*** for 3 min at the same temperature. The supernatant was immediately used for spectrophotometric determination of H_2O_2 at 590 nm using a peroxidase-based assay. The reaction mixture contained 12 mM 3-dimethylaminobenzoic acid in 0.375 M

phosphate buffer (pH 6.5), 1.3 mM 3-methyl-2-benzothiazolidone hydrazone, 20 μl (0.25 U) horseradish peroxidase (Sigma; http://www.sigmaaldrich.com/) and 50 μl of the collected supernatant to a total volume of 1.5 ml. The reaction was started by the addition of peroxidase. Increase in absorbance at 590 nm was monitored after 5 min at 25°C and compared with the absorbance obtained with known amounts of H_2O_2. The results are expressed as nmol H_2O_2 g^{-1} DW and correspond to means of the values obtained with five different extracts ± SD.

Subcellular localization of hydrogen peroxide

The localization of H_2O_2 was determined by $CeCl_3$ staining as described by Bestwick *et al.* (1997). Sections of axes (approximately 5 mm^3) were imbibed in 50 mM MOPS buffer (pH 7.2) containing 5 mM $CeCl_3$ under vacuum until they were fully infiltrated. $CeCl_3$-treated sections and control sections (without staining) were fixed in 6% glutaraldehyde in 25 mM sodium phosphate buffer (pH 7.2) for 24 h at room temperature, washed in the same buffer and post-fixed in 1% osmium tetroxide in 25 mM sodium phosphate buffer (pH 7.2) for 18 h at room temperature. After several washes in double-distilled water, the tissues were progressively dehydrated in ethanol, then soaked in propylene oxide and embedded in Araldite (Sigma). The blocks were sectioned with glass knives at 120 nm using a Reichert Ultractu S (Leica, www.leica-microsystems.com), stained with lead citrate and 2% uranyl acetate (Reynolds, 1963), and viewed with a LEO912 transmission electron microscope (Leo Electron Microscopy, www.stm.zeiss.com).

Malondialdehyde measurements

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) content from 0.5 g FW of embryonic axes, according to the method of Heath and Parker (1968). The results are expressed as μmol g^{-1} DW of seeds and correspond to means of measurements carried out with five extracts ± SD.

Preparation of protein extracts

Axes from dry dormant and non-dormant seeds (140 mg FW, corresponding approximately to 30 axes) were ground in liquid nitrogen using a mortar and pestle. Soluble proteins were extracted from the resulting powder at 4°C in 1.0 ml of a buffer containing 10 mM HEPES, 1 mM EDTA, the protease inhibitor cocktail 'complete Mini' from Roche Molecular Biochemicals, 60 U DNAse I (Roche Diagnostics, www.roche-applied-science.com), and 6 U RNAse A (Sigma, www.sigmaaldrich.com). After 10 min at 4°C, 20 mM dithiothreitol (DTT) was added, and the protein extracts were stirred for 20 min at 4°C and then centrifuged (20 000 ***g***, 15 min) at 4°C. The final supernatant corresponded to the soluble protein extract. Protein concentrations in the various extracts were measured according to the method described by Bradford (1976) using a Bio-Rad assay kit (http://www.bio-rad.com/). Bovine serum albumin was used as a standard.

One- and two-dimensional electrophoresis

One-dimensional SDS–PAGE of seed protein extracts (5 μg protein) was performed using 12% w/v polyacrylamide resolving gels, as described by Laemmli (1970).

Proteins were also analyzed by two-dimensional gel electrophoresis as described previously (Görg *et al.*, 1987; Job *et al.*, 2005). Isoelectrofocusing (100 μg protein) was carried out using gel strips forming an immobilized non-linear pH gradient from 3 to 10 (Immobiline DryStrip, pH 3–10 NL, 18 cm; Amersham Pharmacia Biotech; http://www5.amershambiosciences.com/). Strips were rehydrated for 14 h at 22°C with the thiourea/urea lysis buffer as described previously (Harder *et al.*, 1999), containing 2% v/v Triton X-100, 20 mM dithiothreitol and the protein extracts. Isoelectrofocusing was performed at 22°C in the Multiphor II system (Amersham Pharmacia Biotech) for 1 h at 300 V and 7 h at 3500 V. Then, the gel strips were equilibrated for 2 × 20 min in 2x 100 ml of equilibration solution containing 6 M urea, 30% v/v glycerol, 2.5% w/v SDS, 0.15 M bis-Tris and 0.1 M HCl (Görg *et al.*, 1987; Harder *et al.*, 1999). DTT (50 mM) was added to the first equilibration solution, and iodoacetamide (4% w/v) was added to the second (Harder *et al.*, 1999). Separation in the second dimension was carried out in polyacrylamide gels (10% w/v acrylamide, 0.33% w/v piperazidine diacrylamide, 0.18 M Trizma base, 0.166 M HCl, 0.07% w/v ammonium persulfate and 0.035% v/v Temed). Electrophoresis was performed at 10°C in a buffer (pH 8.3) containing 25 mM Trizma base, 200 mM taurine and 0.1% w/v SDS for 1 h at 35 V and for 14 h at 110 V. Ten gels (200 × 250 × 1.0 mm) were run in parallel (Isodalt system; Amersham Pharmacia Biotech). For each treatment analyzed, 2D gels were run in triplicate.

One-dimensional gels were stained with the GelCode blue stain from Pierce (www.piercenet.com). Two-dimensional gels were stained with silver nitrate according to the methods described by Blum *et al.* (1987) for densitometric analyses or Shevchenko *et al.* (1996) for mass spectrometry analyses. Stained gels were scanned with a UMAX Powerlook III scanner equipped with MagicScan version 4.5 from UMAX Data Systems (Amersham Biosciences, www.amershambiosciences.com).

