TESIS DOCTORAL

ROLE OF NITRIC OXIDE (NO) IN PLANT RESPONSE TO CADMIUM AND *FUSARIUM OXYSPORUM*: POSSIBLE CROSSTALK

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Programa de Doctorado en Biología Fundamental y de Sistemas

UNIVERSIDAD DE GRANADA

UNIVERSIDAD DE GRANADA

ESTACIÓN EXPERIMENTAL DEL ZAIDÍN (EEZ)

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC)

Departamento de Bioquímica, Biología Celular y Molecular de Plantas

MINISTERIO DE CIENCIA, INNOVACIÓN Y UNIVERSIDADES

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Granada 2020

Editor: Universidad de Granada. Tesis Doctorales
Autor: Laura Carmen Terrón Camero
ISBN: 978-84-1306-553-3
URI: http://hdl.handle.net/10481/63325

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Memoria de Tesis Doctoral presentada para optar al grado de Doctor por la Universidad de Granada

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El trabajo que se presenta en esta memoria de Tesis Doctoral ha sido realizado en el Departamento de Bioquímica, Biología Celular y Molecular de Plantas de la Estación Experimental del Zaidín (CSIC) de Granada en la línea de investigación: Señalización por Especies de Oxígeno y Nitrógeno Reactivo en Situaciones de Estrés en Plantas.

La doctoranda ha sido financiada por el Ministerio de Educación, Cultura y Deporte mediante la beca de "Formación de Profesorado Universitario" (FPU 2014), para la realización de la tesis doctoral (2014/2019). Además, recibió la beca "Ayudas a la movilidad para estancias breves y traslados temporales" financiada por el Ministerio de Educación, Cultura y Deporte (2016) que permite optar al grado de Doctor con Mención Internacional.

La investigación ha sido cofinanciada por el Fondo Europeo de Desarrollo Regional y el Ministerio de Economía, Industria y Competitividad de España (proyectos BIO2015-67657-P y PGC2018-098372-B100) y por la Junta de Andalucía (proyecto EX12-BIO296; grupo no. BIO-337).

Parte de los resultados de este trabajo han sido presentados en los siguientes congresos y forman parte de las mencionadas publicaciones:

a. Congresos:

1. Plant Oxygen Group (POG). 2019. Munich, Alemania.

Póster: Identification of NO-dependent signalling in plant response during Cd stress. **Laura C. Terrón-Camero**, A Rodríguez-González, Coral del Val, Luisa M. Sandalio and Maria C. Romero-Puertas.

2. XXIII Meeting of the Spanish Society of Plant Physiology and the XVI Hispano-Portuguese Congress of Plant Physiology. 2019. Pamplona, España.

Póster: Nitric Oxide shape Arabidopsis-*Fusarium oxysporum* interaction. **Laura C. Terrón-Camero**, Eliana Molina-Moya, M. Ángeles Peláez-Vico, Luisa M. Sandalio and María C. Romero-Puertas.

Comunicación Oral: How to elaborate a poster for scientific dissemination and Roundtable moderator in scientific dissemination meeting. **Laura C. Terrón-Camero.**

3. 7th Plant Nitric Oxide International Meeting. 2018. Niza, Francia.

Comunicación Oral 1: Unraveling the Role of Nitric Oxide in the Arabidopsis-*Fusarium oxysporum* interaction. **Laura C. Terrón-Camero**, Luisa M. Sandalio and María C. Romero-Puertas.

Comunicación Oral 2: Role of nitric oxide in peroxisome dynamics under Cd stress. **Laura C. Terrón-Camero**, Coral del Val, Luisa M. Sandalio and María C. Romero-Puertas.

4. XIV Reunión de Biología Molecular de Plantas. 2018. Salamanca, España.

Póster 1: Unravelling the role of nitric oxide in seedling growth under cadmium stress. **Laura C. Terrón-Camero**, Coral del Val, Luisa M. Sandalio and María C. Romero-Puertas.

Póster 2: Identifying key genes dependent on peroxisomal ROS. **Laura C. Terrón-Camero**, Coral del Val, Luisa M. Sandalio and María C. Romero-Puertas.

5. II Young Researchers Science Symposium. 2017. Granada, España.

Comunicación Oral: Función del NO en la respuesta al estrés en Arabidopsis producida por la infección por Fusarium. **Laura C. Terrón-Camero**, Luisa M. Sandalio and María C. Romero-Puertas.

6. 6th Plant Nitric Oxide International Meeting. 2016. Granada, España.

Comunicación Oral: Nitric oxide functions in plants growing under cadmium stress. **Laura C. Terrón-Camero**, Luisa M. Sandalio and María C. Romero-Puertas.

7. I Young Researchers Science Symposium. 2016. Granada, España.

Comunicación Oral: Identificación de componentes de la red de señalización dependiente de óxido nítrico (NO) en respuesta a estrés biótico y abiótico. **Laura C. Terrón-Camero**, Luisa M. Sandalio and María C. Romero-Puertas.

b. Publicaciones:

1. **Laura C. Terrón-Camero**, María Rodríguez-Serrano, Luisa M. Sandalio and María C. Romero-Puertas. Nitric oxide is essential for peroxule production and peroxisome proliferation and signalling in response to Cd stress. Under review.

2. **Laura C. Terrón-Camero**, Coral del Val, Luisa M. Sandalio and María C. Romero-Puertas (2020). Low endogenous NO levels in roots and antioxidant systems are determinants for the resistance of Arabidopsis seedlings grown in Cd. Environmental Pollution, In press: https://doi.org/10.1016/j.envpol.2019.113411

3. **Laura C. Terrón-Camero**, M. Ángeles Peláez-Vico, Coral del Val, Luisa M. Sandalio and María C. Romero-Puertas (2019). Role of nitric oxide in plant responses to heavy metal stress: exogenous application vs. endogenous production. Journal of Experimental Botany 70 (17), 4477-4488.

4. María C. Romero-Puertas, **Laura C. Terrón-Camero**, M. Ángeles Peláez-Vico, Adela Olmedilla and Luisa M. Sandalio (2019). Reactive oxygen and nitrogen species as key

indicators of plant responses to Cd stress. Environmental and Experimental Botany. 161, 107-119

5. **Laura C. Terrón-Camero,** Eliana Molina-Moya, María Sanz-Fernández, Luisa M. Sandalio, María C. Romero-Puertas (2018) Detection of Reactive Oxygen and Nitrogen Species (ROS/RNS) during Hypersensitive Cell Death. Methods in Molecular Biology 1743:97-105. doi: 10.1007/978-1-4939-7668-3_9.

c. Estancia:

- Caracterización de la respuesta a *Fusarium oxysporum* en *Arabidopsis thaliana*. University of Guelph, Canadá (1/5/2017-31/07/2017).

La doctoranda Laura C. Terrón Camero y las directoras de la tesis, Dra. María C. Romero Puertas y la Dra. Luisa Mª Sandalio:

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección de las directoras de la tesis y, hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados cuando se han utilizado sus resultados o publicaciones.

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Granada 25 de mayo de 2020

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A todas las científicas olvidadas

«La ciencia es una parte integral de la cultura. No es esa cosa extranjera, realizada por un arcano sacerdocio. Es una de las glorias de la tradición intelectual humana»

Stephen Jay Gould


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¡Muchas gracias!

Agradecimientos

Summary

Plants are sessile organisms closely linked to their habitat and have thus developed a series of strategies that facilitate the adaptation of their metabolism to changing environmental conditions. A change in the environment can cause stress in plants and trigger metabolic changes, beginning with stress perception in order to optimize the plant response. All this alters gene expression, as well as the regulation of secondary metabolites and protein modifications, which are fundamental to a plant's efficient response to stress. The production of reactive oxygen and nitrogen species (ROS/RNS) plays a key role in the plant's response to stress. Nitric oxide (NO) and ROS are capable of interacting with each other, as well as with other signal molecules, such as hormones. In addition, the concentration and subcellular location of RNS/ROS in plant tissues, which are essential for the functioning of these molecules, can have a signalling effect on many processes or be cytotoxic at high concentrations, leading to cellular damage. Plants have developed a series of highly regulated mechanisms that enable them to produce and eliminate NO and ROS in due measure. Furthermore, peroxisomes, which are highly dynamic and metabolically active organelles present in almost all eukaryotic cells, play an essential role in ROS/RNS homeostasis under control conditions and in stress responses. These organelles can interact with mitochondria and chloroplasts, share metabolic pathways and signalling and are involved in ROS detoxification, signalling and ROS/RNS sensing, as well in the import and transport of proteins to other organelles.

Over the last twenty years, NO has been shown to be a key signal molecule in plant responses to stress. However, the role of NO in plant responses to cadmium is not fully understood, while little is known about its role in plant-pathogenic fungus interactions. On the other hand, transcriptomic analyses carried out in our laboratory showed a possible relationship between the plant's response to Cd and biotic stress, that can also be regulated by NO, which may play a key role in plant responses to both these stress conditions.

Given the diversity of treatments in terms of criteria such as the metal concentrations used, plant species, plant growth and time of treatment, it is difficult to draw conclusions as to the role of NO in plant responses to heavy metals. In this thesis, we made a bioinformatics analysis of articles published over the last ten years on the production and/or function of NO in plant responses to heavy metals, including Cd. This analysis showed that exogenous applications of NO to the plant protect against heavy metals, particularly Cd, and that, in response to exposure to heavy metals, particularly Cd, plants initially produce NO, which can act as a signal molecule. At a later stage, the plant appears to be equipped with mechanisms to control NO levels, thus preventing further symptoms of toxicity. Using both biochemical techniques, involving modifications in NO levels through chemical donors and scavengers, and molecular approaches, involving the use of

mutants with altered levels of NO, we subsequently analysed the role of NO in Arabidopsis seedling responses to Cd stress and its relationship to ROS. We also show that prolonged production of NO in plant responses to Cd can indeed affect antioxidant systems and induce oxidative stress, suggesting yet again that NO levels need to be strictly regulated in plant responses to Cd stress in order to prevent further damage to the plant.

Peroxisomes have recently been shown to play a key role in a plant's early response to Cd stress. While both NO and RNS have been detected in these organelles, little is known about their effect on peroxisomal metabolism and dynamics. In this thesis, we analysed the role of NO in peroxisomal metabolism, distribution and dynamics under control conditions and in response to Cd stress. We showed that NO is involved in changes observed in peroxisomal dynamics which are necessary for the plant to respond to Cd stress. We also demonstrated the effect of NO on the oxidative metabolism of peroxisomes and their cellular distribution, as well as on organelle-dependent signalling.

In addition, we analysed the little-known role of NO in Arabidopsis-*Fusarium oxysporum* interactions. We found that NO metabolism mutants have differential fungal responses with respect to WT in terms of ROS production, phenols, secondary and iron metabolisms; as well as defence gene induction Also, nitrate reductase appears to be essential for adequate cell wall assembly through the regulation of CESA4 and MYB46, given that the cell wall is a key barrier in the plant’s defence against *Fusarium oxysporum*.

Finally, pre-treatment with Cd was found to protect plants against *Fusarium oxysporum* and to increase their survival. This could be explained by a priming effect, as certain genes are common to different plant responses to Cd and fungi, particularly Fusarium, suggesting that crosstalk takes place between both these stress conditions.

Resumen

Las plantas son organismos sésiles ligados estrechamente a su hábitat. Por ello, han desarrollado una serie de estrategias que le permiten adaptar su metabolismo frente a las condiciones cambiantes que las rodean. Una modificación en el entorno de la planta puede ocasionar un estrés en la misma, lo que ocasiona una serie de cambios metabólicos que se inician con la percepción del estrés para optimizar la respuesta. La producción de especies de oxígeno y nitrógeno reactivo (RNS/ROS) son claves en la respuesta de la planta al estrés. Ambas moléculas son capaces de interaccionar entre ellas, así como con otras moléculas señal y hormonas. Todo ello ocasiona cambios en la expresión génica, en la regulación de metabolitos secundarios y modificaciones de proteínas, siendo procesos fundamentales en la planta para una respuesta eficiente frente al estrés. Por tanto, la concentración y localización subcelular de RNS/ROS en los tejidos de la planta son esenciales para la función de estas moléculas, pudiendo tener un efecto señalizador en multitud de procesos, o citotóxico, cuando las concentraciones son elevadas, causando daños celulares. Por ello, las plantas han desarrollado una serie de mecanismos altamente regulados que les permiten producirlas y eliminarlas en su justa medida. Los peroxisomas, que son unos orgánulos muy dinámicos y metabólicamente activos, presentes en las células eucarióticas, tienen una función esencial en la homeostasis de ROS/RNS en condiciones control y en la respuesta al estrés. Además, estos orgánulos pueden interaccionar con mitocondrias y cloroplastos, compartiendo rutas metabólicas y señalización pudiendo cumplir un papel de detoxificación de ROS, señalizador, sensor de ROS/RNS e importación y transporte de proteínas a otros orgánulos.

En los últimos veinte años se ha demostrado que el óxido nítrico (NO) es una molécula señal clave en la respuesta de la planta al estrés. Sin embargo, la función del NO en la respuesta de la planta al cadmio y en la interacción planta-hongo patogénico no está del todo definida y en el último caso, es casi desconocida. Por otro lado, el análisis de un transcriptoma realizado en nuestro laboratorio, de la respuesta de la planta al estrés por Cd mostró una posible relación entre la respuesta de la planta al Cd y al estrés biótico, que además podría estar regulada por NO, lo que sugiere que el NO podría tener una función clave en la respuesta de la planta a ambos tipos de estrés.

Debido a la diversidad de tratamientos (concentración de metal usada, especies utilizadas, crecimiento de la planta, tiempo del tratamiento, etc.), es difícil obtener conclusiones sobre la función del NO en la respuesta de la planta a metales pesados. En esta Tesis, hemos realizado un análisis mediante bioinformática de los artículos publicados en los últimos diez años donde se muestra la producción y/o función del NO en la respuesta de la planta a metales pesados, incluido el Cd; para conocer, en base a los antecedentes disponibles, la posible función del NO en la respuesta de la planta a los metales pesados. Este análisis mostró la función protectora frente a los

metales pesados y en particular frente al Cd, del NO aplicado de manera exógena a la planta. Además, se encontró que tras la exposición a los metales pesados y en particular al Cd, las plantas producen NO en la respuesta temprana que podría actuar como molécula señal. Sin embargo, en etapas posteriores la planta parece que tiene mecanismos para controlar los niveles de NO, evitando así mayores síntomas de toxicidad. Posteriormente, mediante técnicas tanto bioquímicas (alterando los niveles de NO mediante donadores y secuestradores químicos) como moleculares (mediante el uso de los mutantes con niveles alterados de NO disponibles), hemos analizado la función del NO en la respuesta de plántulas de Arabidopsis al estrés por Cd y su relación con las ROS. Así, demostramos que efectivamente, una prolongada producción de NO en la respuesta de la planta al Cd puede afectar a sistemas antioxidantes e inducir un estrés oxidativo, sugiriendo de nuevo que los niveles de NO deberían ser estrictamente regulados en la respuesta de la planta al estrés por Cd para evitar mayores daños en la planta.

Recientemente, se ha demostrado que los peroxisomas tienen una función clave en la respuesta temprana de la planta al estrés por Cd. Si bien, tanto el NO como las RNS se han detectado dentro de estos orgánulos, su función sobre su metabolismo y dinámica es prácticamente desconocida. En esta Tesis, hemos analizado la función del NO en el metabolismo, distribución y dinámica peroxisomal en condiciones control y en respuesta al estrés por Cd. Hemos demostrado que el NO es necesario para que se produzcan los cambios observados en la dinámica peroxisomal en la planta en respuesta al Cd; que el NO afecta al metabolismo oxidativo del peroxisoma y a la distribución de orgánulos dentro de la célula; así como a la señalización dependiente del orgánulo.

Por otro lado, hemos analizado la función del NO en la interacción Arabidopsis-*Fusarium oxysporum*, que es prácticamente desconocida. Hemos observado que los mutantes relacionados con el metabolismo de NO presentan una respuesta al hongo diferencial con respecto al WT, en lo que se refiere a la producción de ROS, fenoles y metabolismo secundario, metabolismo del hierro e inducción de genes de defensa. Además, la nitrato reductasa parece ser fundamental para el ensamblaje adecuado de la pared celular a través de la regulación de CESA4 y MYB46, siendo esta barrera clave para la defensa de la planta frente a *Fusarium oxysporum*.

Finalmente, hemos observado que el pretratamiento con Cd protege a las plantas frente *Fusarium oxysporum*, incrementando su supervivencia lo que podría ser explicado por un efecto "priming" ya que como se ha mencionado anteriormente, la respuesta de la planta al Cd y hongos, en particular a Fusarium, tienen genes en común, sugiriendo una conexión entre ambos tipos de estrés.

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Abbreviations and acronyms

- 8-OHG: 8-hydroxyguanosine
- AA: Aminoacids
- ABA: Abscisic acid
- ABC: ATP-binding cassette
- ACS: 1-aminocyclopropane-1-carboxylic acid synthase
- AG: Aminoguanidine
- AKRs: Aldo-keto reductases
- *AOX1:* Alternative oxidase1
- APX: Ascorbate peroxidase
- Asc: Ascorbate
- ATG: Autophagy-related genes
- ATSDR: Agency for Toxic Substances and Disease Registry
- Avr: Preoginas aviruten
- BCIP: 5-bromo-4-chloro-3-indolyl-phosphate
- BIK1: Botrytis induced kinase 1
- BSA: Bobine serum albumin
- CAPS: N-cyclohexyl-3-aminopropanesulfonic acid
- CAT: Catalase
- CAX: Cation exchanger
- CDF: Cationic diffusion facilitator
- CDPK: Calcium-dependent protein kinase
- cGMP: Cyclic guanosine monophosphate
- Chloro: Choloroplast
- CK: Creatine kinase
- CLSM: Confocal Laser Scanning Microscope
- Col-0: Columbia 0
- COPT: Copper cation transporter
- cPTIO: Carboxy-2-phenyl-4,4,5,5-tetramethy-limidaziline-1-oxyl-3-oxide
- CTAB: Hexadecyltrimethylammonium bromide
- CW: Cell wall
- Cys: Cysteine
- Cyt P450: Cytochrome P450 reductase
- DA: Diacetate
- DAB: 3,3′-Diaminobenzidine
- DAF-2: 4,5-Diaminofluorescein
- DAMP: Damage-associated molecular patterns
- DCF: 2′-7′-dichlorodihydrofluorescein
- DEPC: Diethyl pyrocarbonate
- DHAR: Dehydroascorbate reductase
- DHE: Dihydroethidium
- DPI: Diphenyliodonium chloride
- DTT: Dithiothreitol
- EDTA: Ethylenediaminetetraacetic acid
- EEA: European Environment Agency
- em: Emission
- Epic: Ephicatechin
- ETI: Immunity activated by the effectors

- exc: Excitation
- GA: Gibberellin
- GFP: Green fluorescent protein
- gor*: E. coli* glutathione reductase gene
- GOX: Glycolate oxidase
- GR: Glutathione reductase
- Grx: Glutaredoxins
- GSH: Glutathione
- GSNO: *S*-nitrosoglutathione
- GSNOR: S-nitrosoglutathione reductase
- GSSG-NH3: oxidized glutatione
- GST: Glutathione-*S*-Transferase
- HMA: Heavy-metal-associated domain
- HO1: Heme oxygenase 1
- Hpi: Hours post infection
- HR: Hypersensitive response
- HSF: Heat shock factor
- HSP: Heat shock protein
- IAA: Indoleacetic acid
- IgG: Inmunoglobuline G
- IRT: Iron regulated transporter
- IRT1: Iron-regulated transporter
- JA: Jasmonic acid
- KEGG: Kyoto Encyclopedia of Genes and Genomes
- L-Arg: Levogyre-Arginine
- LCT1: Low-affinity cation transporter
- LOX: Lipoxygenase
- MAMP: Non pathogen-associated molecular patterns
- MAPK: Mitogen-activated protein kinases
- MDAR: Monodehydroascorbate reductase
- MDHAR: Monodehydroascorbate peroxidase
- MDR: Multiple drug resistance
- MG: Methylglyoxal
- MiRNA: MicroRNAs
- Mit: Mitochondria
- mRNA: Messenger RNA
- MRP: Multidrug resistance-associated
- MTP: Metal tolerance protein
- NADPH: Nicotinamide adenine dinucleotide phospate
- NADPH-DH: NADPH dehydrogenases
- NBT: Nitro blue Tetrazolium Chloride
- NDPK: Nucleoside-diphosphate kinase
- NiNOR: Nitrate nitrite reductase
- NIP: Nodulin-26-like intrinsic protein
- NOFNiR: Nitric oxide-forming nitrite reductase
- NOS: Nitric oxide synthase
- NOS-l: Nitric oxide synthase-like
- *Nox*: Nitrogen oxides