Detection of carbonylated proteins and Western blotting

The appearance of carbonyl groups in proteins was analyzed by immunodetection of 2,4-dinitrophenylhydrazone (DNP)-derivatized protein as described previously (Job *et al.*, 2005; Korolainen *et al.*, 2002). SDS was added to the protein extract (100 μl, 10 μg $μl^{-1}$) to a final concentration of 0.8%. Following dialysis, four volumes of 10 mM DNPH (Sigma)/2 M HCl were added. Samples were agitated for 30 min at room temperature, and five volumes of 20/80 ice-cold TCA/acetone containing 1 mM DTT were added to each sample. The samples were centrifuged for 15 min at 15 000 ***g*** at 4°C. The precipitated protein was then washed three times with ice-cold acetone containing 1 mM DTT, then with 1 ml of 1:1 v/v ethanol:ethyl acetate, and resolubilized in the thiourea/urea lysis buffer containing 2% v/v Triton X-100 and 20 mM DTT. Proteins were separated by 1D or 2D SDS–PAGE as described above, and transferred to nitrocellulose sheets (Bio-Rad) using standard procedures. Carbonylated proteins were revealed by incubation with rabbit anti-DNP antibodies (Chemicon, www.chemicon.com) followed by incubation with anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Sigma) and detection with the ECL kit (Roche Diagnostics) (Job *et al.*, 2005). Relative protein carbonyl levels were quantitated by densitometric analyses of the blots as described above.

Protein identification by mass spectrometry

Bands and spots of interest were excised from 1D and 2D SDS–PAGE gels using sterile tips and placed in 1.5 ml sterile tubes. Each

polyacrylamide piece was rinsed with water, then reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and incubated overnight at 37°C with 12.5 ng μl^{-1} trypsin (sequencing grade; Roche Diagnostics) in 25 mM NH_4HCO_3. The tryptic fragments were extracted, dried, reconstituted with 2% v/v acetonitrile, 0.1% formic acid and sonicated for 10 min. Analysis of tryptic peptides by tandem mass spectrometry was performed on a nanoelectrospray ionization quadrupole time-of-flight hybrid mass spectrometer (Q-TOF Ultima Global; Waters Micromass, www.waters.com) coupled with a nano-HPLC (Cap-LC; Waters Micromass). The samples were loaded and desalted on a C18 pre-column (LC-Packings PepMap C18, 5 μm, 100 Å, 300 μm × 5 mm; Dionex Corp., www.dionex.com) at a flow rate of 20 $\mu l\ min^{-1}$ isocratically with 0.1% formic acid. The peptides were separated on a C18 column (Atlantis dC18, 3 μm, 75 μm × 150 mm Nano Ease; Waters). After washing with solvent A (water/acetonitrile 98/2 v/v, 0.1% formic acid), a linear gradient from 5% to 60% of solvent B (water/acetonitrile 20/80 v/v, 0.1% formic acid) was developed over 80 min at a flow rate of 180 $nl\ min^{-1}$. The Q-TOF spectrometer was operated in data-dependent analysis mode using a 1 sec mass spectrometry (MS) survey scan on three different precursor ions. The peptide masses and sequences obtained were either matched automatically to proteins in a non-redundant database National Center for Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov using the Mascot MS/MS ions search algorithm (http://www.matrixscience.com) or manual BLAST searches were performed against the current databases. (NCBI, Swiss-Prot, http://expasy.org/sprot/)

Supplementary material

The following supplementary material is available for this article online:

Table S1 Identification of carbonylated proteins in embryo axes of dormant and non-dormant sunflower seeds.

This material is available as part of the online article from http://www.blackwell-synergy.com.

References

Andersen, M.L. and Skibsted, L.H. (2002) Detection of early events in lipid oxidation by electron spin resonance spectroscopy. *Eur. J. Lipid Sci. Technol.* **104**, 65–68.

Andersen, A.B., Risbo, J., Andersen, M.L. and Skibsted, L.H. (2000) Oxygen permeation through an oil-encapsulating glassy food matrix studied by ESR line broadening using a nitroxyl spin probe. *Food Chem.* **70**, 499–508.

Bailly, C. (2004) Active oxygen species and antioxidants in seed biology. *Seed Sci. Res.* **14**, 93–107.

Baskin, J.M. and Baskin, C.C. (1998) *Seeds: Ecology, Biogeography and Evolution of Dormancy and Germination*. San Diego, CA: Academic Press.

Berlett, B.S. and Stadtman, E.R. (1997) Protein oxidation in aging, disease and oxidative stress. *J. Biol. Chem.* **272**, 20313–20316.

Bestwick, C.S., Brown, I.R., Benneth, M.H.R. and Mansfield, J.W. (1997) Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola*. *Plant Cell*, **9**, 209–221.

Bethke, P.C., Libourel, I.G., Reinohl, V. and Jones, R.L. (2006) Sodium nitroprusside, cyanide, nitrite, and nitrate break Arabidopsis seed dormancy in a nitric oxide-dependent manner. *Planta*, **223**, 805–812.

Bewley, J.D. (1997) Seed germination and dormancy. *Plant Cell*, **9**, 1055–1066.

Bewley, J.D. and Black, M. (1994) *Seeds. Physiology of Development and Germination*, 2nd edn. New York: Plenum Press.

Beyer, W.F. and Fridovich, I. (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* **161**, 559–566.

Blum, H., Beier, H. and Gross, H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, **8**, 93–99.

Bogatek, R., Dziewanowska, K. and Lewak, S. (1991) Hydrogen cyanide and embryonal dormancy in apple seeds. *Physiol. Plant.* **83**, 417–421.

Borghetti, F., Nakamura Noda, F. and Martins de Sá, C. (2002) Possible involvement of proteasome activity in ethylene-induced germination of dormant sunflower embryos. *Braz. J. Plant Physiol.* **14**, 125–131.