- NPR1: NONEXPRESSOR of PR1
- NR: Nitrate reductase
- NRAMP: Natural resistance associated macrophage protein
- NRS: Nitrogen reactives species.
- nsHbs: Non-symbiotic haemoglobins
- NT: Not treated
- OXI1: Type of serine/threonine proteins kinase
- PAMP: Pathogen-associated molecular patterns
- PBL13: Typeo of serine/threonine proteins kinase
- PCD: Programing cell death
- PCs: Phytochelatins
- PDR: Pleiotropic drug resistance
- Perox: Peroxisome
- PEX: Peroxin
- Phy A: Phytochrome A
- PLDA1: phospholipase Da1
- PMP: Peroxisomal membrane protein
- P-N-Tyr: Thyroxine nitrate protein
- POD: Peroxidase
- POX: peroxiredoxin
- PP2A-B'γ: 2A protein phosphatase subunit
- PR: Pathogenesis-Related
- PRR: Pattern recognition receptor
- PRX: Peroxyredoxin
- PSII: Phoyosystem II
- P-SNO: Protein *S*-nitosilated
- PTI: Plant immunity
- PTM: Post-translational modification
- PVDF: Polyvinylidene fluoride
- RBOH: Respiratory burst oxidase homolog
- RLK: Leucine rich repeat receptor like kinase
- RNA: Ribonucleic acid
- RNase: Ribonuclease
- ROS: Reactive oxygen species
- RT: Room temperature
- SA: Salycilic acid
- SAOX: Sarcosin oxidase
- SAR: Acquired systemic resistance
- SDH1-1: Succinate deshidrogenase 1-1
- SDS: Dodecilsulfato sódico
- SGC: Soluble guanylyl cyclase
- SNAP: S-Nitroso-N-acetyl-DL-penicillamine
- *S*-NO: *S*-nitrosothiols
- SNP: Sodium nitroprusside
- SO: Sulphite oxidase
- SOD: Superoxide dismutase
- TAE: Tris Acetate-EDTA buffer
- TBS :Tris-Buffered Saline

- TCA: Tricarboxilic acid
- TF: Transcription factor
- THB: Truncated haemoglobin
- TMP: Tetramethyl piperidinooxy
- Tris: Tris (hidroximetil)aminometano
- Trx: Thioredoxins
- UO: Uricase
- UV-B: Ultraviolet B
- V-ATPase: Vacuolar-type H^+ ATPase
- VIT: Vacuolar iron transporter
- V-PPase: Membrane-bound proton-pumping pyrophosphatase
- WT: Wild type
- XOD: Xanthine oxidase
- XOR: Xanthine oxidoreductase
- YSL: Yellow stripe-like
- ZAT: Zinc transporter of *Arabidopsis thaliana*
- ZIP: IRT-like protein
- ZRT: Zn regulated transporter

1. General introduction

1. General introduction

1.1. Stress in plants

Plants are sessile organisms closely linked to their habitat. That is way, environmental an anthropogenic factors can threaten their survival. Plants have developed a series of specific mechanisms that allow them to grow and adapt to changeable adverse conditions. In 1938 Seyle developed the concept of stress applied to living organisms, and establishes the terms of "stress generating agent" and "stress response", which could be generic or specific. Levitt (1980) defined stress as those potentially unfavorable factors for living organisms. Subsequently, Larcher (1995) applied the term stress specifically referring to plants, clarifying that stress can produce an initial destabilization in the plant, followed by normalization and improved resistance in some cases (Gaspar *et al*., 2002; Kollist *et al*., 2019). On the contrary, if the stress exceeds the limits of tolerance and adaptability, the plant can be permanently damaged or even die (Gaspar *et al*., 2002; Sade *et al*., 2018; Hasanuzzaman *et al*., 2019). Stress can be therefore considered as a powerful evolutionary factor (Karanja *et al*., 2019; Li *et al*., 2019).

Stress, abiotic and biotic, begins with a constraint or with highly unpredictable fluctuations that modify regular metabolic pattern. Plants can respond to stress by acclimatizing their metabolism, with specific adaptations, as well as by developing certain stress tolerance mechanisms (Pagliarani *et al*., 2019). However, sudden stress events, can reduce plant growth, and even can cause cell death if the repair mechanisms are not effective. It will depend on the intensity and duration of the stress, when a certain threshold of stressor exposure is exceeded. The stress tolerance threshold would depends not only on the plant species, but also on the predisposition of the plant, the growth conditions and the plant vitality prior to the action of the stressor (Gaspar *et al*., 2002; Kranner *et al*., 2010; Zhu, 2016).

1.1.1. Plant stress syndrome response

The stress response can be divided in a simplified way into four phases (Fig. 1.1):

1. Response phase: alarm reaction, accompanied by a decrease in conventional physiological functions (beginning of the stress).

2. Restitution phase: adaptation, repair, reactivation of plant metabolism (continuous stress).

3. Final phase: exhaustion stage, where overload of adaptive capacity predominates with possible irreversible damage (long-term stress).

4. Regeneration phase: can be partial or total (elimination of stress).

At the beginning of stress responses, physiological functions of the plant could be altered. Damages can affect metabolic transport, ion uptake and translocation, and photosynthesis. Plants deviate from their physiological state and, as a consequence, their vitality decreases. Those plants with limited tolerance mechanisms have low resistance, resulting after stress in severe damages and senescence (Sade *et al*., 2018). In the alarm phase however, most plants will activate a series of mechanisms to cope with stress such as, acclimation of metabolic flows, activation of repair processes, long-term metabolic and morphological adaptations. This will cause a new physiological state of the plant (Hussain *et al*., 2019; Xiong *et al*., 2019). Long-term stress or high doses can take plant to the depletion phase or final phase, where the vitality of the plant is progressively lost, while causing severe damage and can also lead to cell death (Agathokleos *et al*., 2018; Li *et al*., 2019). However, when the stressors are eliminated, before the senescence processes became dominant, plants regenerate and move to new physiological states (regeneration phase; Lichtenthaler, 1998; Gaspar *et al*., 2002; Fig. 1.1).

Stressful and fluctuating environments are routine events to which plants are exposed. However, continuous stress and tension do not necessarily give rise plant damages. If the intensity and duration of stress are not too high, the plants will be oriented within the range established by the resistance and damage may not occur (Gaspar *et al*., 2002).

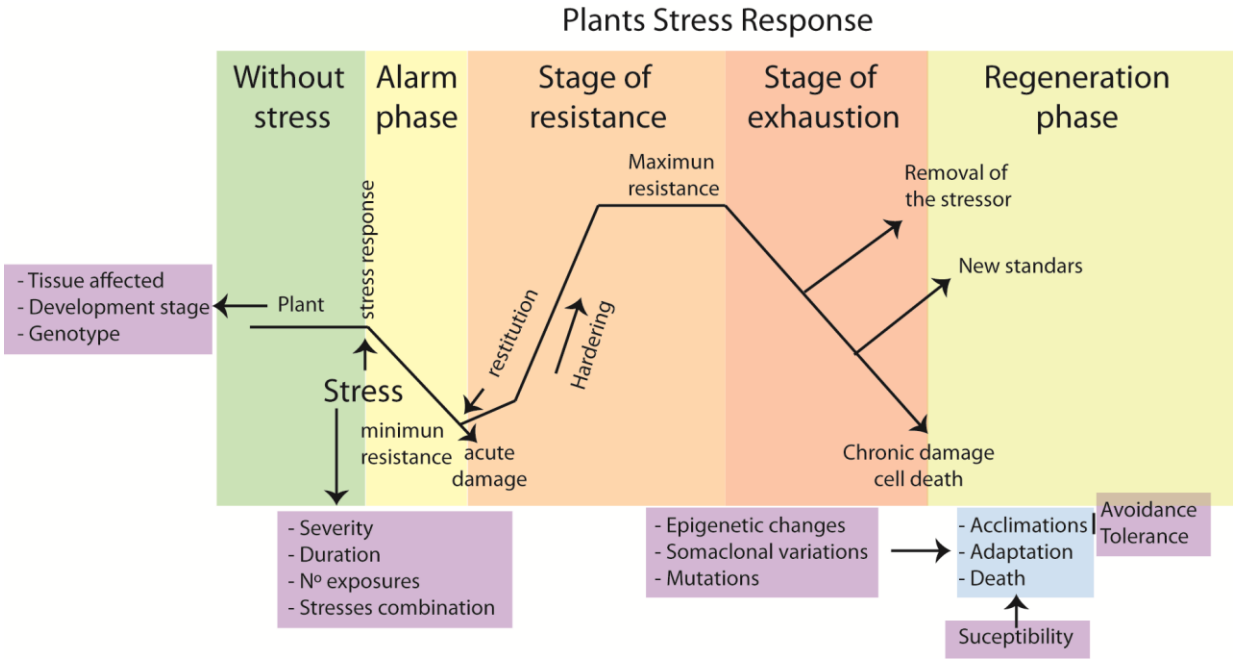

Fig. 1.1. Plant stress response. Scheme of plant responses to stress and characteristics that condition the response. Adapted from Lichtenthaler (1998) and Gaspar *et al*., (2002) .

During the first phase of both biotic and abiotic stress, plants have developed mechanisms to perceive external signals that optimize responses to environmental conditions. The production

of reactive nitrogen (RNS) and oxygen (ROS) species is a key process in both types of stress (Waszczak *et al*., 2018; Molina-Moya *et al*., 2019; Romero-Puertas *et al*., 2019). Also, phytohormones, which are endogenous molecules of low molecular weight that mainly regulate the protective response of the plant, are in turn controlled by ROS/RNS and *vice versa* (Ku *et al*., 2018; Cortleven *et al*., 2019; Li *et al*., 2019). Besides, other well known signalling molecules, such as calcium (Ca), and new ones, such as hydrogen sulfide (H_2S) and cyanide (CN^-), are involved in plant response to stress (Gotor *et al*., 2019; Marcec *et al*., 2019). Subsequently, mitogen-activated-protein kinase (MAPK) cascade is activated by these signalling moleculles, which in turn activates transcription factors essentials to iniciate plant stress response, also modulated by post-translational protein modifications (PTMs; Mittler, 2017; Fig. 1.2).

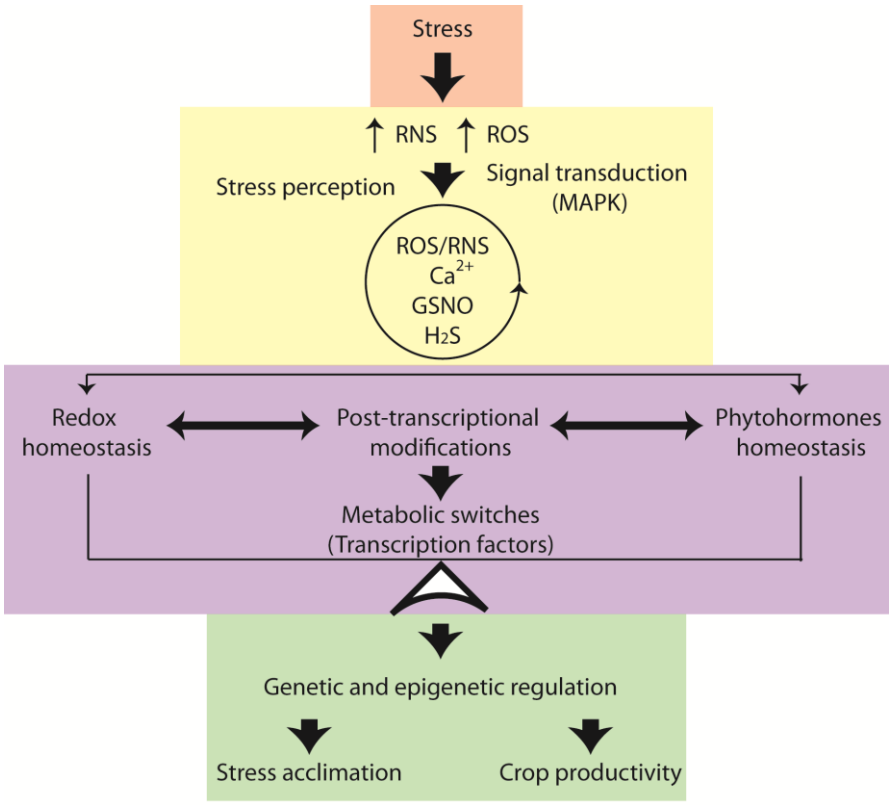

Fig. 1.2. Plant stress response signalling. Schematic diagram showing the central roles of reactive nitric species (RNS) and reactive oxygen species (ROS) in the signal transduction pathways that facilitate acclimation to stress allowing high crop productivity over a wide range of environmental conditions (adapted from Considine *et al*., 2015).

1.1.2. Reactive oxigen and nitrogen species (ROS/RNS): Signalling molecules in response to stress

Plants have developed mechanisms to perceive external signals that optimize responses to environmental conditions. The production of RNS and ROS is a key process in this response (Waszczak *et al*., 2018; Molina-Moya *et al*., 2019; Romero-Puertas *et al*., 2019). ROS are molecules derived from oxygen reduction (Mittler *et al*., 2011; Halliwell and Gutteridge, 2015) and include free radicals, such as superoxide ($O_2^{\cdot-}$), hydroxyl (·OH), alkoxy ions ($RO^{\cdot}$), peroxyl

($ROO^{\cdot}$) and hydroperoxyl (HO_2^{-}); and non-radicals, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3), and hypochlorous acid (HOCl). RNS are molecules derived from the reduction of nitrogen compounds, including nitric oxide (NO), which coexist with the molecules with a energetically more favourable electron structure, the nitrosonium cation (NO^+) and the nitroxyl radical (NO^-); and also, higher nitrogen oxides compounds (NO_2, N_2O_3, and N_2O_4) and peroxynitrite ($ONOO^-$). High nitrogen oxide compounds and $ONOO^-$ are the products of the reaction between NO and ROS (Bellin *et al*., 2013).

Concentration and subcellular location of RNS and ROS in plant tissue is essential for the function of these molecules (Mittler, 2017). A finely tuned balance between ROS and RNS scavenging and production is necessary to determine their level and impact as damaging or signalling molecules (Baxter *et al*., 2014; Romero-Puertas and Sandalio, 2016; Astier *et al*., 2018). In fact, high concentration of these molecules could be cytotoxic causing cellular damage mainly due to the reaction with lipids, proteins and nucleic acids. (Beligni and Lamattina, 2001; Foyer and Noctor, 2005; Neill *et al*., 2008; Romero-Puertas and Sandalio, 2016; Hancock and Neill, 2019). Instead, low concentration of ROS and RNS may function as signalling molecules in a variety of processes including growth and development, ion transport, defence, and cell death (Besson-Bard *et al*., 2009; Gibbs *et al*., 2014; Romero-Puertas and Sandalio, 2016; Turkan, 2018).

Other function of ROS and RNS is the regulation of gene expression and activation of secondary metabolism in response to environmental stimuli (Turpaev, 2002; Palmieri *et al*., 2008; Gaupels *et al*., 2011; Noctor and Foyer, 2016;). In addition to transcription regulation, others levels of regulation are also affected by these molecules. Post-transcriptional, translational and post-translational modifications make the plant's response system much more complex (Martínez-Ruiz *et al*., 2011; Choudhury *et al*., 2017; Mittler, 2017; Fig. 1.3). For example, H_2O_2 could oxidize sulphur-containing residues of protein in *–SH* cysteine group, alter protein structure and function mediated by the formation of disulphide bounds and can alter the transcription factors binding to DNA and affecting transcription (Dietz, 2016). Therefore, metabolic ROS production could directly alter the redox state of regulatory proteins, and control metabolic fluxes in the cell, in order to counter stress effects or altered transcription and/or translation of some genes and protein (Miller *et al*., 2010; Noctor and Foyer, 2016). A study which analyzes different 79 Affymetrix ATH1 microarray studies of redox homeostasis perturbation experiments showed differential expression of genes, specifically, 874 were up-regulated and 313 were down-regulated connected with ROS metabolism (Willems *et al*., 2016). In adittion, ROS and RNS are capable to self-regulation and regulating its partners affecting production and elimination of these molecules (Romero-Puertas and Sandalio, 2016).

Plants have developed antioxidant system to balance the acummulation of ROS and RNS (Miller *et al*., 2010; Wrzaczek *et al*., 2013; Schmitt *et al*., 2014; Sewelam *et al*., 2016). These include enzymatic and non enzymatic mechanisms. Several antioxidant enzymes have been described, such as superoxide dismutase (SOD), which dismutate $O_2^{\cdot-}$ to H_2O_2; catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and peroxiredoxins (PRXs), which remove H_2O_2. Non-enzymatic mechanisms include molecules, such as ascorbic acid, glutathione (GSH), carotenoids, and α-tocopherol (Noctor and Foyer, 1998; Asada, 1999; Mittler, 2002; Choudhury *et al*., 2017). Moreover, a network of more than 152 genes is involved in controlling ROS level in Arabidopsis (Mittler *et al*., 2004).

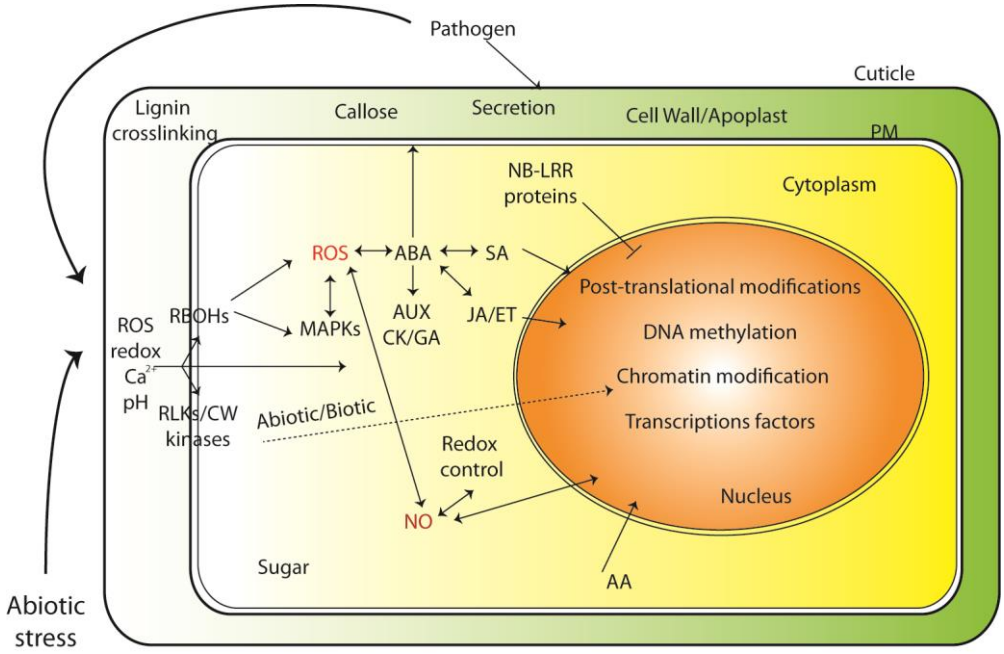

Fig. 1.3. Interaction and overlapping signalling pathways in plant response to abiotic and biotic stress. Homeostasis of signals are affected by stress. Signalling nodes such as RBOHs and RLKs and other cell wall kinases and MAPKs are activated with downstream signal specificity under stress. Hormones are positive or negative regulated depending on the biotic or abiotic stress affected by multiple strategies against damage in plant cells. Nuclear translocation of genes, redox state, effect of chromatin, DNA methylation and transcription factor expression are regulated in response to stress (adapted from Kissoudis *et al*., 2014; Sewelam *et al*., 2016). AA, aminoacid; NO, nitric oxide; ROS, reactive oxygen species; ABA, abscisic acid; SA, salicilic acid; JA, jasmonic acid; ET, ethylene; AUX, auxins; CK, citoquinins; GA, gibberellins; RBOH, respiratory burst oxidase homolog; RLKs, leucine rich repeat receptor like kinase CW, cell wall; MAPK, mitogen-activated protein kinases; NB-LRR, binding domain leucine-rich repeats.

1.1.3. Peroxisomes as a source of ROS/RNS

Peroxisomes are small round organelles with a single lipid bilayer membrane present in eukaryotic cells (Sandalio and Romero-Puertas, 2015; Kao *et al*., 2018; Olmedilla and Sandalio, 2019). These organelles are very dynamic and metabolically active, which participate in development, morphogenesis, senescence and plant responses to stress (Sandalio *et al*., 2013; Sandalio and Romero-Puertas, 2015). Peroxisomes have a close relationship with other organelles

such as mitochondria and chloroplasts sharing many metabolic and signalling pathways (Shai *et al*., 2016; Linka and Theodoulou, 2013; Mathur *et al*., 2018).

The main functions of peroxisomes are associated with metabolic pathways such as photorespiration, glyoxylate cycle, ureide metabolism, fatty acid β-oxidation, auxins, jasmonic acid (JA) and salicylic acid (SA) hormones biosynthesis and polyamine catabolism (Sandalio and Romero-Puertas., 2015; Kao *et al*., 2018; Olmedilla and Sandalio, 2019). Recently, other functions associated with β-oxidation have been described in non-storage tissue by proteomic approaches, such as benzoic acid, biotin, isoprenoid and vitamin K1 biosynthesis (Hu *et al*., 2012; Linka and Theodoulou, 2013; Reumann, 2013; Cassin-Ross and Hu, 2014).

In particular, these organelles are involved in ROS and RNS homeostasis allowing the regulation of cell metabolic pathways (Foyer and Noctor, 2003; Yun *et al*., 2012; Su *et al*., 2019). Different ROS including H_2O_2, $O_2^{\cdot-}$ and ·OH; and RNS including NO, $ONOO^-$, and nitrosoglutatione (GSNO) have been reported to be produced in peroxisomes (del Río, 2011; Sandalio and Romero-Puertas, 2015; Fig. 1.4). The production of H_2O_2 in peroxisomes is mediated by the enzymes glycolate oxidase (GOX) in the photorespiration cycle and acyl Co-A oxidase (ACX) in the β-oxidation route; the spontaneous or enzymatic dismutation of $O_2^{\cdot-}$ produced by different enzymes. $O_2^{\cdot-}$ production takes place in a short electron chain associated with NADH/NADPH-driven peroxisomal membrane, and by the enzymes xanthine oxidoreductase (XOD/XDH) and uricase in the peroxisomal matrix, both important in the catabolism of nucleic acids and ureide metabolism, respectively and polyamine oxidase involved in polyamine catabolism. OH is produced due to the presence of haem-and non haem iron-containing proteins and ascorbate mediated by Fenton reactions (Sandalio and Romero-Puertas, 2015). NO can be produced by nitric oxide synthase (NOS)-like activity in peroxisomes (del Río, 2011). Additionally, peroxisomes contain xanthine oxidase (XOD), polyamine oxidases and amine oxidases, which might be NO sources in these organelles (Antonenkov *et al*., 2010; Wimalasekera *et al*., 2011). Furthermore, $O_2^{\cdot-}$ and GSH may react with NO promoting the formation of $ONOO^-$ and GSNO, respectively, both detected in pea and Arabidopsis peroxisomes (Ortega-Galisteo *et al*., 2012; Corpas and Barroso, 2014; Sandalio and Romero-Puertas, 2015).

In addition, peroxisomes are able to regulate ROS levels by antioxidant enzymes such as CuZn-SOD, Mn-SOD, CAT, and the enzymes of Ascorbate-Glutathione (ASC-GSH) cycle containing ascorbate peroxidase (APX), monodeydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and gluthatione reductase (GR); and non enzymatic antioxidants such as ascorbate (ASC) and glutathione (GSH; Sandalio and Romero-Puertas, 2015). In Arabidopsis peroxisomes, it has been reported the presence of glutathione *S*-transferase, which participate in the detoxification of xenobiotic compounds and lipid peroxide products by removing

H_2O_2 (Dixon *et al.*, 2009). Glutathione peroxidase, peroxiredoxins, and thioredoxins may also be present in peroxisomes (Dietz, 2003; Du *et al.*, 2010; Fig. 1.4).

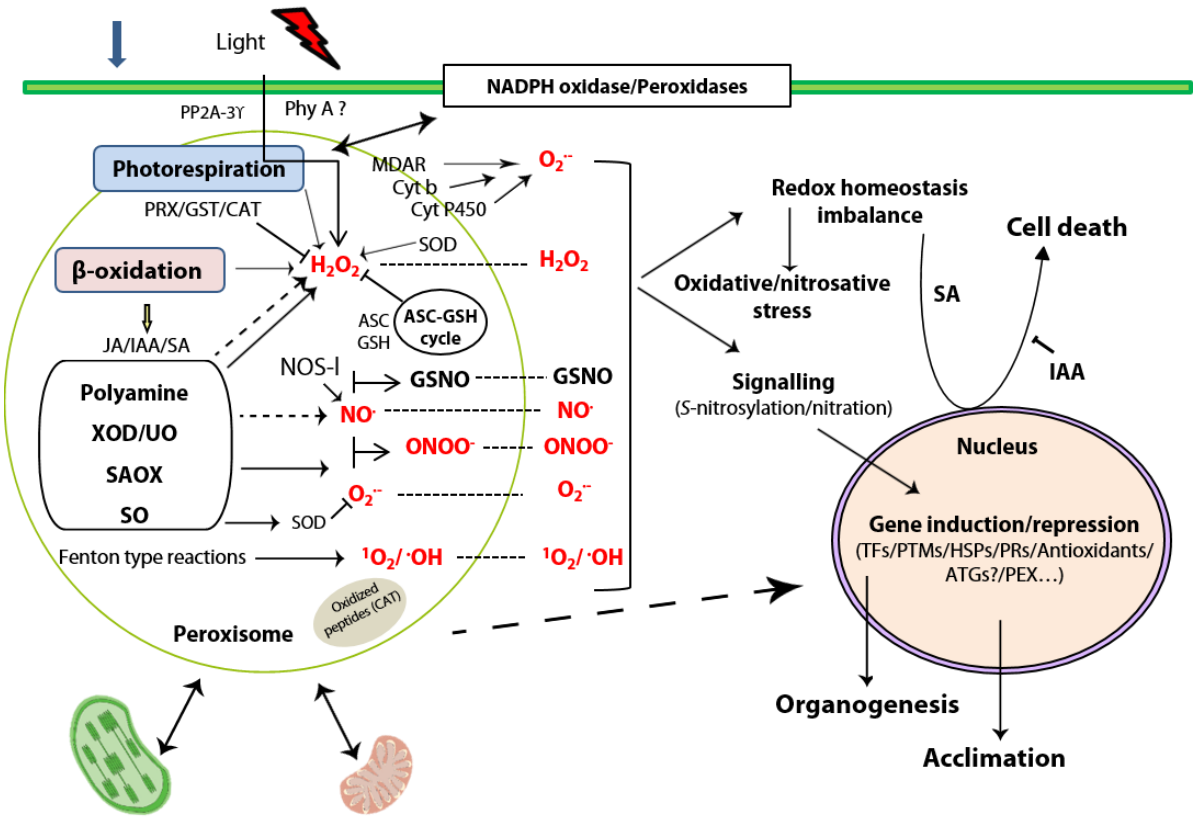

Fig. 1.4. Signalling networks involving ROS and RNS in peroxisomes. Electron transport chain and different metabolic pathways produce H_2O_2 and $O_2^{\cdot-}$. ROS are regulared by catalase (CAT), ascorbate-gluthation cycle (ASC-GSH), superoxide dismutases (SODs) and probably peroxiredoxin (PRX). ROS/RNS can diffuse to the cytosol, alter redox homeostasis and regulate gene transcriptions in the nucleus in order to produce acclimatization or cell death depending on the stimuli. H_2O_2 from peroxisomal photorespiration can be regulated by light causing changes in redox homeostasis, SA-dependent lessions and pathogenesis related protein induction. Hormones are produced by peroxisomes regulating cell response to stress. Peroxisomes produce NO, probably by NOS-like activity, xanthine oxidase (XOD) and/or polyamines. $ONOO^-$, GSNO and GSH are produced also in peroxisomes and can act as signalling molecules regulating gene expression and protein acivity throughout post-translational modifications (PTMs). Homeostasis and peroxisomal quality may be regulated by oxidized peptides. Also, ROS produced in peroxisomes promote cross-talk with different organelles (Sandalio and Romero-Puertas 2015).

1.1.4. Peroxisomes as a stress sensor

Peroxisomes are able to detect changes in their environment and modify their metabolism and dynamics in processes that appears to be regulated by ROS (Rodríguez-Serrano *et al.*, 2009; Sinclair *et al.*, 2009; Hu *et al.*, 2012). A "ROS/RNS signature", which is specific for localization, levels and timing of ROS/RNS production, is capable of triggering a specific response, although the mechanisms and components involved in recognizing and transmitting the information to the nucleus are unclear (Mittler *et al.*, 2011; Rosenwasser *et al.*, 2011; Sewelam *et al.*, 2014); however, peroxisomes appear to play an important role in this whole process.

Peroxisomes are highly dynamic organelles. The movement of the organelles is essential to communicate between each other and with other organelles for a good cellular functioning and signalling processes (Rodríguez-Serrano *et al*., 2009; Suzuki *et al*., 2012). When plants suffer a stress such as heavy metals, the peroxisome number increases through a complex process that involves lengthening, constriction and fission in a process termed proliferation (Oksanen *et al*., 2003; Mitsuya *et al*., 2010; Hu *et al*., 2012; Sandalio and Romero-Puertas, 2015; Fig. 1.5). Peroxisomes proliferation seems to be governed by H_2O_2 in animals and plants (López-Huertas *et al*., 2000; Rodríguez-Serrano *et al*., 2016) and can be a protection mechanism against oxidative stress. In response to H_2O_2 and diferent stresses, peroxisomal extensions called peroxules have been observed in Arabidopsis plants. Although their function is not clear, it has been suggested that peroxules might be involved in the lengthening of peroxisomes (Sinclair *et al*., 2009), and regulation of ROS dependent signalling pathways (Rodríguez-Serrano *et al.,* 2016). Peroxisomes can also change their speed under different conditions, among others in response to cadmium (Cd), being these changes regulated by ROS and Ca^{2+} (Rodríguez-Serrano *et al*., 2009).

Several functions have been proposed to explain the role played by the changes in the dynamics of peroxisomes, including the improvement of detoxification of H_2O_2 in different parts of the cell, improvement of signalling processes and also to facilitate the importation and transport of protein to other organelles (Rodríguez-Serrano *et al*., 2009). Therefore, peroxisomes can act as ROS and RNS sensors triggering a very specific and rapid response to environmental signals, although the mechanisms underlying this process remain unknown.

To maintain cell homeostasis, it is necessary to control the proliferation of peroxisomes by eliminating excess or damaged peroxisomes. Autophagy is a catabolic process which allows to remove, degrade, and recycle damaged and unnecesarry cells components and organelles. Selective authophagy of peroxisome is called pexophagy (Avin-Witternberg *et al*., 2018; Olmedilla and Sandalio, 2019; Fig. 1.5) and might be controled by the peroxisomal protease (Shibata *et al*., 2013; Baker and Paudyal, 2014).

Studies have shown that peroxisome proliferation is involved in fast cell response to several stress such as excessive light (Desai and Hu, 2008), salinity (Mitsuya *et al*., 2011), ozone (Oksanen *et al*., 2003), H_2O_2 application (López-Huertas *et al*., 2000), xenobiotics (Castillo *et al*., 2008) and the heavy metal Cd (Rodríguez-Serrano *et al*., 2016) by and therefore peroxisomal population needs to be regulated. The protein Neighbor of Brca1 gene 1 (NBR1) could act as receptor of pexophagy. Proteases such as caspase 6, legumain and cathepsin seem to have also an importan role in pexophagy. All this process is triggered by ROS and peroxisomal protein oxidation (Calero-Muñoz *et al*., 2019).

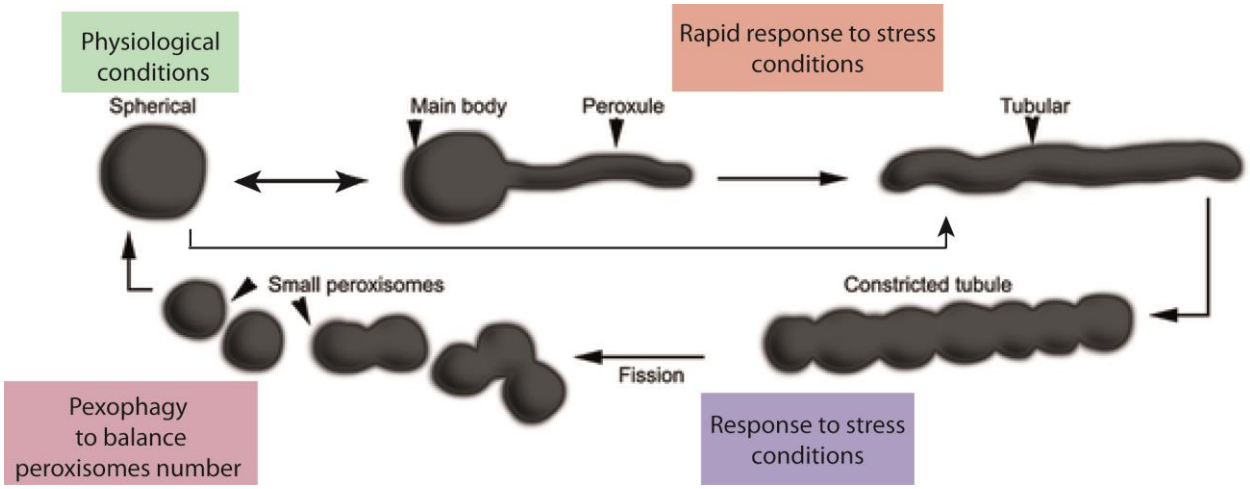

Fig. 1.5. Representation of peroxisome forms observed in plants. In control conditions, peroxisomes present spherical forms. However, when plants suffer stress conditions, peroxisomes produce peroxule as a quick response to stress, followed by elongation and constriction producing an increment in the number of peroxisomes. Finally, peroxisomal population is regulated by pexophagy. Modified from Mathur *et al.,* (2018) and Calero-Muñoz *et al*., (2019).

Under oxidative stress conditions, many peroxisomal proteins can undergo carbonylation processes (Romero-Puertas *et al*., 2002). These proteins may have dangerous effects within peroxisomes and therefore must be degraded by proteases (Romero-Puertas *et al*., 2002; Farmer *et al*., 2013; Quan *et al*., 2013; Shibata *et al*., 2014). Nitration and *S*-nitrosylation have also been shown to affect some peroxisomal proteins mainly involved in photorespiration, β oxidation and antioxidant defences (Ortega-Galisteo *et al*., 2012; Sandalio *et al*., 2019).

1.2. Nitric oxide as an essential molecule in abiotic and biotic stress response

NO is a small, gaseous, radical medium-short-lived and low molecular weight molecule essential in the physiology of organisms. It is also soluble although, to a lesser extent may be in hydrophilic environments and due to its physico-chemical properties, NO may cross biological membranes by passive transport (Neill *et al*., 2003). From a redox point of view, NO is a weak oxidizing agent and a strong reducing agent (Trapet *et al*., 2015). NO has a high affinity for transition metals, molecular oxygen, and $O_2^{\cdot-}$ and also interacts with the nitrogen (N) and sulfur (S) presents in proteins, being the chemical basis of the functions that NO exerts in organisms.

1.2.1. Nitric oxide metabolism

The main sources of NO in animals is a well-characterized enzyme family, called nitric oxide synthase (NOS; Bruckdorfer, 2005). In contrast, the biosynthesis of NO in plants is a more complex process and several mechanisms have been described. Therefore, as oxidation state of N in NO is intermediate between the oxidized forms such as nitrate and nitrite; and the reduced forms such as the amino groups of amino acids and ammonium, plants are able to produce NO through either oxidative and reductive pathways (Astier *et al*., 2018). A wide collection of data on NO

production in several plant species and locations within the plant under different situations and with different temporal patterns point to a coexistence of different pathways for NO production and to a probably self- and cross-regulation between them (Rasul *et al*., 2012; Du *et al*., 2016; León *et al*., 2019; Terrón-Camero *et al*., 2019).

Within the reductive pathways, the nitrate reductase (NRs), which is capable of reducing nitrite to NO under normoxic conditions and depending of nitrite concentration and pH (Yamasaki and Sakihama., 2000; Rockel *et al*., 2002), and the mitochondrial electron transport chain (mETC) by using nitrite as electron acceptor under anaerobic/hypoxic conditions (Gupta *et al*. 2005; Stoimenova *et al*., 2007; Gupta and Igamberdiev, 2011; Kumari *et al*., 2019; Fig. 1.6) are the main sources of NO in higher plants. There are two loci coding for nitrate reductase, in Arabidopsis plants, *NIA1* and *NIA2*. Recent studies showed that NIA2 may functions mainly in nitrate reduction and NIA1 in NO synthesis (Mohn *et al*., 2019). *nia1 nia2* Arabidopsis mutant, with less than 1% of nitrate reductase activity, is a handful tool to analyse NO sources and NR function in different conditions (Yamasaki and Sakihama, 2000; Desikan *et al*., 2002; Rockel, 2002; Guo *et al*., 2003; Modolo *et al*., 2005; Moreau *et al*., 2008). Other ways to produce NO by the reductive route are the Nitric Oxide-Forming Nitrite Reductase (NOFNiR), which belongs to the Amidoxine Reducing Component protein family in the mitochondria (mARC); the xanthine dehydrogenase-oxidase, which may produce NO under anaerobic conditions or phosphate deficiency (Godber *et al*., 2000; Wang *et al*., 2010; Gupta *et al*., 2011) and the non-enzymatic production described in the apoplast from roots in an acidic ambient (Wendehenne *et al*. 2001; Störh and Stremlau, 2006; Bethke *et al*., 2004; Fancy *et al*., 2017). Although it has been recently shown the interaction of NR with NOFNiR to produce NO in *Chlamydomonas* (Chamizo-Ampudia *et al*., 2017) this mechanism still need to be ascertained in higher plants.

Concerning oxidative pathways of NO production, activities similar to mammalian arginine-dependent NOS (NOS-1) have been analysed in plants, showing the functionality of this pathway through the use of NOS inhibitors and by heterologous expression of mammalian NOS (Yamasaki and Cohen, 2006; Moreau *et al*., 2010; Frungillo *et al*., 2014; Astier *et al*., 2018). However, the identification of the gene and/or protein involved in this activity in higher plants has not been successful yet (Cheng *et al*., 2015; Jeandroz *et al*., 2016). Different bionformatics approaches in Arabidopsis, rice and in more than 1000 land plants with sequenced genomes showed no NOS gene/protein in higher plants (Jeandroz *et al*., 2016; Hancock and Neill, 2019) and the only NOS enzymes described in the plant kingdom belong to algae (Foresi *et al*., 2015; Astier *et al*., 2019). Furthermore, it has been also questioned the NO-cGMP signalling works in plant as in mammals (Astier *et al*., 2019; Fig. 1.6).

Although NO-dependent genes have been associated with the oxidative pathway, different mutants with altered NO levels are useful tools for analising NO role in different conditions. Therefore, a guanosine triphosphatase (GTPase) encoded by *Arabidopsis thaliana* NO-associated protein 1 (AtNOA1) has been proposed to contribute indirectly to NO production in response to abscisic acid (ABA; Fancy *et al*., 2017) and the NO-deficiency in the Arabidopsis mutants *Atnoa1/rif1* appears to be related with a disfuntion in chloroplast (Gas *et al*., 2009; Misra *et al*., 2014). Two NO-overproducers, which show increased levels of arginine have been described in Arabidopsis: *nox1/cue1*, which have altered a chloroplast phosphoenolpyruvate/phosphate translocator (Streatfield *et al*., 1999; He *et al*., 2004) and *argh1-1*, which lacks arginase gene (Flores *et al*., 2008; Fig. 3.6).