Bove, J., Lucas, P., Godin, B., Oge, L., Jullien, M. and Grappin, P. (2005) Gene expression analysis by cDNA-AFLP highlights a set of new signaling networks and translational control during seed dormancy breaking in *Nicotiana plumbaginifolia*. *Plant Mol. Biol.* **57**, 593–612.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254.

Bucharov, P. and Gantcheff, T. (1984) Influence of accelerated and natural aging on free radical levels in soybean seeds. *Physiol. Plant.* **60**, 53–56.

Burcham, P.C. and Kuhan, Y.T. (1996) Introduction of Carbonyl groups into proteins by the lipid peroxidation product, malondialdehyde. *Biochem. Biophys. Res. Commun* **220**, 996–1001.

Cabiscol, E., Piulats, E., Echave, P., Herrero, E. and Ros, J. (2000) Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275**, 27393–27398.

Cadman, C.S.C., Toorop, P.E., Hilhorst, H.W.M. and Finch-Savage, W.E. (2006) Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant J.* **46**, 805–822.

Chastain, C., Heck, J.W., Colquhoun, T.A., Voge, D.G. and Gu, X.Y. (2006) Posttranslational regulation of pyruvate, orthophosphate dikinase in developing rice (*Oryza sativa*) seeds. *Planta*, **224**, 924–934.

Chibani, K., Ali-Rachedi, S., Job, C., Job, D., Jullien, M. and Grappin, P. (2006) Proteomic analysis of seed dormancy in Arabidopsis. *Plant Physiol.* **142**, 1493–1510.

Claassens, M.M., Verhees, J., van der Plas, L.H., van der Krol, A.R. and Vreugdenhil, D. (2005) Ethanol breaks dormancy of the potato tuber apical bud. *J. Exp. Bot.* **56**, 2515–2525.

Cohn, M.A., Jones, K.L., Chiles, L.A. and Church, D.F. (1989) Seed dormancy in red rice. 7. Structure–activity studies of germination stimulants. *Plant Physiol.* **89**, 879–882.

Côme, D., Corbineau, F. and Lecat, S. (1988) Some aspects of metabolic regulation of cereal seed germination and dormancy. *Seed Sci. Technol.* **16**, 175–186.

Corbineau, F., Bagniol, S. and Côme, D. (1990) Sunflower (*Helianthus annuus* L.) seed dormancy and its regulation by ethylene. *Isr. J. Bot.* **39**, 313–325.

Corbineau, F., Gouble, B., Lecat, S. and Côme, D. (1991) Stimulation of germination of dormant oat (*Avena sativa* L.) seeds by ethanol and other alcohols. *Seed Sci. Res.* **1**, 21–28.

Dalle-Donne, I., Giustarini, D., Colombo, R., Rossi, R. and Milzani, A. (2003) Protein carbonylation in human diseases. *Trends Mol. Med.* **9**, 169–176.

Davies, M.J. (2005) The oxidative environment and protein damage. *Biochim. Biophys. Acta*, **1703**, 93–109.

Ding, Q., Dimayuga, E. and Keller, J.N. (2006) Proteasome regulation of oxidative stress in aging and age-related diseases of the CNS. *Antioxid. Redox Signal.* **8**, 163–172.

Donohue, K., Dorn, L., Griffith, C., Kim, E., Aguilera, A., Polisetty, C.R. and Schmitt, J. (2005) Niche construction through germination cueing: life-history responses to timing of germination in *Arabidopsis thaliana*. *Evolution* **59**, 771–785.

Drapron, D. (1985) Enzyme activity as a function of water activity. In *Properties of Water in Food* (Simatos, D. and Multon, J.L., eds). Dordrecht: Martinus Nijhoff Publishers, pp. 171–190.

Dunlop, R.A., Rodgers, K.J. and Dean, R.T. (2002) Recent development in the intracellular degradation of oxidized proteins. *Free Radic. Biol. Med.* **33**, 894–906.

Elstner, E.F. and Heupel, A. (1976) Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. *Anal. Biochem.* **70**, 616–620.

Esashi, Y., Isuzugawa, K., Matsuyama, H. and Hasegawa, R. (1991) Endogenous evolution of HCN during pre-germination periods in many seed species. *Physiol. Plant.* **83**, 27–33.

Esashi, Y., Ogasawara, M., Górecki, R. and Leopold, A.C. (1993) Possible mechanisms of afterripening in *Xanthium* seeds. *Physiol. Plant.* **87**, 359–364.

Finch-Savage, W.E and Leubner-Metzger, G. (2006) Seed dormancy and the control of germination. *New Phytol.* **171**, 501–523.

Gallie, D.R., Le, H., Caldwell, C. and Browning, K.S. (1998) Analysis of translation elongation factors from wheat during development and following heat shock. *Biochem. Biophys. Res. Commun.* **245**, 295–300.

Gay, C., Corbineau, F. and Côme, D. (1991) Effects of temperature and oxygen on seed germination and seedling growth in sunflower (*Helianthus annuus* L.). *Environ. Exp. Bot.* **31**, 193–200.

Görg, A., Postel, W., Weser, J., Günther, S., Strahler, J.R., Hanash, S.M. and Somerlot, L. (1987) Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. *Electrophoresis*, **8**, 122–124.

Halliwell, B. and Gutteridge, J.M.C. (1999) *Free Radicals in Biology and Medicine*, 3rd edn. New York: Oxford University Press.

Harder, A., Wildgruber, R., Nawrocki, A., Fey, S.J., Larsen, P.M. and Görg, A. (1999) Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. *Electrophoresis*, **20**, 826–829.

Heath, R.L. and Parker, L. (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoechiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **125**, 189–198.

Hendry, G.A.F. (1993) Oxygen, free radical processes and seed longevity. *Seed Sci. Res.* **3**, 141–153.

Job, C., Rajjou, L., Lovigny, Y., Belghazi, M. and Job, D. (2005) Patterns of protein oxidation in Arabidopsis seeds and during germination. *Plant Physiol.* **138**, 790–802.

Johansson, E., Olsson, O. and Nyström, T. (2004) Progression and specificity of protein oxidation in the life cycle of *Arabidopsis thaliana*. *J. Biol. Chem.* **279**, 22204–22208.

Koornneef, M., Bentsink, L. and Hilhorst, H. (2002) Seed dormancy and germination. *Curr. Opin. Plant Biol.* **5**, 33–36.

Korolainen, M.A., Goldsteins, G., Alafuzoff, I., Koistinaho, J. and Pirttila, T. (2002) Proteomic analysis of protein oxidation in Alzheimer's disease brain. *Electrophoresis*, **23**, 3428–3433.

Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of the bacteriophage T4. *Nature*, **227**, 680–685.

Leubner-Metzger, G. (2005) Beta-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *Plant J.* **41**, 133–145.

Liu, W. and Wang, J.Y. (2005) Modification of protein by polyunsaturated fatty acid ester peroxidation products. *Biochim. Biophys. Acta.* **1752**, 93–98.

McDonald, M.B. (1999) Seed deterioration: physiology, repair and assessment. *Seed Sci. Technol.* **27**, 177–237.

Murthy, U.M.N. and Sun, W.Q. (2000) Protein modification by Amadori and Maillard reactions during seed storage: roles of sugar hydrolysis and lipid peroxidation. *J. Exp. Bot.* **51**, 1221–1228.

Murthy, U.M.N., Kumar, P.P. and Sun, W.Q. (2003) Mechanisms of seed ageing under different storage conditions for *Vigna radiata* (L.) Wilczek: lipid peroxidation, sugar hydrolysis, Maillard reactions and their relationship to glass state transition. *J. Exp. Bot.* **54**, 1057–1067.

Nelson, K.A. and Labuza, T.P. (1992) Relationship between water and lipid oxidation rates: water activity and glass transition theory. In *Lipid Oxidation in Foods* (St Angelo, A.J., ed). Washington, DC: American Chemical Society, pp. 93–103.

Newman, J.W., Morisseau, C. and Hammock, B.D. (2005) Epoxide hydrolases: their roles and interactions with lipid metabolism. *Prog. Lipid Res.* **44**, 1–51.

Nyström, T. (2005) Role of oxidative carbonylation in protein quality control and senescence. *EMBO J.* **24**, 1311–1317.

O'Kane, D., Gill, V., Boyd, P. and Burdon, R. (1996) Chilling, oxidative stress and antioxidant responses in *Arabidopsis thaliana* callus. *Planta*, **198**, 371–377.

Pellinen, R.I., Korhonen, M.S., Tauriainen, A.A., Palva, E.T. and Kangasjärvi, J. (2002) Hydrogen peroxide activates cell death and defense gene expression in birch. *Plant Physiol.* **130**, 549–560.

Priestley, D.A. (1986) *Seed Aging. Implications for Seed Storage and Persistence in the Soil*. Ithaca, NY: Cornell University Press.

Pukacka, S. and Ratajczak, E. (2005) Production and scavenging of reactive oxygen species in *Fagus sylvatica* seeds during storage at varied temperature and humidity. *J. Plant Physiol.* **162**, 873–885.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208–212.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–858.

Slooten, L., Capiau, K., Van Camp, W., Van Montagu, M., Sybesma, C. and Inzé, D. (1995) Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide dismutase in the chloroplasts. *Plant Physiol.* **107**, 737–750.

Sun, W.Q. and Leopold, A.C. (1995) The Maillard reaction and oxidative stress during aging of soybean seeds. *Physiol. Plant.* **94**, 94–104.

Tamarit, J., Cabiscol, E. and Ros, J. (1998) Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress. *J. Biol. Chem.* **273**, 3027–3032.

Taylorson, R.B. and Hendricks, S.B. (1973) Promotion of seed germination by cyanide. *Plant Physiol.* **52**, 23–27.

Vertucci, C.W. and Roos, E.G. (1993) Theoretical basis of protocols for seed storage II. The influence of temperature on optimal moisture levels. *Seed Sci. Res.* **3**, 201–213.

Wilson, D.O. and McDonald, M.B. (1986) The lipid peroxidation model of seed aging. *Seed Sci. Technol.* **14**, 269–300.

Xiong, Y., Contento, A.L., Nguyen, P.Q. and and. Bassham, D.C. (2007) Degradation of oxidized proteins by autophagy during oxidative stress in Arabidopsis. *Plant Physiol.* **143**, 291–299.

Supplemental Table S1

Oracz et al., After-ripening in sunflower seeds

Identification of carbonylated proteins in embryo axes of dormant and non-dormant sunflower seeds.
Peptide sequences identified by MS-MS sequencing. MM, molecular mass (kDa)

N°	Identification	Protein name	MM	pI	Identified peptides by mass spectrometry
3	gi|6056373	Elongation factor EF-2 (Arabidopsis thaliana)	93.9	6.1	K.FSVSPVVR.V R.GGGQVIPTAR.R R.VIYASQITAKPR.L R.IRPVLTVNKMDR.C R.RVIYASQITAKPR.L K.NATLTNEKEVDAHPIR.A
4	**gi|3024423**	Pyruvate, phosphate dikinase chloroplast precursor (Flaveria browni)	93.9	5.1	R.SINQITGLK.G R.AALIADEIAK.E K.VGTMIEIPR.A R.AMDGLPVTIR.L R.FAYDSYRR.F R.NNGAQGIGLCR.T + Carbamidomethyl (C) K.VMANADTPNDALTAR.N
	gi|32541281	HaDisDM710vsCt Helianthus annuus cDNA clone (Pyruvate, phosphate dikinase)			R.SINQITGLK.G K.ELVENCEILER.H + Carbamidomethyl (C)
	gi|7378614	transitional endoplasmic reticulum ATPase (Arabidopsis thaliana)			K.GILLYGPPGSGK.T K.GVLFYGPPGCGK.T + Carbamidomethyl (C)