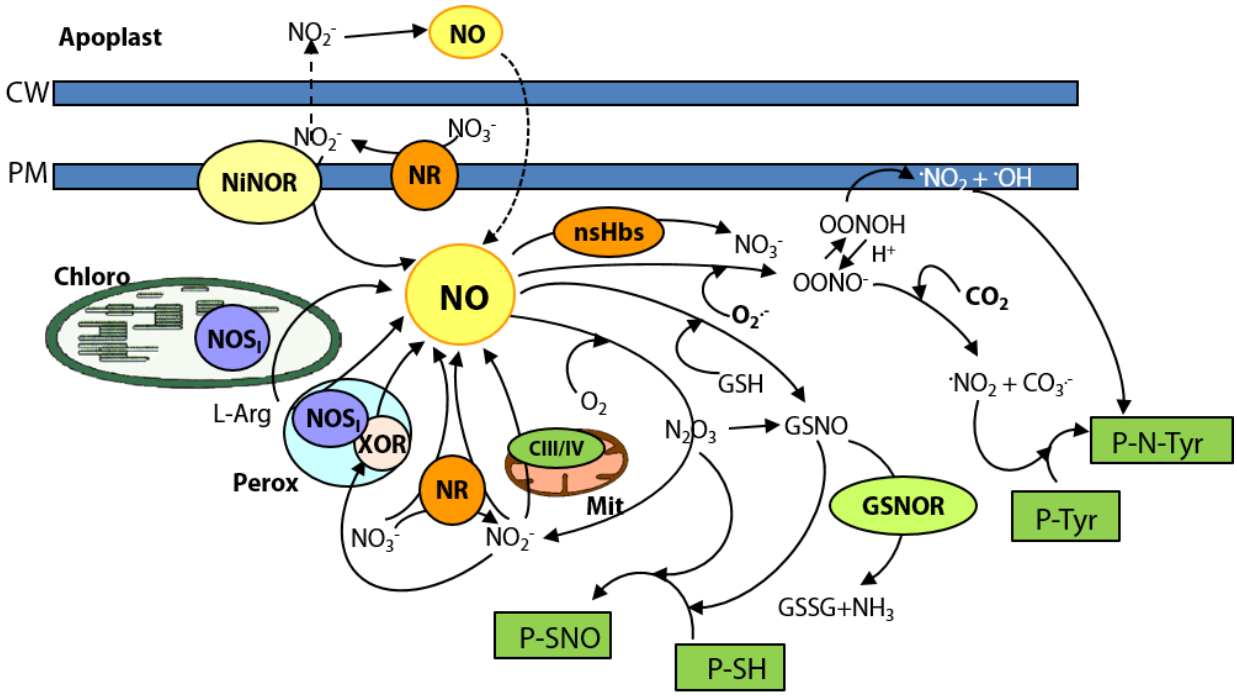

Fig. 1.6. Overview of NO production, metabolism and scavenging in plants. The figure shows a diagram of the main sources described for NO production, including oxidative (arginine- or hydroxylamine-dependent) and reductive (nitrate-dependent) pathways. The main scavengers for NO including haemoglobins, oxygen, GSH and superoxide ion is also showed. Abbreviations: GSH, glutathione; GSNO, nitrosoglutathione; GSNOR, GSNO reductase; nsHbs, non-symbiotic haemoglobins; NOSl, activity that resemble NO production as catalyzed by the animal enzyme NOS; NiNOR, plasma membrane-bound NiNOR; NR, nitrate reductase; XOR, xanthine oxidoreductase (Romero-Puertas and Sandalio, 2016).

As a signalling molecule, NO levels should be strongly regulated having in mind that an excess of NO is toxic for the cell. Therefore, NO levels are tightly regulated by different molecules and enzymes. NO reacts rapidly with O_2 and its derivatives ($O_2^{\cdot-}$), producing with the first nitrogen dioxide (NO_2), which degrades to nitrite and nitrate in aqueous solution (Neill *et al*., 2008). The reaction between NO and $O_2^{\cdot-}$ will form $ONOO^-$, which is a powerful oxidant agent leading to lipid peroxidation and protein nitration, (Ischiropoulos and Al-Mehdi, 1995; Radi, 2004). The nitration of tyrosine (Tyr) residues is a NO-dependent PTM consisting in the addition of a nitro group

(−NO_2) to a Tyr side chain (Radi, 2004; Rubbo and Radi 2008). $ONOO^-$ can also induce the production of oxygenated forms of Cys residue, such as sulfenic, sulfinic and sulfonic acids (-SOH; -SO_2H and -SO_3H), and *S*-glutathionylation (Martínez-Ruiz *et al*., 2013). NO can react with lipid peroxyl radical (LOO·) to produce nitro-fatty acids (NO_2-FAs), although the mechanism is not well known (Rubbo, 2013).

NO also reacts with glutathione (GSH) producing nitrosoglutation (GSNO). This molecule is considered a reservoir of NO, although it appears to have free NO-independent functions in plant response to stress (Yun *et al*., 2016). GSNO levels are regulated by GSNO reductase (GSNOR; Liu *et al*., 2001, Sakamoto *et al*., 2002), which produce oxidized glutathione (GSSG) and ammonia, being a key enzyme in different processes regulated by NO from development to stress (Rusterucci *et al*., 2007; Feechan *et al*., 2005; Frungillo *et al*., 2014; Jahnová *et al*., 2019). On the other hand, NO can also react with transition metals with the formation of complex bonds to heme groups (so-called nitrosylation), such as guanylate cyclase, Cyt p450 and haemoglobin (Martínez-Ruiz and Lamas, 2009) being the nitrosylation of haemoglobin heme group also described in plants (Perazzolli *et al*., 2004; Igamberdiev *et al*., 2006).

Finally, non-symbiotic hemoglobins (ns-Hbs; so-called phytoglobins) may function as NO dioxygenases metabolizing NO to nitrate (Perazzolli *et al*., 2004; Seregelyes *et al*., 2004; Hill *et al*., 2016). NO may induce Phytoglobin 1 (*PHYTOGB1*) gene expression in several species and different conditions (Perazzolli *et al*., 2004; Bustos-Sanmamed *et al*., 2011; Bai *et al*., 2016). Transgenic lines with altered levels of nsHbs showed an important role for NO bioactivity under stress conditions, especially during plant-microbe interaction (Fukudome *et al*., 2016; Martínez-Medina *et al*., 2019a). It has been shown that the induction of *PHYTOGB1* expression under stress conditions could produce an increase in NR-dependent NO (Gupta *et al*., 2011; Trevisan *et al*., 2011; Fig. 1.6).

As far as we know, NO's principal mode of action in plants has been reported to be dependent on covalent protein post-translational modifications (PTMs). These PTMs are carried out by a series of RNS produced by the reaction of NO with other free radicals. The best known NO-dependent PTM in plants is *S*-nitrosylation, also called *S*-nitrosation (Martínez-Ruiz and Lamas, 2004), which involves the formation of a nitrosothiol in a Cys residue and changes the function, location and/or stability of a number of proteins (Sandalio *et al*., 2019; Sánchez-Vicente *et al*., 2019). *S*-nitrosylation facilitates gene regulation through the modification of transcription factors (TFs; Spoel *et al*., 2010; Cui *et al*., 2018) and the DNA methylation index by altering enzymes involved in the methylation cycle (Engel *et al*., 2018). NO also interacts with most phytohormone-dependent signalling pathways through the *S*-nitrosylation of enzymes involved in phytohormone-dependent regulatory proteins and/or biosynthetic pathways (Gibbs *et al*., 2014;

Albertos *et al*., 2015; Castillo *et al*., 2018; Sánchez-Vicente *et al*., 2019). It has been described also in plants that *S*-nitrosylation may result in the establishment of a disulfide bond, which is a more stable modification (Serrato *et al*., 2018). In large-scale proteomic studies, over 1000 proteins have been shown to be putative targets of *S*-nitrosylation in plants (Kovacs *et al*., 2013; Hu *et al*., 2015), although the functional impact of this PTM has only been analysed in approximately 2% of these proteins (Sánchez-Vicente *et al*., 2019). Less is known about the NO-dependent PTM tyrosine nitration in plants (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2019). Fewer putative targets of nitration have been identified in plants as compared to those of *S*-nitrosylation (Lozano-Juste *et al*., 2011; Vandelle and Delledonne, 2011).

Interestingly, NO and its derivatives are able to regulate NO levels, mainly through PTMs of enzymes involved in NO metabolism. Thus, GSNO inhibits nitrate uptake preventing NR-dependent NO production (Frungillo *et al*., 2014). In addition, it has been suggested that *S*-nitrosylation of NR may decrease its activity under different stress conditions (Cheng *et al*., 2015; Du *et al*., 2016; Fu *et al*., 2018). In a similar way, GSNOR activity is regulated by *S*-nitrosylation controlling GSNO and nitrosothiol levels in the plant (Frungillo *et al*., 2014). In addition, nsHbs have been shown to be target of *S*-nitrosylation (Perazzolli *et al*., 2004; Rubio *et al*., 2019) being the AtnsHb1 able to scavenge NO through the production of *S*-nitrosohemoglobin in plants under hypoxic stress (Perazzolli *et al*., 2004). On the other hand, *S*-nitrosylation of PrxIIE leads to inhibition of its activity diminishing its ability to detoxify $ONOO^-$ during the incompatible interaction (Romero-Puertas *et al*., 2007).

1.2.2. NO-ROS interactions

ROS and NO interact each other producing other compounds that will induce different effects on the plant. One of the first event where the interaction between NO and ROS was shown during the hypersensitive response (or programmed cell death in the incompatible interaction), since it requires a tight regulation of H_2O_2 and NO ratio (Delledonne *et al*., 2001).

In addition to regulate its own level, NO is also able to control both, ROS-producing enzymes and antioxidant system (Romero-Puertas and Sandalio, 2016). Therefore, NO is able to affect CAT activity by nitration, *S*-nitrosylation and by reacting directly with the hemo group (Clark *et al*., 2000; Lozano-Juste *et al*., 2011; Ortega-Galisteo *et al*., 2012; Chaki *et al*., 2015). On the other hand, NO is also able to regulate the production of H_2O_2 by *S*-nitrosylation of GOX, which is one of the main sources of H_2O_2 in the peroxisome in response to Cd (Ortega-Galisteo *et al*., 2012) and APX activity appears to be inhibited by nitration (Begara-Morales *et al*., 2014). In contrast, *S*-nitrosylation of APX seems to be a process dependent on the environment, species and/or stress conditions, since different results have been found (Bai *et al*., 2011; Correa-Aragunde

et al., 2013, 2015; de Pinto *et al*., 2013; Yang *et al*., 2015). Moreover, NO can react with the hemo group of APX reversibly during the resistance response to modulate its activity (Clark *et al*., 2000). On the other hand, $ONOO^-$ inhibits by Tyr nitration the mitochondrial Mn-SOD1, peroxisomal Cu/Zn-SOD3, and chloroplastidial Fe-SOD3 (Holzmeister *et al*., 2015).

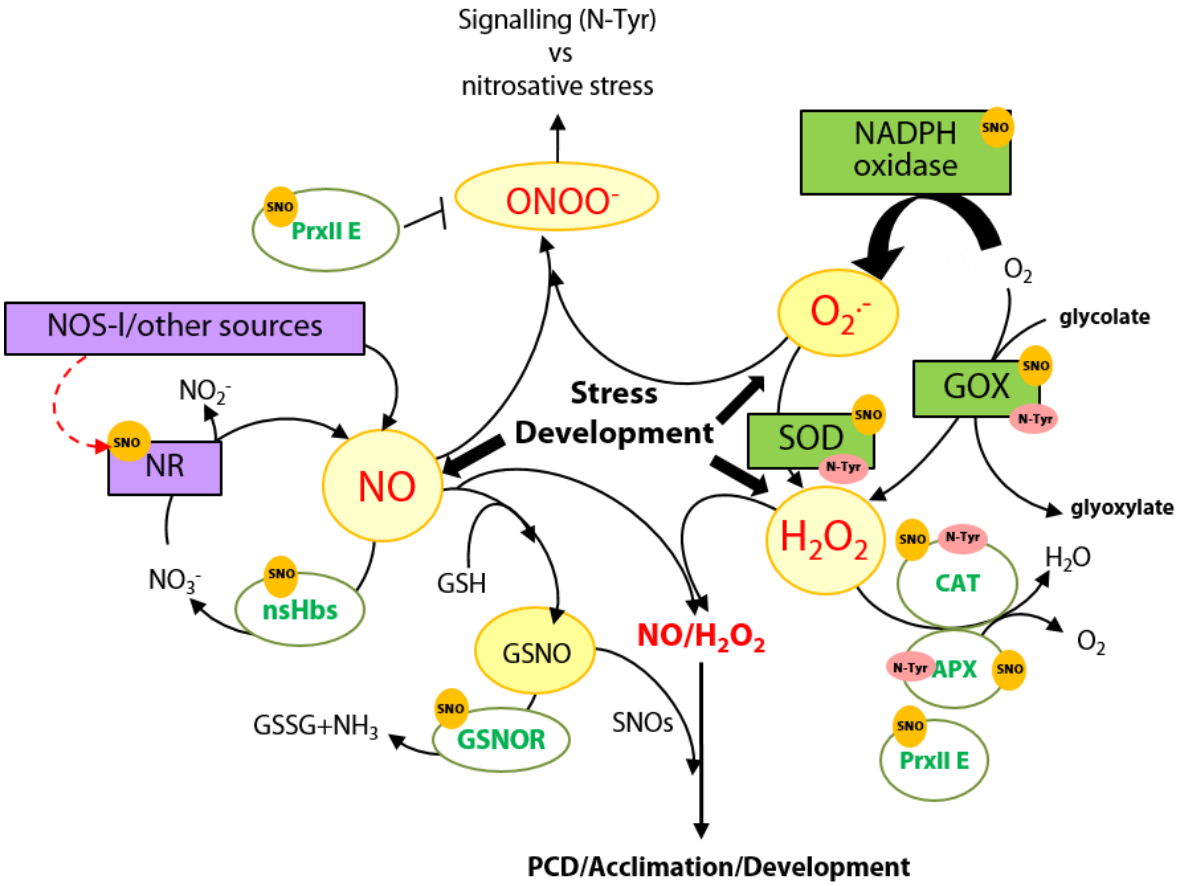

Fig. 1.7. Overview of NO and ROS level regulation by NO. The figure shows a diagram of the main targets of *S*-nitrosylation (SNO), nitrosylation (Haem-NO), or nitration (N-Tyr) described in plants that affected NO and ROS production and regulation. CAT, catalase; GSH, glutathione; GOX, glycolate oxidase; GSNO, nitrosoglutathione; GSNOR, GSNO reductase; nsHbs, non-symbiotic hemoglobins; NOS-l, activity that resembles NO production as catalyzed by the animal enzyme NOS; NiNOR, plasma membrane-bound NiNOR; NR, nitrate reductase; XOR, xanthine oxidoreductase (Romero-Puertas and Sandalio, 2016).

1.3. Nitric oxide in abiotic stress

NO is a highly reactive molecule that plays an important role in practically all processes in plants, such as seed germination, growth, tuber formation, stomatal closure, pollen self incompatibility, pollen development and senescence processes (Hancock and Neill, 2019; León and Costa-Broseta *et al*., 2019). In particular, NO has been involved in plant responses to abiotic stress (Sánchez-Vicente *et al*., 2019; Terrón-Camero *et al*., 2019).

It is well-documented that exogenous application of NO in non toxic concentrations plays a protective role during salt, drought, low-temperature, heat, ozone and heavy metal stresses where

NO-dependent PTMs afecting NO-gene regulation and NO interaction with hormones and ROS, as described above, are the mechanisms underlying this protection (Fancy *et al*., 2017; Terrón-Camero *et al*., 2019; Chapter 4.1). Therefore, NO has been shown to enhance antioxidant enzymes, peptidases, photosynthesis rate and hormones metabolism (Zhao *et al*., 2001; Lei *et al*., 2007; Simontacchi *et al*., 2015). In addition, an analysis of the effect of an NO donor on Arabidopsis transcriptome highlights the importance of *S*-nitrosylation, pointing to about 700 NO dependent TFs which are potentially involved in physiological processes including multiple stresses responses (Imran *et al*., 2018; Sami *et al*., 2018).

On the other hand, although NO has been extensively documented as a key signalling molecule in plants, the function of endogenous NO sources and NO-dependent pathways involved in plant response to stress are not fully understood. NO production has been shown in plant response to abiotic stress extensively (Fancy *et al*., 2017; Terrón-Camero *et al*., 2019). Genetic and biochemical approaches, such as available mutants with NO metabolism altered and NO donors/sequesters have been used to get a deeper insight into NO role in plant response to stress. Thus, *Atnoa1* mutants have been analised under salt stress showing the importance of NO in antioxidant regulation (Zhao *et al*., 2007a, 2007b). In addition, *nia1 nia2* has been studied in plant response to drough stress showing the key role of NO in ABA-dependent stomata clousure (Desikan *et al*., 2002; Lozano-Juste and León, 2010). *nox1* and *AtGSNOR1* mutants have been shown to be sensitive to heat stress implying that NO/SNOs level may correlate with thermotolerance (Hong *et al*., 2003; Lee *et al*., 2008).

In sumary, endogenous NO may operate by using the same mechanisms of exogenous NO althogh the effect of exogenous NO could differ since concentration, timing and location is fundamental in plant response to stress (Terrón-Camero *et al*., 2019). Further work is required to fully understand NO-dependent mechanisms in plant response to abiotic stress.

1.3.1. Cadmium as an environmental problem

Cd is a heavy metal not essential for life (Ismael *et al*., 2019; Zhang and Reinolds, 2019) and even it is toxic for living organisms at low concentrations (Li *et al*., 2019; Zhang and Reinolds, 2019). Cd toxicity is due to its chemical properties as it has a type II oxidation state, being an electronegative element that can form complexes with a great variety of ligands, mainly with weak donors such as sulfur, nitrogen and selenium (Cabot and Poschenrieder, 1988; Salt and Wagner, 1993; Ismael *et al*., 2019). In addition, Cd affects different ecosystems causing problems at atmospheric, terrestrial and marine levels (Pinto *et al*., 2004; Gupta and Sandalio, 2012; Li *et al*., 2019). Cd first enters roots through the cortex and then is translocated to whole plant tissues through the vascular tissue. Cd can entry into the food chain, which constitutes both an

environmental and health risk wordwide (Nawrot *et al*., 2006; Dong *et al*., 2010; Clemens *et al*., 2013).

Although Cd is not abundant in the earth's crust (between 0.08 to 0.1 ppm), its concentration has been increasing in the last century as a consequence of human action (Rudnick and Gao, 2003; Gupta and Sandalio, 2012; Cullen and Maldonado, 2013). The European Environment Agency (EEA) published in 2018 a report comparing emissions from 1990 to 2016, showing a decrease of about 64% in this period mainly due to a decrease in Cd emission in agriculture processes and waste. Even so, the Agency for Toxic Substances and Disease Registry (ATSDR) in 2017 considered Cd, as the seventh most important toxic substance due to its toxicity and potential exposure to humans (http: //www.atsdr.cdc. gov /). The main sources of Cd emissions are shown in Fig. 1.8A, highlighting energy use in industry (29%), industrial processes and products use (28%), and commercial, institutional and households (21%; https://www.eea.europa.eu/; Fig. 1.8B).

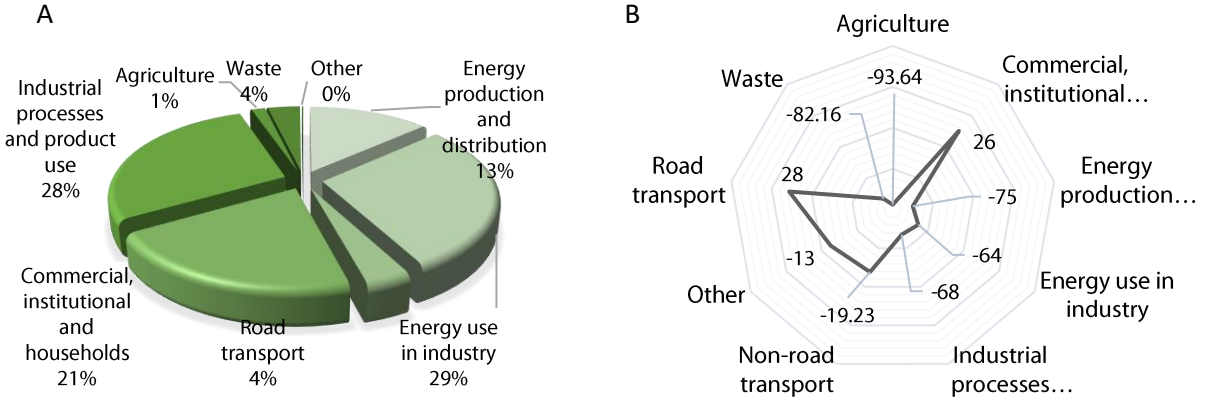

Fig. 1.8. Cadmium emissions to the environment. (A) Sector split of emissions of Cd and (B) changes in Cd emissions for each sector. Data was obtained from European Environment Agency actualized in 2019 https://www.eea.europa.eu/.