N°	Identification	Protein name	MM	pI	Identified peptides by mass spectrometry
7	gi|217855	81kDa heat-shock protein [Arabidopsis thaliana]	84.0	4.9	K.SFENLCK.T + Carbamidomethyl (C) K.AVENSPFLER.L K.ADLVNNLGTIAR.S
9	gi|2827002	HSP70 (Triticum aestivum)	73.4	5.0	R.TTPSYVAFTDTER.L K.NAVVTVPAYFNDSQR.Q R.IINEPTAAAIAYGLDKK.I
	gi|19878	heat shock protein 70 (Nicotiana tabacum)			K.DAGAISGLNVMR.I R.QATKDAGAISGLNVMR.I K.NAVVTVPAYFNDSQR.Q R.IINEPTAAAIAYGLDKK.A
12	gi|22273	enolase (Zea mays)	56.9	5.5	K.TCNALLLK.V + Carbamidomethyl (C) K.MGVEVYHNLK.S
	gi|1041245	enolase (Alnus glutinosa)			K.ISADQLKDLYK.S
15	gi|58735933	DH0AQA1ZD02RM1 HaDevS1 Helianthus annuus cDNA clone [7S globulin (Sesamum indicum)]	56.9	6.9	R.YEQVSSELRR.G K.ATMVLMVSNGGGR.F R.FEMACPHLAEQGTR.G + Carbamidomethyl (C)
18	gi|22460345 or gi|22461458 or gi|22462909	QHF6E13.yg.ab1 QH_EFGHJ sunflower RHA280 Helianthus annuus cDNA clone (>gi|1458098 globulin-like protein Daucus carota)	38.0	5.2	K.DLKAGDLYR.I R.IGSIYKDNLVEK.D R.IGSIYKDNLVEKDLK.A

N°	Identification	Protein name	MM	pI	Identified peptides by mass spectrometry
46	gi|32530040	DH0AB43ZF02RM1 HaDevR1 Helianthus annuus cDNA clone HaDevR143F02, mRNA sequence	37.0	6.6	R.ECQCEAVQEVAR.R + 2 Carbamidomethyl (C) R.ALLEVVESGGK.N

IS THERE A ROLE FOR CYANIDE IN SUNFLOWER SEED GERMINATION ?

Christophe Bailly, Norbert Mamadou, Krystyna Oracz*, Daniel Côme, Françoise Corbineau

Physiologie Végétale Appliquée, Université Pierre et Marie Curie, case 152, 4 place Jussieu, 75252 Paris cedex 05, France

*permanent address: Department of Plant Physiology, Warsaw Agricultural University, Rakowiecka 26/30, 02-528 Warsaw, Poland

Introduction

Endogenous ethylene has been shown to play a role in the regulation of sunflower seed germination and dormancy (Corbineau and Côme, 2003), but until now little attention has been paid to a possible role of cyanide, a co-product of ACC conversion to ethylene, in sunflower seed dormancy.

The aims of this work were to determine whether exogenous cyanide could improve germination of dormant sunflower seeds and to try to elucidate its putative mechanisms of action. In particular, the possible interaction of cyanide with ethylene, the activity of β-cyanoalanine synthase, an enzyme involved in cyanide detoxication, and the changes in H_2O_2 content and catalase expression have been investigated.

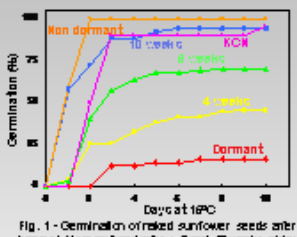


Fig. 1 - Germination of naked sunflower seeds after harvest (dormant) and after 4, 6 and 10 weeks of dry storage (20°C, 70 % RH) at 15°C on water, and germination of dormant seeds at 15°C on 1 mM KCN.

Table 1 - Germination of naked dormant sunflower seeds incubated on water (control) or in the presence of various solutions at 1 mM at 15°C.

	Germination (%) at 15°C after		
	3 d	7 d	14 d
Control	12	20	20
KCN	90	90	90
KCN+SHAM	72	72	78
NaN_3	0	24	32

Results

1- Cyanide alleviates dormancy of freshly harvested seeds

Immediately after harvest, naked (ie without pericarp) sunflower seeds germinated poorly at 15°C, thus revealing an embryo dormancy (Fig. 1). Dry storage progressively allowed their germination, and after 10 weeks at 20°C and 75 % RH they fully germinated at 15°C (Fig. 1).

When incubated in the presence of 1 mM potassium cyanide, naked dormant seeds became abble to germinate at 15°C (Fig. 1), whatever the duration of application of KCN (not shown).

The effect of cyanide on seed germination did not result from an inhibition of respiration since NaN_3, an other respiratory inhibitor, did not improve germination, nor from an activation of the cyanide insensitive pathway, since SHAM, an inhibitor of this pathway, did not suppress the effect of KCN on germination (Table 1).

2 - Towards an understanding of cyanide effect on germination

Cyanide action requires functional ethylene receptors

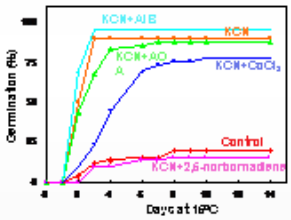


Fig. 2 - Germination of dormant naked sunflower seeds at 15°C on various solutions at 1 mM. AIB, aminoisobutyric acid; AOA, amino-oxyacetic acid.

Cyanide stimulated germination of dormant seeds in the presence of the inhibitors of ACC oxidase, AOA, $CoCl_2$ and AIB, but this beneficial effect was suppressed in the presence of 2,5-norbornadiene, an inhibitor of ethylene action.

Cyanide effect is not related to β-cyanoalanine synthase activity

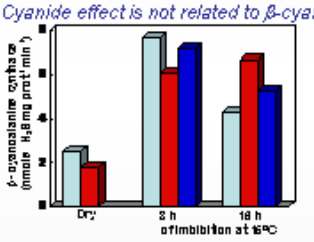


Fig. 3 - Activity of β-cyanoalanine synthase in dry dormant and non-dormant seeds, and during their imbibition at 15°C in water, and in dormant seeds imbibed at 15°C in the presence of 1 mM KCN.

Activity of β-cyanoalanine synthase remained almost unchanged during imbition of dormant and non dormant seeds, and was not stimulated by cyanide (Fig. 3)

Germination is associated with changes in H_2O_2 content and catalase expression

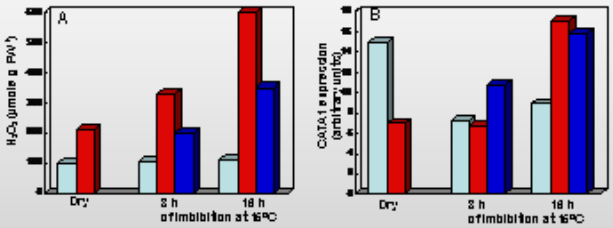


Fig. 4 - H_2O_2 content (A) and CATA1 expression (B) in dry dormant and non-dormant seeds, and during their imbibition at 15°C in water, and in dormant seeds imbibed at 15°C in the presence of 1 mM KCN.

Alleviation of seed dormancy during dry storage was associated with a decrease in CAT mRNA content and with an increase in H_2O_2 content (Figs 4A,B).

H_2O_2 level was always higher in germinating seeds, ie in non dormant seeds incubated in water or in dormant seeds incubated in the presence of KCN than in non germinating dormant seeds (Fig. 4A).

CAT expression was clearly stimulated in seeds that were undergoing germination, as soon as after 8 h in the presence of KCN, or after 16 h on water (Fig. 4B)

Conclusion

Freshly harvested seeds are dormant which mainly results from an embryo dormancy. This dormancy progressively disappears during dry storage.

Cyanide allows germination of dormant seeds but this effect is probably not related to an effect on respiratory metabolism nor to an activation of the pentose phosphate pathway.

Although the effect of cyanide on germination does not require ACC oxidase activity, it requires functional ethylene receptors.

Activity of β-cyanoalanine synthase during seed imbibition is not affected by cyanide, which suggests that the conversion of cyanide to H_2S and then to asparagine does not play a role in this mechanism.

There seems to exist a relationship between H_2O_2 metabolism and seed germination. Cyanide could interfere with this mechanism since this compound is also known to stimulate active oxygen species production and to affect catalase activity.

REFERENCE: Corbineau, F. and Côme, D. (2003) - Germination of sunflower seeds as related to ethylene synthesis and sensitivity - an overview. In Biology and Biotechnology of the Plant Hormone Ethylene III, M. Vendrell, H. Klee, J.C. Pech, F. Romojaro, eds, IOS Press, NATO Science Series, Amsterdam, 116-117.

Effect of cyanide on germination of dormant sunflower (*Helianthus annuus* L.) seeds

Krystyna Oracz [1], Christophe Bailly [2], Francoise Corbineau [2]

[1] Warsaw Agricultural University

[2] Laboratoire de Physiologie Végétale, Université Pierre et Marie Curie, Paris

Introduction

Endogenous ethylene has been shown to play a role in the regulation of sunflower seeds germination and dormancy (Corbineau and Côme, 2003), but until now little attention has been paid to a possible role of cyanide, a co-product of ACC conversion to ethylene, in sunflower seeds dormancy.

The aims of this work were to determine whether exogenous cyanide could improve germination of dormant sunflower seeds and to try to elucidate its putative mechanisms of action. In particular, the possible interaction of cyanide with ethylene, the activity of β-cyanoalanine synthase, an enzyme involved in cyanide detoxication, and the changes in H_2O_2 content and catalase expression have been investigated.

Results

1 - Short pretreatment with hydrogen cyanide alleviates dormancy of freshly harvested seeds

Freshly harvested, naked (ie without pericarp) sunflower seeds are considered to dormant because they fail to germinate at relatively low temperature 10°C (Fig. 1). This dormancy progressively disapears during dry storage and is broken by ethylene.

When incubated in the presence of 1 mM hydrogen cyanide, naked dormant seeds became able to germinate at 10° C (Fig. 1), whatever the duration of application of HCN (not shown).

The effect of cyanide on seed germination did not result from an inhibition of respiration since NaN_3, an other respiratory inhibitor, did not improve germination, nor from an activation of the cyanide insensitive pathway, since SHAM, an inhibitor of this pathway, did not suppress the effect of HCN on germination (Tab. 1).