Phytoremediation has demonstrated to be an alternative tool to decontaminated soils, which it is a cost effective and environmentally friendly. Approximately 500 taxa have been described as plant hyperaccumulators of one or more metals (Li *et al*., 2019). In addition, further studies mixing biotechnological approaches with multidisciplinary research is needed to improve plant tolerance and decrease the accumulation of toxic metals in soils (Sanz-Fernández *et al*., 2017; Romero-Puertas *et al*., 2019; Li *et al*., 2019; Chapter 6.1).

1.3.2. NO role in plant response to cadmium stress

Several works have described NO production in plant response to Cd stress, although the NO role and NO-dependent mechanisms underlying this response are not clear. From the literature,

results can be divided in two groups: one dealing with the role of NO supplied exogenously before and/or during metal exposure and the other devoted to endogenous NO production (produced by the plant) in plant response to the metal. Therefore, the use of NO donors have mainly shown that NO induces antioxidant system avoiding ROS production and oxidative damage although other possible effects of protection by NO have been described (reviewed in Terrón-Camero *et al*., 2019, Chapter 4.1; Romero-Puertas *et al*., 2019, Appendix 8.1). On the other hand, endogenous NO production during plant responses to Cd stress has also been showed mainly after short-term treatments, while a general decrease has been shown after long-term Cd treatment. The role of endogenous NO in plant response to Cd stress is still not clear, having in mind that NO levels should be tightly regulated as an excess of NO appears to promote Cd-dependent root-growth inhibition, and disturbances in Cd uptake and antioxidant system. Different studies also shown that NO-dependent PTMs regulate ROS metabolism, phytochelatins and proteolysis in plant responses to heavy metal stress. Furthermore, transcriptomic analyses have revealed the presence of signalling pathways such as Ca, MAPKs, hormones and TFs (reviewed in Terrón-Camero *et al*., 2019, Chapter 4.1; Romero-Puertas *et al*., 2019, Appendix 8.1).

1.4. Plant-pathogen interactions: role of ROS and RNS

Plants coexist with a variety of microbies, which can be pothentialy pathogenic or beneficial for them (Lenk and Thordal-Christensen, 2009; Dangl *et al*., 2013) and have developed an immune system able to discriminate among them. Plants have pattern recognition receptors (PRRs), which recognize molecular patterns (chitin in the case of fungus) called pathogen-associated molecular patterns (PAMPs), microbe-associated molecular patterns (MAMPs) or damage associated molecular patterns (DAMPs; Mackey and McFall, 2006; Boller and Felix, 2009;). These molecules trigger an active response called PAMP/MAMP activated plant immunity (PTI). Pathogens may evade this PTI or block plant defence mechanisms through effectors which promote infection (Chisholm *et al*., 2006; da Cunha *et al*., 2006; Couto and Zipfel, 2016). However, plants have a second layer of perception mechanism that may recognize effectors by NB-LRR with a high variability that allows coevolution with microbes (Dodds and Rathjen, 2010). This recognition induces immunity activated by the effectors (ETI; Couto and Zipfel, 2016). Both, ETI and PTI responses are carried out by Ca^{+}/other ions, MAPK signalling cascade, and a ROS/RNS balance. ETI response is faster and stronger than PTI and can lead to programed cell death (PCD) of invaded tissue producing the so-called hypersensitive response (HR) and avoiding pathogen dispersion. In contrast, PTI response is effective for adapted pathogens, although it will depend on each pathogen (Dodds and Rathjen, 2010; Dangl *et al*., 2013; Fig. 1.9.).

After plant-pathogen recognition, a rapid ions flow (K^{+}, NO_3, Ca^{2+} and H^{+}) is established in plasma membrane (Wendehenne *et al*., 2002; Boller and Felix, 2009), followed by an oxidative

burst produced by RBOH D and F proteins, which are PRR dependent (Simon-Plas *et al*., 2002; Torres *et al*., 2002, 2010; Yoshioka *et al*., 2003; Zhang *et al*., 2007; Chinchilla *et al*., 2007; Kadota *et al*., 2015). RBOHD regulation is needed to avoid cellular damage and Ca is required to activate its phosphorylation, mediated by Ca-dependent protein kinases (CDPK; Ogasawara *et al*., 2008; Boudsocq *et al*., 2010; Kadota *et al*., 2015). *Botrytis* induced Kinase 1 (BIK1) and is able to phosphorylate RBOHD directly in the cytoplasm as a specific response (Kadota *et al*., 2014; Li *et al*., 2014; Qi *et al*., 2017). Although RBOHD is involved in the oxidative burst, Arabidopsis *rbohD* mutants are not more susceptible to pathogen in incompatible interactions and it has been hypothesized that RBOHD might be involved in preventing spread of PCD (Torres and Dangl, 2005). On the other hand, peroxidases class III can produce H_2O_2 in the apoplast (Brown *et al*., 1998; O'Brien *et al*., 2012) and may also be involved in the HR, callose content and the expression of MAMP-dependent defence genes (Bindschedler *et al*., 2006; Daudi *et al*., 2012; O'Brien *et al*., 2012; Qi *et al*., 2017). Different functions have been asigned to ROS produced in response to pathogens such as cell wall reinforcements, phytoalexin production, stomatal closure, hormones signalling, TFs expression and acquired systemic resistance (SAR; Kotchoni and Gachomo, 2006; Torres *et al*., 2010; O'Brien *et al*., 2012; Daudi *et al*., 2012; Kadota *et al*., 2014 Skelly *et al* 2016; Fig. 1.9)

A concomitant NO burst has been observed during ETI responses. It has been described in different species such as soybean, Arabidopsis and tobacco in response to effectors (Delledonne *et al*., 1998; Lamotte *et al*., 2004; Zeidler *et al*., 2004; Courtois *et al*., 2008; Gaupels *et al*., 2011; Rasul *et al*., 2012). MAMPs, and DAMPs such as lipopolysacaccharides, cryptogein or oligogalacturonides also induced a NO increment in plants (Trapet *et al*., 2015). This NO peak is important for reprograming gene expression, secondary metabolites production and finally, the HR response and SAR developing (Bellin *et al*., 2013b; Wendehenne *et al*., 2014). During PTI response both pathways, oxidative (L-Arginine dependent) and reductive (nitrate reductase, NR) have been involved. Furthermore, it has been shown that mutants with altered NO levels, such as *nox1* and *nia1 nia2* are more susceptible to *Pseudomonas syringae* (Oliveira *et al*., 2010; Vitor *et al*., 2013; Yun *et al*., 2016). On the other hand, dioxygenase activity of phytoglobins has been shown to modulate NO levels in plant immunity (Hebelstrup *et al*., 2014). During incompatible interaction, NO has been shown to be essential for HR (Delledonne *et al*., 1998, 2001; Wang *et al*., 2013; Trapet *et al*., 2015). Main sources of NO in this case appears to be NOS-l dependent (Delledonne *et al*., 1998; Romero-Puertas *et al*., 2004; Chen *et al*., 2014) and to a lesser extent NR (Modolo *et al*., 2005, 2006; Chen *et al*., 2014).

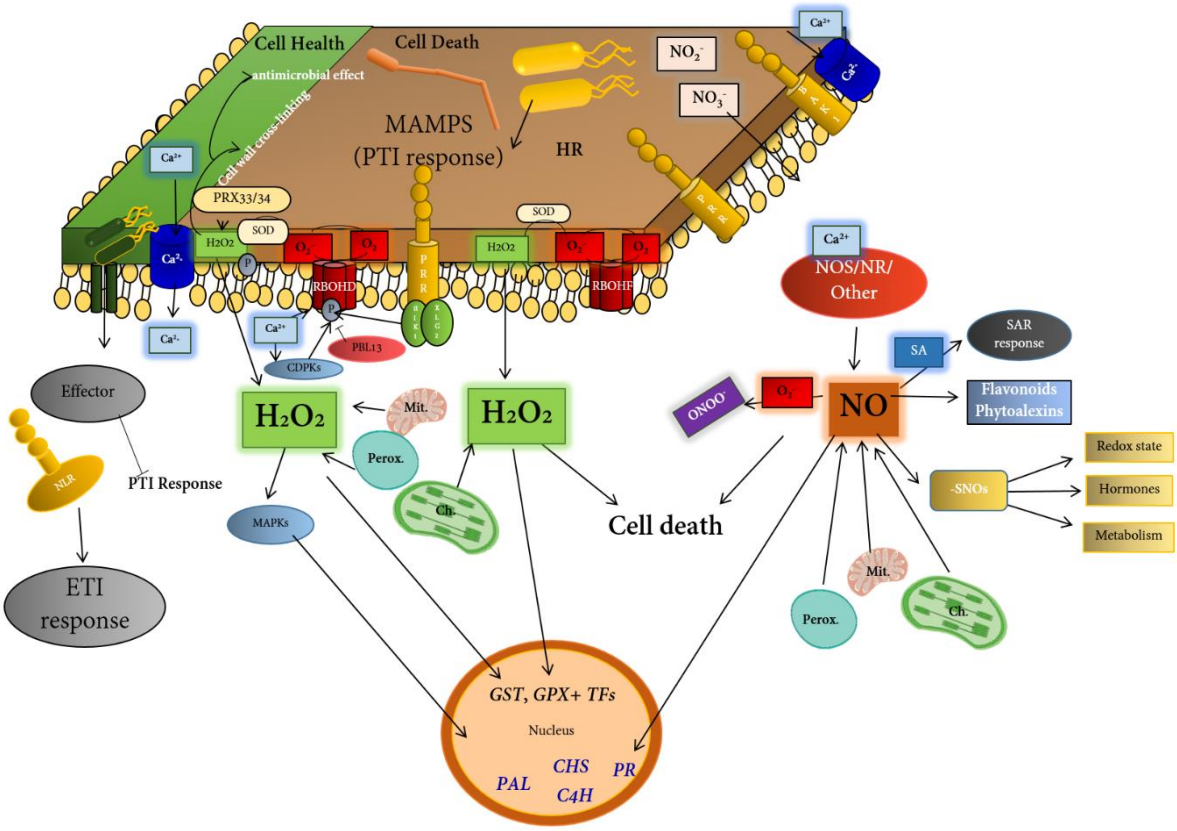

Fig. 1.9. Schematic overview of the ROS and RNS sources, regulation and signalling during defence response. After pathogen recognition due to their microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) or their effectors, plants respond in the so-called MAMP-triggered immunity (PTI) or the effector-triggered immunity (ETI), respectively. Then, a production of reactive oxygen species (ROS) and nitric oxide (NO) take place as an early event, leading to the plant response and avoiding pathogen spread and to the cell death (HR) of the invaded tissue in the ETI response. ROS are mainly produced by respiratory burst oxidase homolog proteins (RBOHs) and class III apoplastic peroxidases (PRXs) while nitric oxide synthase like (NOS-l) is one of the main sources of NO production and nitrate reductase (NR) and nitrite-dependent NO production is also involved. Both, ROS and RNS induced gene expression and production of antimicrobial metabolites to avoid the infection (Molina-Moya *et al*., 2019).

As described before, NO-dependent PTMs is the main mode of action for NO being essential within the plant-pathogen interaction context. Differential *S*-nitrosylated proteins in plants undergoing HR or not, showed proteins related with hormone-dependent signalling, intermediary metabolism, ROS producing enzymes, antioxidant defences proteins, programmed cell death and TFs (Feechan *et al*., 2005; Romero-Puertas *et al*., 2007, 2008). For example, *S*-nitrosylation facilitates bonds between NPR1 monomers and stabilizes the oligomeres in the cytosol under normal conditions (Després *et al*., 2003; Tada *et al*., 2008). NPR1 has an important role activating pathogenesis related genes (PR) and regulating DNA binding of its transcription factor interactor TGA1 (Tada *et al*., 2008; Lindermayr *et al*., 2010). SA binding protein, peroxiredoxin IIE (PRxII E) and RBOH-D also suffer *S*-nitrosylation during plant defence regultating ROS levels and hormone signalling (Romero-Puertas *et al*., 2007; Yun *et al*., 2011; Wang *et al*., 2013). Glyceraldehyde-3-phospate dehydrogenase (GAPDH) and metacaspase 9 proteins, related with PCD has been shown to be also *S*-nitrosylated in incompatible interactions

(Belenghi *et al*., 2007; Kwon *et al*., 2012). Recently, Cui *et al*. (2018) demostrated that the zinc-finger transcription factor salt tolerance during germination (STG1), is *S*-nitrosylated during plant immunity response leading to a decrease in this response. In addition, nitrosothiols levels also regulate plant immunity and gene expression related to SA (Feechan *et al*., 2005; Rusterucci *et al*., 2007; Yun *et al*., 2016).

Tyr nitration has also been described in plant-pathogen interaction although its function is not well known (Romero-Puertas *et al*., 2007; Cecconi *et al*., 2009; Vandelle and Delledonne, 2011). Proteomic analysis showed that photosynthesis, nitrate assimilation and glycolysis processes may be affected by nitration during incompatible interaction (Cecconi *et al*., 2009). In addition, MAPK signalling and phosphorylation cascade may be regulated by nitration during plant response to pathogen infection (Vandelle and Delledonne, 2011; Fig 1.9).

1.4.1. Adapted response to fungal pathogens

Pathogenic fungal microorganisms use diverse mechanisms to infect plants and have been classified according to their mode of action. Therefore, necrotrophic pathogens kill plant cells and take up nutrients from dead tissues using among other strategies, cell wall degrading enzymes, ROS and toxins (Wolpert *et al*., 2002; Martínez-Medina *et al*., 2019b). Even, some necrotrophic pathogens induced an NO overproduction in order to accelerate the infection (Van Baarlen *et al*., 2004; Sarkar *et al*., 2014; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2016;), although according to NO intensity and timing, this could activate plant defences (Asai and Yoshioka, 2009). Plants also activate other signalling pathways such as JA an ethylene (ET) dependent defence genes (Thomma *et al*., 2001; Kunkel and Brooks, 2002; Broekaert *et al*., 2006). Other phytohormones such as GAs have an important role in plant resistance to necrotrophic pathogens due to the degradation of a DELLA repressor, which activates plant growth (Achard *et al*., 2008) and interacts with a JA signalling repressor (Zhang *et al*., 2017).

Biotrophic fungus pathogens usually have an especific host and they are able to secrete effectors to suppress plant immune system (Perfect and Green, 2001). Besides, fungus may derive nutrients from living cells by maintaining host viability through specialized structutal and biochemical relations (Gebrie, 2016). In some cases, the fungus synthetize CKs into the plant to attract nutrients from the plant to the infected tissues and decrease SA levels produced by plant to activate defence genes of plant biotrophic fungus (Choi *et al*., 2011; Zhang *et al*., 2017).

Conversely, plants develop several mechanisms in order to resist to biotrophic fungal infection. One of them is the penetration resistance, trough strengthens cell wall and membrane to halt spore germination and avoid the formation of haustorium. Furthermore, plant may activate PCD accompanied with a burst of NO leading to the HR when fungus penetrated epidermal cell to avoid nutrient supply to fungus (Koeck *et al*., 2011; Fig 1.10B).

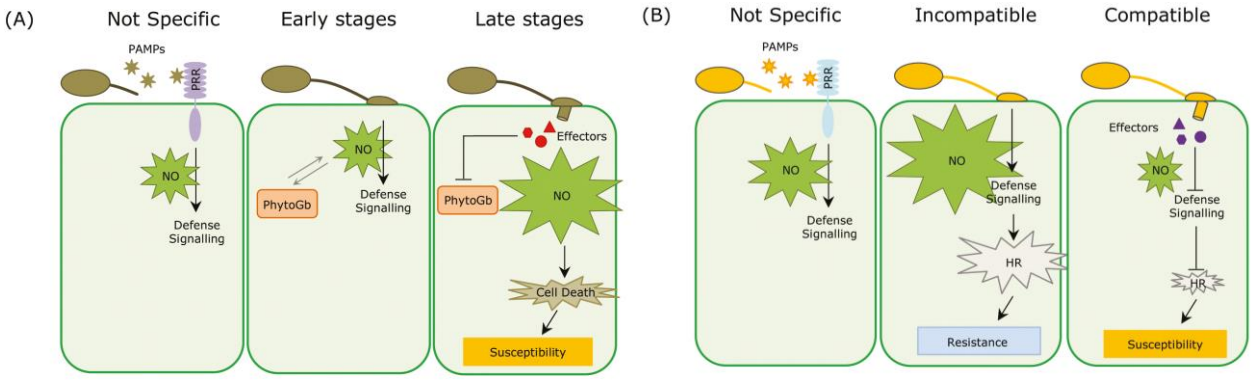

Fig. 1.10. Model of NO functioning in plant–fungal interactions. (A) Plant pattern-recognition receptors (PRRs), during interactions with necrotrophic fungus, triggers a rapid and unspecific NO burst, which activates plant response at the early stages. At later stages, the pathogen expands the lesions promoting a rapid and unspecific NO burst associated with cell death. (B) Plant perception of fungal PAMPs, in the interactions with biotrophic pathogens, triggers a rapid and unspecific burst of NO to activiate plant response. A second NO burst, during incompatible interaction, leads to the hypersentive response (HR) and cell death which prevent the pathogen from spreading along the plant. However, NO levels rapidly decrease after initial burst, in compatible interaction, due to the activation of effector-mediated suppression of defences by the fungus, which leads to susceptibility (Martínez-Medina *et al*., 2019).

Then, levels of NO decrease due to the activation of effector-mediated suppression of defences by the fungus, which leads to susceptibility. Once the fungal effector avoids plant defence mechanism, the plant will not resist. Hemibiotrophic pathogens are intermediate between necrotrophic and biotrophic lifestyles, growing initially as biotrophs and adopting later on a necrotrophic lifestyle (Koeck *et al*., 2011).

1.4.2. Fusarium as an environmental problem

Plants activate defence mechanisms to avoid pathogenic fungus, and in general, plants are resistant to the infection (Staskawicz, 2001). In other cases, fungus can thrive plants causing important economic loses in crop production and natural ecosystems. Fusarium spp. are included within the group of pathogens that produce the highest productivity loses (Dean *et al*., 2012; Geiser *et al*., 2013). In particular, *Fusarium oxyporum* is a pathogen that are able to infect 150 of economically important crops cultivated around the world such as tomatoes, cotton and bananas causing vascular wilt diseases (Summerell, 2006; Laurence *et al*., 2012; Leslie and; Ma *et al*., 2013; Rana *et al*., 2017; Gordon, 2017).

The genus *Fusarium* belongs to the *Ascomycota* phylum belonging to the Fungi kingdom. Molecular phylogenetic studies showed at least 300 different species, but half of these species

have not been formally described (Aoki *et al*., 2014; O'Donnell *et al*., 2018). Fusarium have diverse life cycles (necrotrophic, biotrophic and hemibiotropic), niche specialization, host adaptation and specificity (Rana *et al*., 2017). Fusarium is a filamentous fungus able to live in soil in association with plants being abundant in the environment. Most species are facultative pathogens capable of survive in soil water feeding on decomposing material. Other species are phytopathogenic causing diseases in plants such as wilt, blight, rot, and canker diseases (Berrocal-Lobo and Molina, 2008; Ma *et al*., 2013). In addition, Fusarium causes infection in humans as an opportunistic species, which are able to infect immune-compromised patients. Diseases like keratomycosis, onychoycosis and neutropenia are the most common diseases caused by *Fusarium oxysporum* in humans (Askun, 2018).

Several strategies for controlling diseases caused by Fusarium have been evaluated, including cultural, biological and chemical ones. Despite efforts to reduce the effects of Fusarium, the proposed methods are very limited (Pirgozliev *et al*., 2003; Bubici *et al*., 2019), especially because Fusarium produce resting spores called chlamydospores that can survive for many years (Akhter *et al*., 2016), being the more efficient the use of resistant species as far as possible (Bai and Shaner, 2004; Chen *et al*., 2019).

1.4.3. Fusarium infection and plant response.

Fusarium vary in reproduction strategy, some species are asexual, others are both asexual and sexual with either self-fertility or obligate out-crossing. Fusarium species produce sexual spores and at least 3 types of asexual spores (microconidia, macroconidia, chlamydospores). However, all Fusarium species do not produce all type of spores. In the case of *F. oxysporum*, only presents asexual reproduction. *F. oxysporum* reprensents most abundant and ubiquitous soil-borne fungus, able to degrade lignin and complex carbohydrate associated with soil debris (Rana *et al*., 2017). Moreover, *F. oxysporum* have been differentiated into opportunistic, true pathogens and obligate pathogens based on the level/specialization of fungal-plant interactions (Scheffer, 1991). Almost, individual *F. oxysporum* isolates are characterized by a high degree of host specificity. Remarkably, large genetic diversity has been shown by molecular phylogenetic studies amongst isolates, suggesting that *F. oxysporum* represents a complex species (Rana el al., 2017).

The infection of plant by Fusarium is characterized by different steps, including: 1) contact with the host plant; 2) fungal attachment to host; 3) entry and colonization of plant tissues and 4) fungal reproduction (Lo Presti *et al*., 2015; Fig. 1.11).

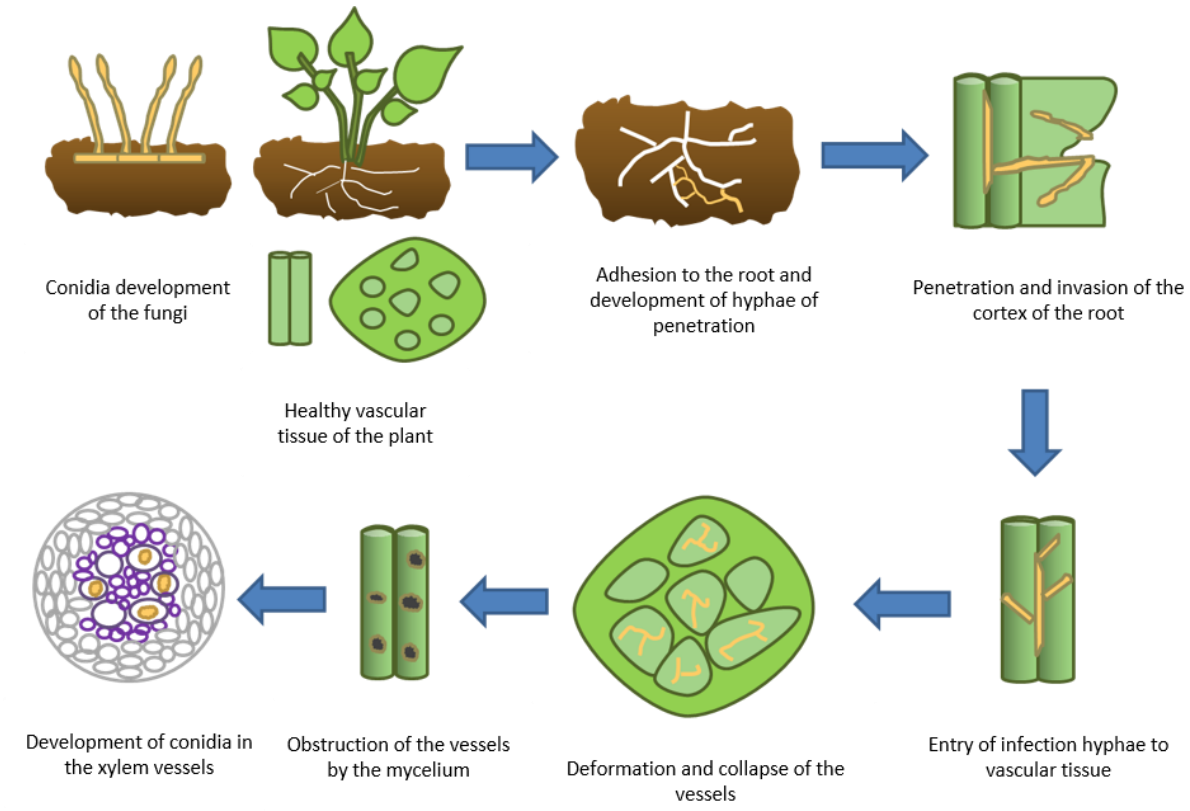

Fig 1.11. *Fusarium oxysporum* infection mechanisms. *F. oxysporum* requires well-regulated mechanisms to make the infection process successful, including: (1) the recognition of plant signals,(2) the adhesion to the root surface and the differentiation of penetrating hyphae, (3) the invasion of the root cortex and degradation of physical barriers to vascular tissue, (4) adaptation to the adverse environment of plant tissue, including tolerance to antifungal compounds, (5) hyphae proliferation and conidia production in the xylem vessels and, (6) the secretion of virulence factors such as enzymes, peptides or phytotoxins (Di Pietro and González Roncero, 2004).

It has been described that chemotropism is the mechanism used by Fusarium to approach to the plant roots. Studies in *F. oxysporum* showed that fungus hyphae recognize chemical signals from the plant and orient their growth towards them. Chemoattractants, such as sugar, amino acids, peptides, pheromones and peroxidases from plant root exudates can redirect the growth of hyphae towards a gradient of these substances (Turrà and Di Pietro, 2015; Nordzieke *et al*., 2019). Membrane receptors followed by a signalling cascade, which include the activation of a three kinase modules, MAPK, MAPKK and MAPKKK that are activated sequentially by phosphorylation (Pérez-Nadales and Di Pietro, 2012), facilitate the pathogenesis process. This signalling pathway is involved in the perception and response to stimuli mediating adhesion processes, structure differentiation, lytic enzymes secretion, secondary metabolites and evasion mechanisms against the plant defensive response (Lengeler *et al*., 2000; Lo Presti *et al*., 2015). The secretion of lytic enzymes plays an important role degrading physical barriers and depolymerizing cell wall compounds such as pectin and cellulose (Lo Presti *et al*., 2015; Turrà and Di Pietro, 2015). Moreover, changes in morphology and biochemistry lead that pathogenic fungi take over and utilize host metabolic pathways for their development (Zeilinger *et al*. 2015;

Rana *et al*., 2017). Meanwhile, plants respond to fungal entry by signalling compouds including ions such as Ca, ROS/RNS, phytohormones and small RNAs (Mur *et al*., 2006; Pieterse *et al*., 2012; Weiberg *et al*., 2014; Pozo *et al*., 2015; Waszczak and Carmody, 2018). Some *F. oxysporum* hyphaes are able to penetrate the root and subsequently, the fungus colonize vascular tissue and cause massive wilting, necrosis and chlorosis of aerial plant (Gordon, 2017).

Numerous studies have focused in specific mechanisms developed by plants or Fusarium species to prevent fungal infection or colonice plants, respectively. For example, *F. graminearum* and *F. culmorun* are able to infect Arabidopsis, brachypodium and wheat, causing severe damages on production (Peraldi *et al*., 2011; Blümke *et al*., 2015). These two species of Fusarium among others species, may produce the micotoxin deoxinivalenol (DON) which dramatically increases the severity of the disease due to an increment in H_2O_2 leading to cell death, and a decrease in defence genes expression (Chen *et al*., 2006; Desmond *et al*., 2008; Pasquet *et al*., 2014; Schmeitzl *et al*., 2015). However, it has been shown that pre-treatment with low levels of DON reduces the infection (Diamond *et al*., 2013; Blümke *et al*., 2015). Other study showed that secondary metabolites associated with iron uptake govern the potential virulence in *F. graminearum* (Oide *et al*., 2015).

Hormones such as SA, JA and ET are also essential in plant infection by Fusarium. Therefore, SA appears to play an importan role in *F. oxysporum*, *F. solani* and *F. asiaticum* plant resistance (Pritsch *et al*., 2000; Makandar *et al*., 2010, 2012; Qi *et al*., 2012; Wang *et al*., 2018). Plants induce PRs genes, among other SA-dependent genes, often 24-48 h of infection, which play a role in plant defence response (Asano *et al*., 2012). On the other hand, JA may induce plant susceptibility at the beginning of the infection but may promote plant resistance to *F. oxysporum* and *F. sporotrichioides* as disease progresses (Makandar *et al*., 2010, 2012; Luu *et al*., 2015). It has been shown that JA dependent genes such as *PDF1.2* is expresses at 48h in *F. sporotrichioides* infection in *A. thaliana* (Asano *et al*., 2012). Moreover, a comparative proteomic aproach on cucumber roots following infection by *F. oxysporum* showed the involvement of JA and redox signalling in modulating plant resistance (Zhang *et al*., 2017). Furthermore, oxylipins (an oxygenated lipid), which may be produced by plant and fungus, could act by inducing JA-responsive genes in response to *F. oxysporum* in *A. thaliana* (Thatcher *et al*., 2009). On the contrary, high levels of ET increase damages in Arabidopsis, wheat and barley plants infected by *F. graminearum* (Chen *et al*., 2009). Furthermore, Arabidopsis mutants with disrupted ethylene signalling (*ert1-1*), showed more resistance to *F. oxysporum* and *F. raphani* (Pantelides *et al*., 2013).

1.4.3.1. NO production and function in plant interaction with root-fungal pathogens: *Fusarium oxysporum*.

NO role in plant interaction with root-fungal pathogens has not been examined closely (Shelef *et al*., 2019). Both organisms, plants and Fusarium are able to produce NO in the interaction process (Turrión-Gómez and Benito, 2011; Sarkar *et al*., 2014). NO production and function by the fungal side have been well analised in recent reviews (Arasimowicz-Jelonez and Floryszak-Wieczorek, 2016; Cánovas *et al*., 2016). Fungus have different NO biosynthetic and regulation routes. Fungus does not contain mammalian NOS enzymes (Takaya, 2002), but in low oxygen condition denitrification and ammonium fermentation processes NO may be generated and activates the fungal response to nitrosative stress (Hillmann *et al*., 2015). Denitrification was identified in Fusarium and involves the action of a NR, nitrite reductase (NiR), and a nitric oxide reductase (Nor), to transform nitrate into nitrite and further reduce in to N_2O (Kobayashi and Shoun, 1995), however it is not well known how this processes produced NO. Lasts studies showed that NR gene is required in some fungal species to produce NO (Schinko *et al*., 2010; Marcos *et al*., 2016). Flavohemoglobins, porphobilinogen deaminases, nitrosothioneins and GSNO may be involved in NO levels regulation in several fungal species but the mechanisms are still unknown (de Jesús-Berrios *et al*., 2003; Hromatka *et al*., 2005; Turrión-Gomez *et al*., 2010; Zhou *et al*., 2012, 2013; Lapp *et al*., 2014; Zhang *et al*., 2015).

From the plant side, different studies have shown NO production in plant-fungal interaction. However, NO function and regulation remain almost unknown in the case of root pathogen (Martínez-Medina *et al*., 2019; Shelef *et al*., 2019). *Verticillum dahliae* in olive seedling showed an increase of NO in roots 1 h post infection (hpi). Subsequently, this increment was spread across cell walls epidermal and cortical cells, concomitant with an increment in phenolic compounds. This NO burst may be related with the activation of the plant immune reponse to the pathogen (Espinosa *et al*., 2014). *Rizoctonia solani* in tomate also increment NO levels at 2 hpi which may be related with cell wall denfese response, octadecanoid and phenylpropanoid pathways which are associated with cell wall modification. Exogenous NO was adding using sodium nitroprusside (SNP) and 1,1-Diethyl-2-hydroxy-2-nitroso-hydrazine sodium, 2-(N,N-Diethylamino)-diazenolate 2-oxide sodium salt hydrate (DEANONOate) in tomato roots. This NO increment, regulated malondialdehyde, H_2O_2 and $O_2^{\cdot-}$ levels in plant cells, and raise callose deposition, phenolics and lignification, as defence responses related to cell wall modification lipoxygenase (LOX) and phenylalanine ammonialyase (PAL) may have relevant role in this defence responses in tomato plants (Noorbakhsh and Taheri, 2016). In addition, elicitors such as chitosan increase NO level in guard cells in *Pisum sativum* (Srivastava *et al*., 2009). In a study in cell of *Taxus chinensis* using different fungal elicitors it was observed an increase of NO at 30

min, 6 h, 8 h and stabilized at 12 h (Wang and Wu, 2004). NO-dependent signalling pathway also may modulate PCD induced by fusaric acid, a non-host selective toxin, produced by *F. oxysporum* in tobacco suspension cells (Jiao *et al*., 2013).

Thus, an early strong and transient NO burst was observed in tomato roots infected by *F. oxysporum*, followed by an uncontrolled and sustained NO accumulation concomitant with plant cell death (Martínez-Medina *et al*., 2019a). Interestingly, Phytogb1 was down regulated as the infection progresses in tomato plants, explaining the increment of NO levels, which probably promote cell death. In fact, tomato mutants overexpressing Phytogb1, leading to a decrease in plant NO levels, showed more resistance to *F. oxysporum* infection (Martínez-Medina *et al*., 2019a). Similarly, an early NO burst was found in Arabidopsis 1 hpi with *F. oxysporum* (Gupta *et al*., 2014). Other study suggested that exogenous NO and sucrose may modulates phenylpropanoids pathways in yellow lupine embrios infected with *F. oxysporum* (Morkunas *et al*., 2013).

Althoguh several data have shown the importance of NO in the regulation of stablisment of plant-fungus interaction and plant defence against fungal pathogens, the mechanisms underlying NO function remain unclear. NO has also been recently shown to be produced by the plant after the recognition of root fungal symbionts, and optimal levels of NO are required for the control of mycorrhizal simbiosis (Martínez-Medina *et al*., 2019). Experimental data also show that different regulation patterns and functions for NO exist between plant interactions with pathogenic and mutualistic fungus (Martínez-Medina *et al*., 2019b). Aparently, NO may play a dual role in the interaction between plants and root pathogenic fungus. Therefore, NO may act as a signal molecule that activate defence response and also may be used by the pathogen in order to propagate the infection in the compatible interaction (Martínez- Medina *et al*., 2019b).

1.5. Interactions between biotic and abiotic stress response: ROS and NO function

Large-scale transcriptomic analysis through the use of microarrays and RNA-seq technologies support the existence of interferences between different signalling networks (Cheong *et al*., 2002; Cohen and Leach, 2019). As signalling molecules, RNS and ROS can directly or indirectly influence the expression of different gene sets with more or less specificity depending on the stress intensity (Vaahtera; *et al*., 2014; Imran *et al*., 2018; Siaucinaite *et al*., 2019). Narusaka and collaborators (2004) described that treatment of *A. thaliana* with copper (Cu), a heavy metal, or the infection with the necrotrophic pathogens *Alternaria alternate* and *Alternaria brassicicola* cause a significant overlaping related to P450 genes, suggesting that ROS are the common signals triggering the similar response.

Apparently, initial stimuli activate overlapping signalling pathways being some of them often redundant. Plants require an early choice in order to priorize and regularize pathways. In this

case, the perception of damage-associated molecular patterns (DAMP) and signalling networks associated play an important role although molecular mechanisms underlying are still unknown (Saijo and Loo., 2019). In general, abiotic stress increases susceptibility to hemi-biotrophic or necrotrophic pathogens, but reduce susceptibility to biotrophic pathogens. Gradual application of the abiotic stressor will improve the result of the interaction (Zhang and Sonnewald, 2017).

Suzuki and collaborators (2014) analised plant responses to different combined stressors trying to simulate enviromental conditions (Fig. 1.12). Although studies in this field have increased to date, there is still much to investigate about regulation and interaction between different signalling pathways in plant response to stress.

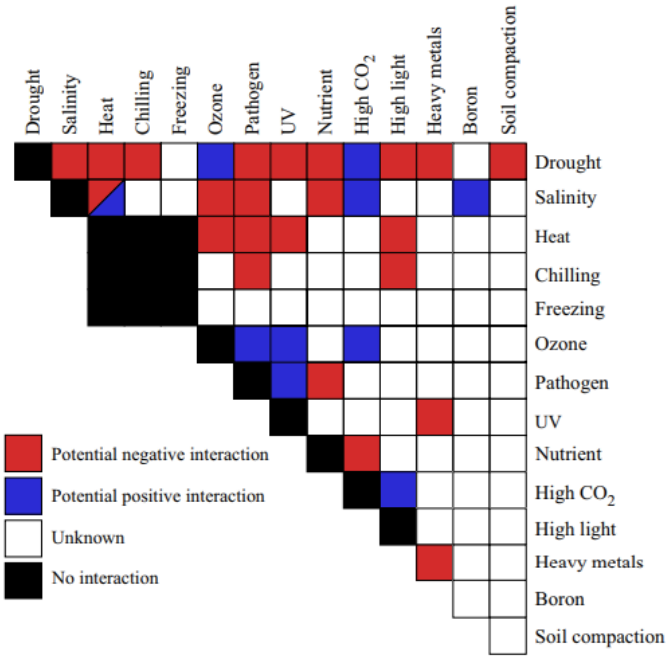

Fig. 1.12. The stress matrix. Potential combinations of stresses that can affect crops are shown in a matrix. Colours showed indicate overall effect on plant growth (Suzuki *et al*., 2014).

Some previous studies have used different microorganisms and compounds in order to avoid *F. oxysporum* infection. *Trichoderma harzianum* which is an opportunistic fungal improve cucumber plants by attenuating oxidative and nitrosative stress in cucumber roots due to the increment of antioxidant capacity and reduction of NO synthesis via both NR- and NOS-dependent pathways. Furthermore, ASC-GSH cycle and oxidative pentose phosphate (OPP) pathway are induced by Trichoderma inoculation, which potentially helps plant to maintain redox balance and increase tolerance to Fusarium (Chen *et al*., 2019). Moreover, *Trichoderma asperelloides* also improve Arabidopsis plants response to *F. oxysporum*, where NO timing production was suppress and disturbances of some NO-responsive genes were observed. Receptor-like genes such as *RLP30* and a-like lectin protein kinase reduced their expression, which may be required for *F. oxysporum* disease development (Gupta *et al*., 2014).

Other molecules, such as $CaCl_2$ foliar application in tomato may suppress Fusarium infection due to the induction of different defensive responses and the improvement of tomato plant growth (Chakraborty *et al*., 2017).

2. Objectives

2. Objectives

The mechanisms involved in plant response to both, biotic and abiotic stress, with special interest in the role of nitric oxide as a signal molecule is one of the main goals of the group "Reactive Oxygen and Nitrogen Species Signalling under Stress Conditions in Plants", from the Biochemistry, Molecular and Cellular Biology of Plants Department in the Estación Experimental del Zaidín-CSIC, Granada.

Plant response to Cd has been largely analysed in the group for more than twenty years, focused mainly in the role of reactive oxygen species as essential signalling molecules in plant adaptation and survival. In particular, special interest has been paid to peroxisomal dynamics in plant response to Cd stress, and peroxisomal role as stress sensor of redox changes. Therefore, we have shown very early dynamic peroxisomal extensions, called peroxules, in plant response to Cd, followed by peroxisome proliferation. We have also shown specific autophagy of the organelles, called pexophagy to regulate the number of peroxisomes, as an excessive amount of these organelles can produce severe disturbances in redox homeostasis. Finally, we have found an increase in speed of the organelles after one-day Cd treatment. NADPH oxidases-dependent reactive oxygen species are involved in regulation of these changes in peroxisomal dynamic in plant response to Cd stress, being the peroxin 11a (PEX11a) essential to produce peroxules. Our results demonstrate that PEX11a and peroxule formation play a key role in regulating stress perception and fast cell responses to environmental cues. Although initial studies suggested a key role for nitric oxide (NO) in plant response to Cd stress, a deeper insight is needed to unravel NO-dependent underlying mechanism, especially those related with endogenous (produced by the plant) NO. On the other hand, NO and its derivatives, such as *S*-Nitrosoglutathione (GSNO) and peroxynitrite, have been shown to be present in plant peroxisomes under physiological and stress conditions. Although some peroxisomal proteins are targets of NO-dependent post-translational modification, regulating their activity, NO role in peroxisomal metabolism, dynamics and ultrastructure is not well known.

Recently, we have also found a key role of NO in the initial steps of plant responses to both, pathogenic and mutualistic microbes. However, studies dealing with NO function in plant-fungal pathogen interactions and especially with root-fungus are still scarce, despite the huge economic importance as they threaten the production of crops not only when growing in the field, but also at postharvest stage. Furthermore, initial analysis of an in house transcriptome in plant response to Cd stress showed a possible crosstalk between Cd and biotic stress, which further boost for more analysis.

Therefore, the following specific objectives have been established:

1. To gather an updated knowledge by bioinformatics analysis, about the role of NO in plant response to heavy metals, with special interest in cadmium.

2. To evaluate the role of NO as a regulator of seedling responses to cadmium stress and its crosstalk with reactive oxygen species.

3. To analyse the role of NO in regulating peroxisomal ultrastructure, signalling and dynamics in response to cadmium stress.