Fig. 1 – Germination of dormant and non-dormant sunflower seeds (control) and seeds treated by 1mM HCN for 3h at 10°C

Table 1 – Germination of naked dormant sunflower seeds incubated on water (control) or in the presence of various solutions at 1 mM at 10°C

	germination (%) at 10°C after		
	3 days	7 days	14 days
control	4	18	32
HCN	76	96	98
HCN + SHAM	64	78	88
NaN_3	8	40	56

2 - Towards an understanding of cyanide effect on germination

Cyanide action requires functional ethylene receptors

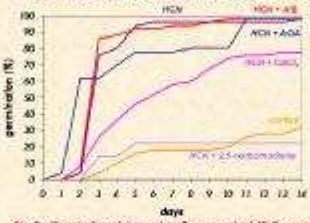


Fig. 2 – Germination of dormant sunflower seeds at 10°C on various solutions at 1 mM. AIB – aminoisobutyric acid; AOA – amino-oxyacetic acid

Cyanide stimulated germination of dormant seeds in the presence of the inhibitors of ACC oxidase: AOA, $CoCl_2$ and AIB, but this beneficial effect was suppressed in the presence of 2,5-norbornadiene, an inhibitor of the ethylene action (Fig. 2).

Cyanide effect is not related to β-cyanoalanine synthase activity

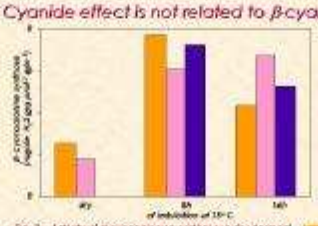


Fig. 3 – Activity of β-cyanoalanine synthase in dry dormant and non-dormant seeds and during their imbibition at 10°C in water, and in dormant seeds imbibed at 10°C in the presence of 1 mM HCN

β-CAS activity remained almost unchanged during imbibition of dormant and non dormant seeds, and was not stimulated by cyanide(Fig. 3)

Germination is associated with changes in H_2O_2 content catalase activity and CAT expression

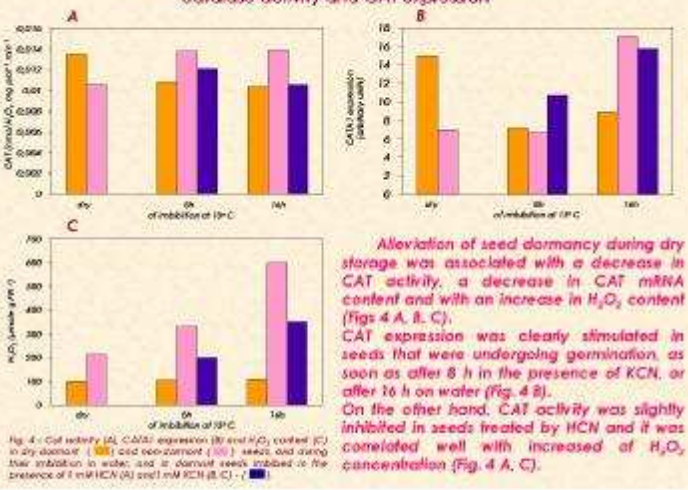


Fig. 4 – Cat activity (A), CAT2 expression (B) and H_2O_2 content (C) in dry dormant ( ) and non-dormant ( ) seeds, and during their imbibition in water, and in dormant seeds imbibed in the presence of 1 mM HCN (A) and 1 mM HCN (B, C) - ( )

Alleviation of seed dormancy during dry storage was associated with a decrease in CAT activity, a decrease in CAT mRNA content and with an increase in H_2O_2 content (Figs 4 A, B, C).

CAT expression was clearly stimulated in seeds that were undergoing germination, as soon as after 8 h in the presence of KCN, or after 16 h on water (Fig. 4 B).

On the other hand, CAT activity was slightly inhibited in seeds treated by HCN and it was correlated well with increased of H_2O_2 concentration (Fig. 4 A, C).

Conclusions:

Freshly harvested seeds are dormant which mainly results from an embryo dormancy. This dormancy progressively disappears during dry storage.

Cyanide allows germination of dormant seeds but this effect is probably not related to an effect on respiratory metabolism nor to an activation of the penthose phosphate pathway.

Although the effect of cyanide on germination does not require ACC oxidase activity, it requires functional ethylene receptors.

Activity of CAS during seeds germination is not affected by cyanide, which suggest that the conversion of cyanide to H_2S and them to asparagine does not play a role in this mechanism.

There seems to exist a relationship between H_2O_2 metabolism and seed germination. Cyanide could interfere with this mechanism since this compound is also known to stimulate active oxygen species production and to affect CAT activity.

Possible interplay of HCN and ROS with ethylene signaling pathway in alleviation of sunflower embryo dormancy

Krystyna Oracz[1,2], Hayat El-Maarouf-Bouteau[2], Renata Bogatek[1], Francoise Corbineau[2], Christophe Bailly[2]

(1) Department of Plant Physiology, Warsaw University of Life Sciences, Nowoursynowska 159, 02-776, Warsaw, Poland; email:
(2) EA2388 Physiologie des Semences, Université Pierre et Marie Curie, Le Raphael, Site d'Ivry, Boîte 152, 4 Place Jussieu, Paris F-75005, France

Introduction

At harvest time, sunflower (*Helianthus annuus* L.) embryos are in a state of deep dormancy and are unable to germinate at temperatures below 10 °C. Endogenous ethylene has been shown to play a role in the regulation of sunflower seeds germination and dormancy, but until now little attention has been paid to a possible role of cyanide, a co-product of ACC conversion to ethylene. There, exist a circumstantial evidence that HCN is an important controlling factor which is involved in alleviation of seed dormancy. How this regulation is achieved is unknown. Considering also the increasing evidence on the role of ROS to various physiological processes, the aim of this work was to investigate whether ROS were generated by exogenous HCN and if they both maight play a role in dormancy removal by participation in regulation of ethylene signal transduction pathway.