4. To evaluate by a genetic approach, the role of NO in the *Arabidopsis thaliana-Fusarium oxysporum* interaction.

5. To assess a crosstalk between plant response to cadmium and Fusarium and the role of NO.

3. General Materials and Methods

3. General materials and methods

3.1. Biological material

Seeds of *Arabidopsis thaliana* L. Heynh, ecotype Columbia (Col-0) have been used in this study. The role of nitric oxide (NO) in response to stress has been analysed using Arabidopsis mutants specified in each chapter and shown in the Fig. 3.1. Mutans *nia1 nia2* and *nox1* were crossed with *px-ck* mutans (Nelson *et al*., 2007), containing a peroxisomal-targeted CFP in order to study peroxisomal dynamics described in Chapter 4.3 *Fusarium oxysporum* f. sp *conglutinans* PHW 699-3 (ATCC 58110; Kistler *et al*., 1987) and a *F. oxysporum* expressing the green flurescent proteins (GFP; Hou *et al*., 2014), from Prof. Antonio di Pietro laboratory (Cordoba University) were used.

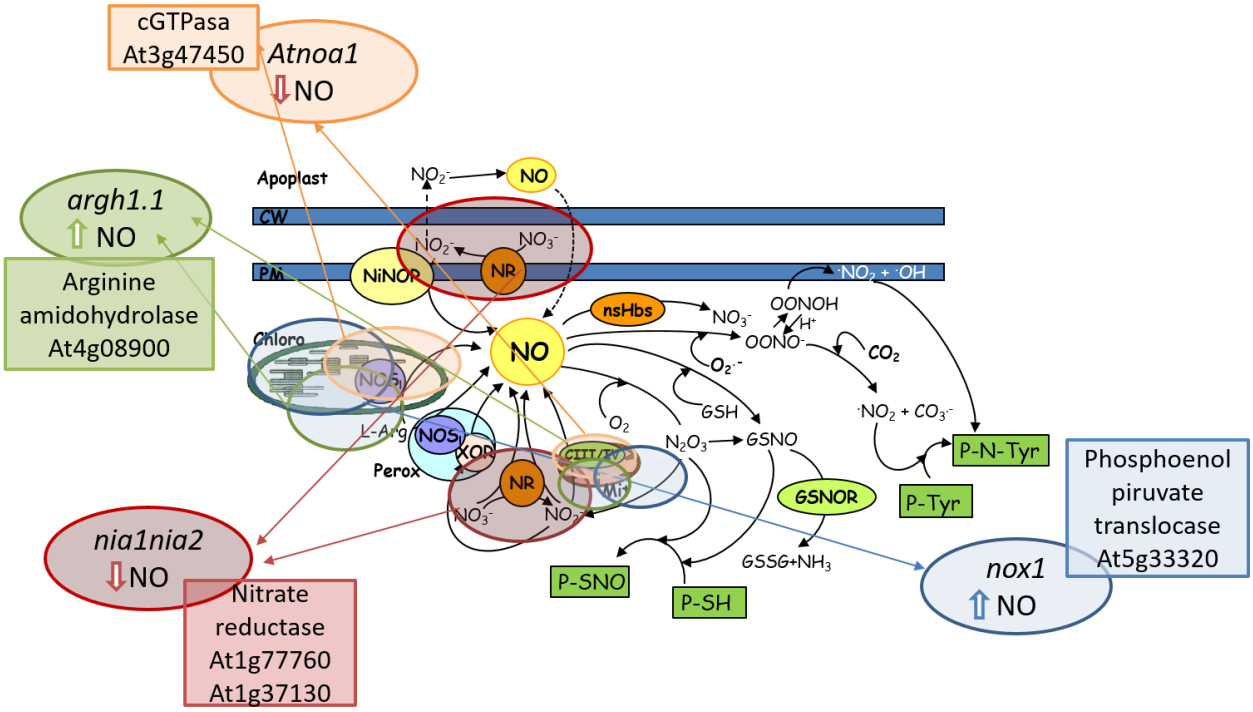

Fig. 3.1. Mutants used and affected routes in NO metabolism. The mutants related with NO metabolism used in this study have been: *Atnoa1*, *nox1*, *argh.1.1* and *nia1 nia2*. According to http//www.bar.utoronto.ca/eplant, the expression of these genes is as follow: *Atnoa1* in mitochondria (score 52), chloroplasts (score 40) and golgi apparatus (score 10); *nox1* in chloroplasts (score 66), mitochondria (score 28) and plasma membrane (score 14); *argh1.1* in chloroplasts (score 40) and mitonchondria (score 30); NIA1 in cytosol (score 32), nucleus (score 6), mitonchondria (score 6), peroxisomes (score 4); NIA2 in cytosol (score 22), vacuole (score 20) plasma membrane (score 20), mitochondria (score 18) and chloroplasts (score 4).

3.2. Growth conditions and media

3.2.1. Plant medium

Hoagland medium pH 5.6 (0.5x; Hoagland and Arnon, 1950; Fig. 3.2) was used for sowing and growing seeds in Petri dishes adding or not specific treatment: Cadmium (Cd), NO donors or scavenger; as specified in each chapter. The medium was supplemented with sucrose (30 g/L), and

phytoagar (8 g/L) to solidify. Medium was autoclaved for 20 min at 121°C and 1 atmosphere of pressure.

Table 3.1. Composition of Hoagland nutrients medium (0,5 x) for Arabidopsis.

Nutrients	Concentration (µM)
$Ca(NO_3)_2$ $4H_2O$	750
KNO_3	625
$H_2O_5Mg(SO)_4$ $7H_2O$	375
KH_2PO_4	25
$FeSO_4$ $7H_2O$ + EDTA	2050+1070
KCl	25
$MnSO_4$ H_2O	5
$CuSO_4$ $5H_2O$	0.75
$ZnSO_4$ $7H_2O$	1
H_3BO_3	25
$(NH_4)_6Mo_7O_{24}$ $4H_2O$	0.0375

3.2.2. In vitro growth cultivation

Seeds were superficially disinfected by consecutive immersion in 70% (v/v) ethanol for 1 min and in 50% (v/v) of commercial sodium hypochlorite for 10 min under sterile conditions; after that, three washes were done in sterile distilled water. Subsequently, seeds were submitted to a stratification process, consisting of immersing seeds in sterile distilled water for two days at 4°C in darkness in order to synchronize their germination. After stratification, seeds were sown with a micropipette in petri dishes in a horizontal laminar flow hood.

Arabidopsis plants were grown vertically on 10 cm^2 square plate dishes containing Hoagland medium (Table 3.1) in a SANYO phytotron under controlled conditions: 20/22ºC with a long day conditions, 16 h light/8 h dark of photoperiod, with a light intensity of 100 µE and 60-65% relative humidity. Figures 3.2-3.4 show a diagram of experimental designs used in this work.

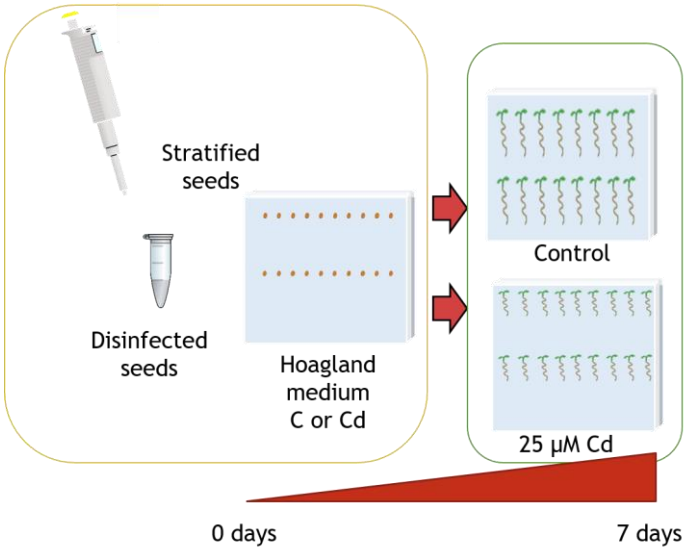

Fig. 3.2. Experimental design to study the role of NO in Arabidopsis under Cd treatment (Chapter 4.2) Arabidopsis seeds were sown in Petri dishes with Hoagland 0,5x or Hoagland with Cd 25 µM. Seedlings grown for 7 days and were harvested and processed for each assay. At least 3 independent replicates were used indepently per experiment with 3 independet experiment.

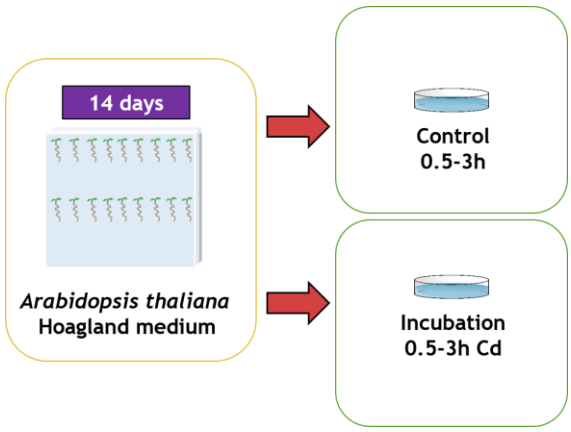

Fig. 3.3. Experimental desing to study the role of NO in Arabidopsis peroxisomes under Cd treatment (Chapter 4.3). Arabidopsis seeds were sown in Petri dishes with Hoagland (0.5x). After 14 days, seedlings were transferred to Hogland liquid during 1 day to acclimatize Arabidopsis. Later, seedling were transfered to Hoagland liquid or Hoagland liquid with Cd 100 µM during 0,5 to 3 h and seedlings were harvested and processed for each assay. At least 3 independent replicates were used indepently per assay.

3.2.3. Growth in soil

To obtain seeds and phenotype characterization in some experiments, different Arabidopsis lines were sown in soil with an universal substrate (Compo-Sana) and vermiculite in a 1:1 or 2:1 ratio (substrate:vermiculite). Subsequently, pots were transferred to a growth chamber at 22°C with photoperiod of 16 h light/8 h dark with a light intensity of 120-150 µE and a relative humidity of 50-60%. After complete life cycle of the plant, seeds were collected and stored at 4°C. *Fusarium oxysporum* was grown at 24ºC (Fig. 3.4).

3.2.4. *Fusarium oxysporum* growth

Microconidia generation of *Fusarium oxysporum* were carried out in potato-glucose medium. 200 g of potato were boiled during 1 h in 1 L of distilled water. Subsequently, 20 g/L of glucose was added to potatoes broth, which was autoclaved at 121°C for 20 min and distributed in 200 mL flasks (Fig. 3.4).

200 µL of *Fusarium oxysporum* microconidia stored in glycerol (30%, v/v) at -80ºC were grown in 100 mL potatoes-glucose medium, at 170 rpm and 28°C for 4 days. *F. oxysporum* microconidia were filtered by placing a sterile gauze pad. Subsequently, microconidia were centrifuged at 6000 g for 10 min and resuspended in autoclaved distilled water, and quantified with a Neubauer chamber by light microscopy (Fig. 3.5).

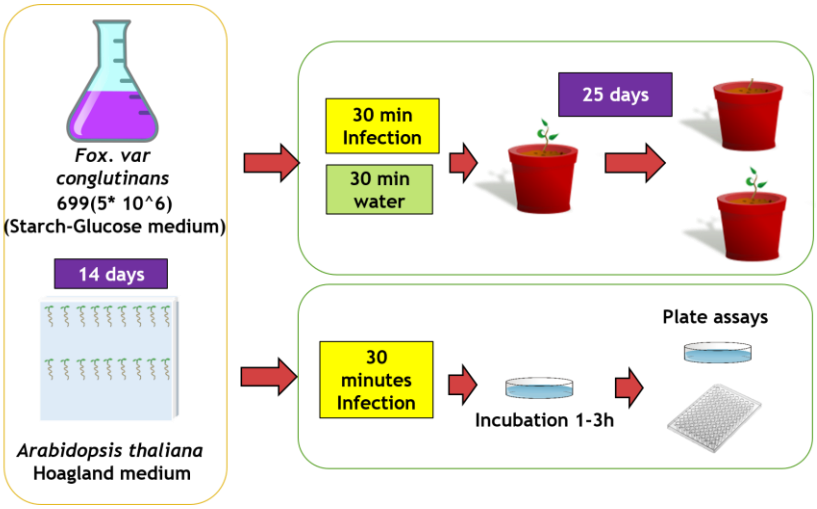

Fig. 3.4. Experimental design to analise NO role in Arabidopsis *F. oxyporum* in (Chapter 4.4). Seedlings roots were immersed in a suspension of 5×10^6 microconidia mL^{-1} for 30 min before sow in soil-vermiculite mixture. Sixty plants were used for each treatment and replicate. Plants were maintained in a growth chamber as described previously in section 3.2.1 during 24 days after infections. At least 3 replicates were done indepently.

$$Concentration = \frac{\text{Total spores counted (3)} * (\text{counted area (mm}^2) \cdot \text{ chamber depth (mm)})}{\text{Nº squares (3)}}$$

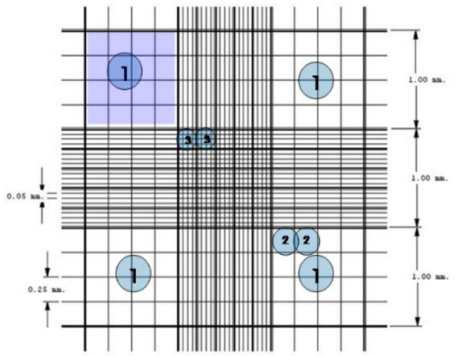

Figure 3.5. *Fusarium oxysporum* of spores quantification. After filtering the microconidia, they were quantified by using the Neubauer chamber**.** Sterile distillated water was prepared with $5*10^6$ microconidia for infection. After that, the seedlings were immersed 30 min in spore suspension.

3.3. Phenotypic characterization of Arabidopsis mutants

3.3.1. Fresh weight measurement

As growth parameter, seedlings weight was used and evaluated at the end of the 7th or 14th day of growth period. After that, plants were harvested, dried and weighed on a Sartorius precision balance (CPA225D).

3.3.2. Root length measurement

The length of the main root was determined by growing the seedlings vertically in 10 cm square Petri dishes for 7 days. Images of each plate were taken by scanner Epson 3200. Subsequently, the length of the main root was measured by Image J sofware and the average value of more than 300 seedlings per treatment and replicate was determined.

3.3.3. Survival rate of plant inoculated with *Fusarium oxysporum*

Every day after inoculation, severity of disease symptoms were recorded using the value 1 (life plant) and 0 (dead plant). Survival rate was calculated as indicated in Chapter 4.4 through Kaplan–Meier method (Grad Pad Prism sofware).

3.4. Protein assays

3.4.1. Total protein determination

Protein concentration was assayed by Bradford method (1976) using bovine serum albumin (BSA) for calibration curve. Samples (1-2 µL) were prepared in 200 µL of distilled water (final volume) containing 40 µL of a Bio-Rad solution (Bio-Rad Protein Assay Reagent). After 5 min incubation at room temperature, samples were analysed in a spectrophotometer (Sunrise, Absorbance microplate reader) at 595 nm.

3.4.2. Enzymatic activities

Plants were homogenized with liquid nitrogen in 50 mM Tris-HCl buffer pH 7.0 containing 0.2% Triton X-100 (v/v), 0.1mM EDTA and a cocktail of 1x protease inhibitors (Sigma). Samples were centrifuged at 5,000 g for 40 min and the supernatants were collected.

3.4.2.1. Catalase (CAT; EC 1.11.1.6)

Catalase was measured spectrophotometrically according to the method described by Aebi (1984), based on the analyses of the decrease in absorbance at 240 nm, caused by the disappearance of H_2O_2. The reaction was carried out at 25°C in a mixture containing 10.6 mM H_2O_2 in 50 mM K-phosphate buffer at pH 7.0 and in a final volume of 1 mL. The reaction was started by adding the sample. The enzymatic activity, expressed in µmoles of H_2O_2 min^{-1} μg^{-1} protein was calculated

from the initial reaction rate and a molar extinction coefficient for H_2O_2 of 39.58 M^{-1} x cm^{-1} (del Río *et al*., 1977).

3.4.2.2. Glycolate oxidase (GOX; EC 1.1.3.1)

Glycolate oxidase was measured spectrophotometrically according to the method of Kerr and Groves (1975). The reaction mixture, in a final volume of 1 mL, contained 50 mM K-phosphate buffer, pH 8.3, 10 mM phenylhydrazine and 5 mM glycolate. The reaction was started by adding the sample, measuring the formation of the glyoxylate-phenylhydrazone complex at 324 nm for 3 min. Enzymatic activity was calculated from the initial reaction rate and a molar extinction coefficient for the complex of $1.7x10^4$ M^{-1} cm^{-1} (Kerr and Groves, 1975).

3.4.2.3. Ascorbate peroxidase (APX; EC 1.11.1.11).

Samples were prepared in the same extraction buffer containing 2 mM ascorbate, to prevent the inactivation of APX. The activity was determined as described by Jiménez *et al*. (1997), measuring at 290 nm the oxidation of ascorbic acid, for 2 min. The reaction mixture contained in 1 mL 50 mM Hepes-NaOH buffer, pH 7.6, 0.2 mM ascorbate, 0.3 mM H_2O_2, and the sample conveniently diluted. The reaction was performed at 25°C and started with the addition of H_2O_2. The enzymatic activity, expressed in µmoles of NADPH min^{-1} μg^{-1} protein was calculated from the initial reaction rate and a molar extinction coefficient for ascorbic acid of 2.8 mM^{-1} cm^{-1} (Hossain and Asada, 1984). Three controls were used, one without H_2O_2, another without ascorbate and one without sample. As a negative reaction control, an APX inhibitor, p-chloromercuriphenylsulfonic acid (pCMS) was used. APX activity was calculated by substracting the value without pCMS to this obtained with pCMS.

3.4.2.4. Superoxide dismutase (SOD; EC 1.15.1.1)

Different superoxide dismutase isoenzymes were individualized by native EGPA in 10% gels (w/v). Enzymatic activity was localized by a photochemical method based on the reduction of NBT by free radicals $O_2^{\cdot -}$ generated by the action of light on a solution of riboflavin and tetramethylethylenediamine (TEMED; Beauchamp and Fridovich, 1971). Thus, blue staining of the gels is obtained due to the formation of formazan blue, except in those areas where there is SOD activity that remain achromatic. Once the electrophoresis was completed, the gels were incubated in a solution with 2.45 mM NBT prepared in 50 mM K-phosphate buffer, pH 7.8, for 20 min in the dark after which, they were incubated in another solution with riboflavin 28 mM and TEMED 28 mM prepared in the same buffer, for 15 min in the dark. Finally, the gels were rinsed in buffer and exposed to light for a few min until the gels became stained in blue color except in those areas where there was SOD activity.

3.4.2.5. Other activities

Peroxidase (Turrà *et al*., 2015) and ferrochelate reductase activities (Schmidt *et al*., 2000; Martínez-Medina *et al*., 2017) were measured in the whole plant (14 d old) grown in an agar/water Petri dishes (Chapter 4.4).

3.4.3. Quantification of specific proteins and postranslacional modification

3.4.3.1. Electrophoresis polyacrylamide gels under denaturing conditions (EGPA-SDS)

The technique described by Laemmli (1970) was followed, using a "Mini-Protean II" device from Bio-Rad. 12% (w/v) polyacrylamide gels 6.5 x 8.5 cm and 1 to 1.5 mm thick were prepared with a 4% polyacrylamide concentrator gel (w/v). Samples for electrophoresis were prepared in 0.063 M Tris-HCl buffer, pH 6.8, containing 2% sodium dodecyl sulfate (SDS; w/v), 10% glycerol (v/v), 0.006% bromophenol blue (w/v), and 10 mM DTT, and heated at 95° C for 5 min. Following, samples (with the same protein concentration) were loaded on the gels and a voltage of 100 V was applied for 20 min and then 200 V for 50 min using 0.025 M Tris-HCl electrode buffer, pH 8.3, with 0.192 M glycine and SDS at 0.1% (w/v). Molecular mass ladders between 14.4 and 97.4 kDa (Bio-Rad) were used.

3.4.3.2. Protein transference (Western blot) and immunodetection

Proteins contained in gels, obtained by polyacrylamide gel electrophoresis (SDS-PAGE), were transferred to a Millipore polyvinyl difluoride (PVDF) membrane, using a semi-dry transfer system from Bio-Rad. For the transfer a 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11, containing 10% methanol (v/v) was used and a current of 1.5 mA per cm^2 during 1 h, was applied.