Results

1- Short pretreatment (3h) with HCN and ROS alleviate dormancy of sunflower embryos

MV (methylviologen) – ROS generating compound

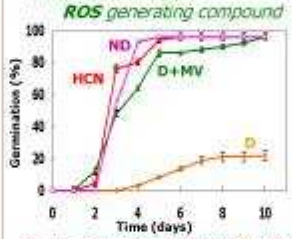


Fig. 1 – Germination of dormant (D) and nondormant (ND) sunflower embryos (control and embryos treated by 1mM HCN or MV, during 3h), in darkness, at 10° C.

2- Effect of HCN on H_2O_2 production

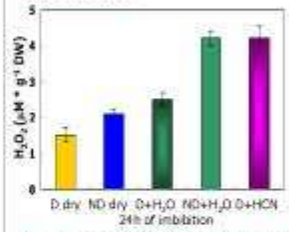
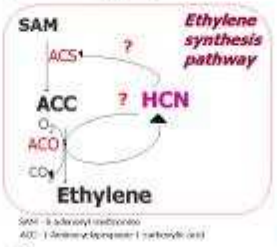


Fig. 2 – H_2O_2 content in axes isolated from dormant and nondormant sunflower embryos (dry, imbibed on water or treated by HCN, at 10° C).

HCN stimulates production of **ROS** (H_2O_2) during alleviation of sunflower embryo dormancy (Fig. 2).

3- Involvement of HCN and ROS in regulation of ethylene synthesis in sunflower embryos

HCN and **ROS** do not increase transcription of ACC synthase (*ACS*) and ACC oxidase (*ACO*) genes (Fig. 4), and this effect on germination is not associated with a stimulation of ethylene production (Fig. 3).



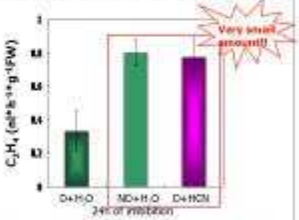


Fig. 3 – Ethylene production by axes isolated from dormant (D) and nondormant (ND) sunflower embryos (imbibed on water or treated by HCN, at 10° C, in darkness).

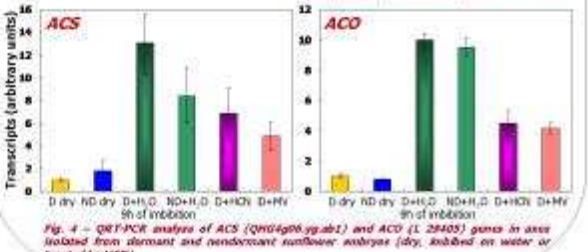
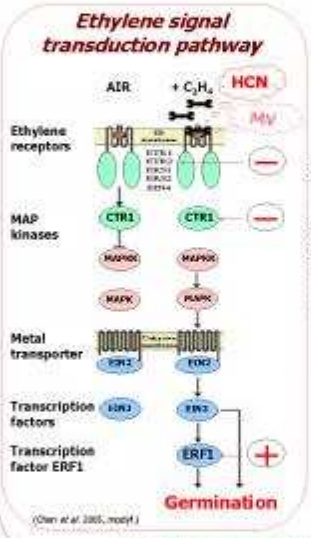


Fig. 4 – QRT-PCR analyze of ACS (QH0140G06.yg.ab1) and ACO (L 28405) genes in axes isolated from dormant and nondormant sunflower embryos (dry, imbibed on water or treated by HCN).

4- HCN and ROS involvement in C_2H_4 signal transduction

HCN and **MV** markedly increase the transcription of *ERF1* but not the *ETR2* and *CTR1* ones.



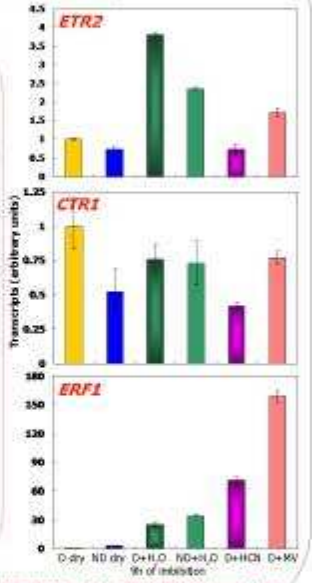


Fig. 6 – QRT-PCR analyze of ETR2 (QHF11C05.yg.ab1), CTR1 (QH01J2010.yg.ab1) and ERF1 (QH_CA_Contig5791) genes in axes isolated from dormant and nondormant sunflower embryos (dry, imbibed on water or treated by HCN).

Conclusions and general remark

- ✓ **HCN** and **ROS** (MV) remove dormancy and stimulate germination of sunflower embryos.
- ✓ **HCN** stimulates production of **ROS** during alleviation of sunflower embryo dormancy.
- ✓ **HCN** as also **ROS** do not increase transcription of *ACS* and *ACO* genes, and this effect on germination is not associated with a stimulation of ethylene production.
- ✓ **HCN** and **ROS** markedly increase the transcription of *ERF1* but not the *ETR2* and *CTR1* ones.
- ✓ **Transduction pathways of HCN and ROS initiate the transcription factor *ERF1* but through different mechanism of that involved by ethylene.**

Nagroda

Dla

zespołu: **K. Oracz, H. El-Maarouf-Bouteau, R. Bogatek F. Corbineau, C. Bailly**

za pracę eksperymentalną

Possible interplay of HCN and ROS with ethylene signalling pathway in alleviation of sunflower embryos dormancy

wyróżnioną w konkursie Młodych Naukowców

Prezydent PTBER

Anna M. Rychter

III Konferencja
Polskiego Towarzystwa Biologii Eksperymentalnej Roślin
Warszawa, 26-30 sierpień, 2007

Wyrażam zgodę na udostępnienie mojej pracy w czytelniach Biblioteki SGGW.

.....
Krystyna Oracz

I am agree for making my manuscript accessible in reading room of Library of University Pierre and Marie Curie .

.....
Krystyna Oracz