For the detection of specific protein, membranes were permeabilized with 100% (v/v) methanol, after which they were washed with distilled water. In order to block the nonspecific binding sites of the immunoglobulin G (IgGs), membranes were incubated for 1 hour at room temperature, or overnight at 4°C in 20 mM Tris-HCl buffer, pH 7.8, and 0.18 NaCl M (TBS) and Tween 20 (0.1%), to which 3% (w/v) skimmed milk powder was added. Subsequently, the membranes were incubated for 1 h with the primary antibody diluted in the concentration specified in each chapter, 1 h at room temperature. After that, the membranes were washed with TBS+Tween 20 (0.1%) and incubated for 1 h with the secondary antibody rabbit or mouse joined to a horseradish peroxidase in the corresponding dilution. The antibodies used were obtained from AgriseraTM or Sigma and are specified in each chapter.

Membranes were incubated in an "ECL Plus Western Blotting detection system" (AmershamTM) following the company instructions. Fluorescence was detected with a BioRad

ChemiDoc. The exposure time change (15 secs to 30 min) depending on the intensity of the signal obtained. 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (NBT/BCIP) solution from Sigma was used as alternative method using a secondary antibody alkaline phosphatase conjugated. BCIP is the alkaline phosphatase (AP) substrate, which after dephosphorylation is oxidized by NBT to yield a dark-blue indigo precipitate dye. NBT is thereby reduced to a dark-blue precipitate dye and serves to intensify the color reaction making the detection more sensitive.

The nitrated protein pattern was also analyzed by Western blot using an anti-nitrotyrosine (N-Tyr; 1:2000 dilution; Sigma).

3.4.3.3. Determination of carbonyls groups

Carbonyl groups were determined as a marker of oxidatively modified proteins. It was carried out according to the method of Romero-Puertas *et al*., 2002 and 2004. The homogenates were incubated with 10 mM of 2,4-dinitrophenylhydrazine (DNPH) prepared in 2M HCl for 1 hour. As controls samples incubated in HCl without DNPH were used. Subsequently, the proteins were precipitated with 10% TCA (w/v) and washed with ethanol/ethyl acetate (1:1) three times as indicated above. Finally, the proteins were resuspended in 10 mM phosphate-Na buffer, pH 7.4, and were loaded on polyacrylamide-SDS gels (12%) and further transfered to a PVDF membrane. This membrane was incubated with commercial antibodies against the DNPH (dilution 1: 40,000 Sigma) and the oxidized proteins were detected by chemiluminescence or by the alkaline phosphatase method, using as substrate of NBT. In addition, oxidized catalase was detected by immunoprecipitation of carbonylated protein (Romero-Puertas *et al*., 2002) as a decribed in Chapter 4.3.

3.4.3.4. Determination of *S*-nitrosylated proteins.

To detect *S*-nitrosylated proteins in plants, we adopted the biotin-switch method, a three-steps procedure that converts *S*-nitrosylated Cys residues into biotinilated Cys residues (Jaffrey *et al*., 2001; Romero-Puertas *et al*., 2007; Fig. 3.6). Subsequently, proteins were subjected to SDS-PAGE and Western blot analysis using an anti-biotin antibody from Sigma-Aldrich (1:10,000 dilution). Otherwise, biotin-labeled proteins were purified by incubation with IPA-anti-Biotin (inmunoprecipitation assay; Sigma), and bound proteins were eluted with the addition of 1,4-dithiothreitol (DTT) to a final concentration of 150 mM, followed by an identification of the specific proteins such as CAT with is antibody (1:5,000; Agrisera) carried out EGPA-SDS and Western blot (Fig. 3.6).

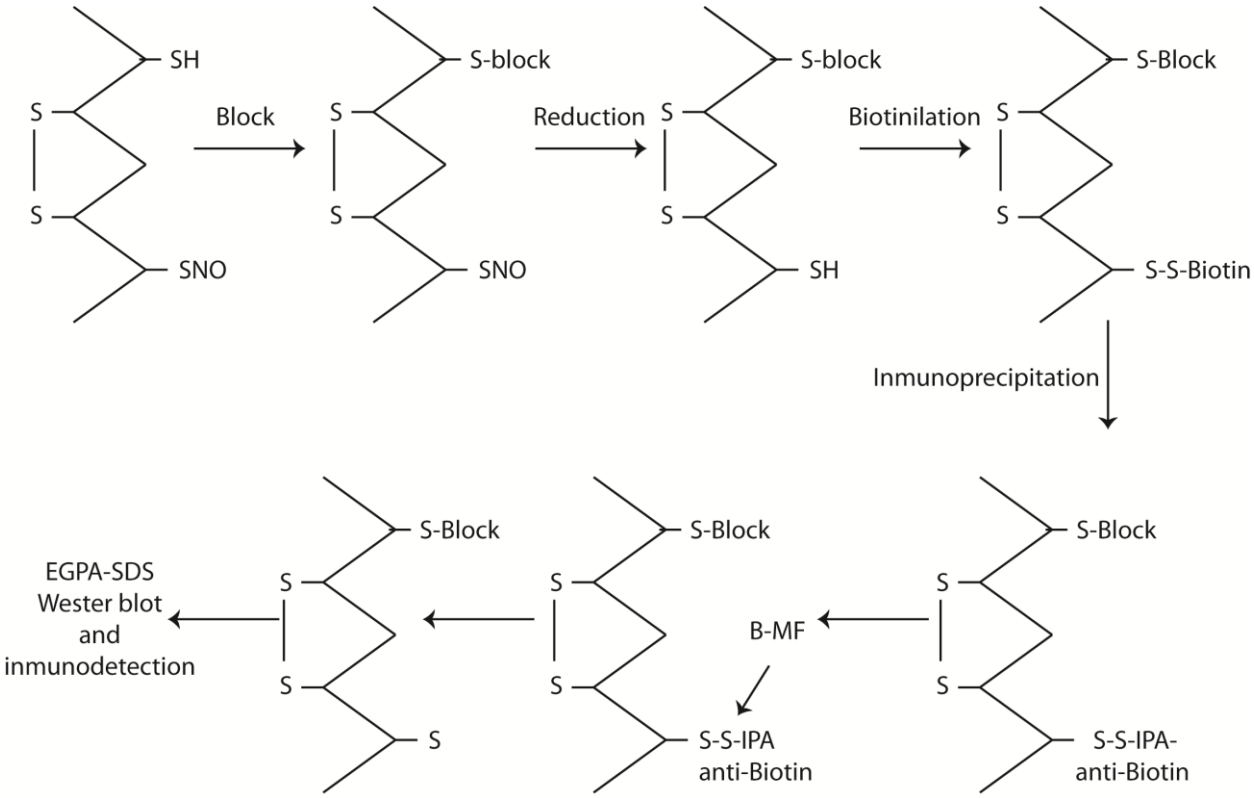

Fig. 3.6. Identification of *S*-nitrosylated proteins. Summary of the biotin-switch method. Adapted from Jaffrey *et al*., 2001 and Romero-Puertas *et al*., 2008.

3.5. Lipid peroxidation

Lipid peroxidation rate was determined using thiobarbituric acid, according to Buege and Aust (1978) method. Reaction mixture was prepared in Milli-Q H_2O_2 adding 15% trichloroacetic acid (w/v), thiobarbituric acid 0.375% (w/v) and butylhydroxytoluene 0.01% (w/v) prepared in 0.25 N HCl. 20 µL sample were added to 100 µL reactive solution. Mixture was heated for 15 min at 95ºC followed by centrifugation of the samples at 2.000g for 2 min. After that, samples were measured using a Varioskan LUX (Thermo Fisher) at 535 nm. Calibration curve were calculated using MDA as standard (0.1 to 10 µM).

3.6. Nucleic acid analysis

3.6.1. Extractions of total plant and *Fusarium oxysporum* DNA

Infected seedlings with *F. oxysporum* microconidial haversted at 2 and 7 days post inoculation, were homogenized with liquid nitrogen in cetyl trimethyl ammonium bromide (CTAB) extraction buffer (Tris 100 µM, EDTA 25 µM, NaCl 13mM, CTAB 55 µM pH 8) and vortexed. 4 µL mercaptoethanol and 1 mL chloroform: octanol (24:1) were added, vortexed again and incubated 30 min at 65ºC. Subsequently samples were incubated at room temperature 15 min and then, were centrifuged at 5,000 g for 5 min. Supernatants were collected, mixed with 1 mL cold ethanol (100%; v/v) and incubated 20 min at -20ºC. After that, samples were centrifuged at

17,000 g for 10 min, the supernatants were discarded, and 1 mL cold ethanol (75%) was added to the pellet, resuspended and centrifuged under the same conditions. Finally, supernatans were discarded, pellets were drained and resuspended in 20-30 µL of Tris-EDTA buffer (TE), 3 µL of RNAse were added to the suspension and incubated for 60 min at 37ºC. DNA obtained was used to calculate fungal burden as described in Chapter 4.4.

3.6.2. Extraction of RNA

50-150 mg of Arabidopsis seedlings were homogenized adding liquid nitrogen. Subsequently, 1 mL of Trizol reagent (MRC) was added (Chomczynski and Sacchi, 1987). After 5 min incubation at room temperature, 0.2 mL of chloroform was added and mixed by inversion for 15 s and incubated 3 min at room temperature. After centrifugation at 12000 g for 15 min at 4°C, to the initial supernatant containing the RNA, 0.5 mL of isopropanol was added per mL of initial Trizol and incubated at room temperature for 10 min. After that, it was centrifuged at 12000 g for 10 min at 4°C and the precipitate, containing the RNA, was washed with 1 mL of 75% ethanol (v/v), centrifuged again at 12,000 g for 5 min and the precipitate was air dried. Finally, the precipitated RNA was resuspended in 1% of diethylpyrocarbonate water (DEPC) free of ribonucleases and stored at -80°C. DNAse treatment was performed by DNA-free Thermo Kit, following commercial instructions.

3.6.3. Integrity of nucleic acids by electrophoresis

The analysis of the DNA and RNA by electrophoresis was made to check their integrity and quantification. Samples were analyzed by electrophoresis in 1% agarose gels (w/v) in 45 mM Tris-HCl buffer (pH 8.0), 45 mM glacial acetic acid and 1 mM EDTA (TAE). The gels were prepared by dissolving the agarose in TAE buffer by heating and subsequently prepare the gels at room temperature. Samples were prepared in loading buffer containing 4% glycerol. The electrophoresis was done at 100 V for 12 min. The bands were stained with ethidium bromide 0.5 µg/ml and visualized using a ChemiDoc (Bio-Rad) system coupled with a high sensitivity CCD camera. The band intensity was quantified with Image J.

3.6.4. Reverse transcription

cDNA from 1,5 µg isolated RNA, was obtained by reverse transcription reaction following the instructions of the commercial company of the enzyme PrimeScript RT Reagent Kit (Takara), with the following reaction conditions: 37°C, 15 min; 85°C, 5 secs using a Mastercycler thermal cycler (Eppendorf).

3.6.5. Oligonucleotides design

The oligonucleotides used for expression studies by quantitative PCR are specificed in each chapter. They were designed using the "Beacon Designer" program. These oligonucleotides amplify a cDNA segment between 50-250 bp, with a G-C content between 50-60% and the temperatures used were between 50-60°C whenever it was possible. The approximated alignment temperature (Ta) was calculated depending on the length and composition of the oligonucleotides used, following the formula: Ta = 2 (A + T) + 4 (G + C). Serial dilutions of pooled samples were prepared to calculate the efficiency of the oligonucleotides. The calculations were made from the slopes of the standard curve obtained by the iQ5 program using the formula E = [10 (1/a) -1] x 100, where "a" is the slope. Primer melting curves with 90-105% efficiency were performed to validate amplification specificity (Bustin *et al*., 2009; Suppl. Tables S3.2 and S3.3).

3.6.6. Polymerase chain reaction (PCR): Real time q-PCR

SYBR Green I molecule, that emits fluorescence when binding to double-stranded cDNA molecules during amplification, has been used as a detection method. For the quantitative PCR analysis, the specifications of the Takara company for "iQ SYBR Green Supermix" were followed. To perform the PCR reaction, 10.5 µL of SYBR Green, 0.5 µL of each 10 µM oligo and 1 µL of cDNA were mixed as a template molecule in 8.5 µL of Milli-Q water. The following program described in Table 3.4. was applied in an iCycler iQ5 thermal cycler (Bio-Rad).

Table 3.4. q-PCR programs used for expression analysis.

Stage	Temperature	Time	Nº cycles
Initial desnaturalization	**95ºC**	**3 min**	**1**
Desnaturalization	**95ºC**	**10 s**	**35**
Annealing	**50-60ºC**	**30 s**	
Extension	**72ºC**	**30 s**	
Final extension	**72ºC**	**10 min**	**1**

Suppl. Table S3.2. Reverse transcription quantitative PCR primers for Chapter 4.2, 4,3 and 4.4.

Gene	Primer sequence	ID	Tº	Amp	Eff.	References
CAT2-s	TGGATCTCTTACTGGTCTCAGGC	AT4G35090	**55**	**257**	**1.91**	(Rodríguez-Serrano., 2016)
CAT2-as	CGAGAGACACAACAACACACAAGG					
Pex11a-s	CCTCGCATCATCACTAATCC	AT1G47750	**60**	**379**	**1.97**	(Rodríguez-Serrano., 2016)
Pex11a-as	GATCGTCGAAGCAACACAAC					
Fes1A-s	AAGTAGCAGCATAGATAA	AT3G09350	**55**	**159**	**1.99**	Beacon desingner sofware
Fes1A-as	GAAGAAGGTTCATTGTAG					
HSP20-like-s	ATAAGAAGAAGGCTCAAGT	AT1G59860	**55**	**122**	**1.98**	Beacon desingner sofware
HSP20-like-as	AACGAAGAACCATAATCATC					
GST-1 s	AAAGCTTGTTTGGGAGCAAGTCTTAAAGC	AT1G02930	**55**	**414**	**2.05**	(Rodríguez-Serrano., 2016)
GST-1as	AACACTCGGCAGCAGAAAAACAGAGTAAAC					
PDF 1.2- s	AGTTGTGCGAGAAGCCAAGT	AT5G44420	**60**	**107**	**1.98**	(Fernández-Calvo *et al*., 2011)
PDF 1.2-as	GTTGCATGATCCATGTTTGG					
VSP2-s	CGTCGATTCGAAAACCATCT	AT5G24770	**55**	**135**	**2.01**	(Fernández-Calvo *et al*., 2011)
VSP2-as	GGCACCGTGTCGAAGTCTAT					
PR1-s	TCCGCCGTGAACATGTGGGTTAG	AT2G14610	**55**	**180**	**2.01**	Beacon desingner
PR1-as	CCCACGAGGATCATAGTTGCAACTGA					
PR5-s	CGGTACAAGTGAAGGTGCTCGTT	AT1G75040	**55**	**312**	**1.94**	Beacon desingner
PR5-as	GCCTCGTAGATGGTTACAATGTCA					
FIT-s	ATCCTTCATACGCCCTCTCC	AT2G28160	**60**	**149**	**1.99**	Beacon desingner
FIT-as	GAGCCGGTGGTGAAGAAG					
IRT1-s	CGGTTGGACTTCTAAATGC	AT4G19690	**55**	**165**	**1.97**	(Besson-Bard *et al*., 2009)
IRT1-as	CGATAATCGACATTCCACCG					
FRO2-s	AAGATGTTGGAGATGGACGG	AT1G01580	**60**	**122**	**1.97**	(Wang *et al*., 2007)
FRO2-as	CTTGGTCATCTCCGTGAGC					
AtCesA4 s	CTCAACATGGTCGGTGTTGTTG	AT5G44030	**55**	**275**	**2.02**	Beacon Desing
AtCesA4 as	TCGACGCCACATTGCTTCAGT					
MYB46-s	GAATGTGAAGAAGGTGATTGGTACA	AT5G12870	**55**	**150**	**1.99**	(Turco *et al*., 2019)
MYB46-as	CGAAGGAACCTCAGTGTTCATCA					
Foxc act1-s	ATGTCACCACCTTCAACTCCA	FOXG_04579	**55**	**300**	**2**	(Masachis *et al*., 2016)
Foxc act1-as	CTCTCGTCGTACTCCTGCTT					
CuZnSOD1-s	AACTCAGCCTGGCTACTGGAAAC	AT1G08830	**55**	**143**	**1.98**	(Rojas-Gonzlez 2015)
CuZnSOD1-as	CACACAACTACCAAACCCAGGTC					
CuZnSOD2-s	ATTCCTCCTTCCTCCAATCC	AT2G28190	**55**	**508**	**1.99**	(Rojas-Gonzalez 2015)
CuZnSOD2-as	CATCCTTAAGCTCGTGAACC					
CuZnSOD3-s	AGTATTCCATACTCGGGAGGGCG	AT5G18100	**55**	**145**	**1.97**	(Rojas-Gonzalez 2015)
CuZnSOD3-as	GCATCCGCAGATGATTGAAGTCC					

Suppl. Table S3.3. Reverse transcription quantitative PCR parameters according to the minimum information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines derived from Bustin *et al*., 2009.

Sample/Template	
Source	*Arabidopsis thaliana* seedling.
Method of preservation	Harvest in liquid nitrogen, storage at -80 °C.
Storage time (if appropriate)	Maximum one week.
Handling	Frozen.
Extraction method	Trizol reagent (MRC).
RNA: DNA-free	DNA-free™ DNA Removal Kit (Thermo DNA free).
	Use of intron-spanning primers.
	Verification of single peak on dissociation curves (melting curve).
RNA: concentration	NanoDrop® ND-1000 spectrophotometer and agarose (1%) electrophoresis gel.
RNA: integrity	NanoDrop® ND-1000 spectrophotometer and agarose (1%) electrophoresis gel.
Assay optimisation/validation	
Accession number	References cited in Materials and methods.
Amplicon details	References cited in Materials and methods.
Primer sequence	References cited in Materials and methods.
In silico	Primer-BLAST (http://www.arabidopsis.org/Blast/index.jsp).
Empirical	Primer concentrations of 250 nM.
	Annealing temperature of 55 or 60 °C.
Priming conditions	Combination of oligo-dT primers and random hexamers.
PCR efficiency	Dilution curves (slope, deviation).
Linear dynamic range	Samples are within the range of the efficiency curve.
RT and qPCR	
Protocols	iCycler iQ Real-Time PCR Detection System (Bio-Rad).
	PrimerScript RT reagent Kit (Takara).
	SYBR Premix Ex Taq™ II (Takara) .
	As described in the Materials and Methods section.
Reagents	As described in the materials and Methods section.
NTC	Cq and dissociation curve verification.
Data analysis	
Specialist software	e iCycler Program (Bio Rad)
Statistical justification	As described in the Materials and Methods section.
Transparent, validated normalisation	Minimum five references genes selected using the GrayNorm algorithm.
	As described in the Materials and Methods section.

The relative expression of each gene was normalized with Tubulin (*TUB-4*) selected from 6 genes according to GrayNorm algorithm as described in Terrón-Camero *et al*., (2019) and Remans *et al*., (2014) , and the results were calculated using the relative expression ratio according to the Pfaffl method (Pfaffl, 2001; Bustin *et al*., 2009).

$$Relative\ expresion = \frac{(\text{Eproblem})^{\Delta \text{CP problem(C}-\text{sample)}}}{(\text{Eref})^{\ \Delta \text{CP ref(C}-\text{sample)}}}$$

- Eproblem: Is the effectiveness of real-time PCR of gene problem transcription of the problem gene.
- ERef: Is the real-time efficiency of the PCR of a transcription of reference gene; in this case *TUB-4*.
- ΔCP problem: Is the CP deviation of the control - gene problem.
- ΔCP ref: CP deviation of the control - reference sample.

The relative expression ratio of a problem gene is calculated based on the efficiency (E) and the point at which the fluorescence rises appreciably above the background fluorescence (CP) of an unknown sample versus a control, and it is expressed in comparison to a reference gene (Suppl. Table S4.2.2, S4.3.2, S4.4.2)

3.7. Reactive oxygen and nitrogen species measurements

3.7.1. Fluorimetric methods

Seedling extracts were prepared in 25 mM HCl (1/3, w/v) and centrifugated at 5,000 g for 5 min. Supernatants were neutralized with NaOH and used to measure the concentration of H_2O_2. 20-100 µL sample was added to Hepes buffer, pH 7.6 (50 mM), homovanillic acid (5 mM) and peroxidase (40 µM). H_2O_2 concentration was determined by measuring the fluorescence in a spectrofluorimeter with excitation at 315 nm and emission at 425 nm, against a standard curve of H_2O_2 (1-80 µM), prepared in 25 mM HCl previously neutralized with NaOH (Creissen *et al*., 1999; Romero-Puertas *et al*., 2004).

For NO detection, seedling extracts were incubated with 20 µM 4,5-diaminofluorescein (DAF-2) for 2h at 37ºC and fluorescence was analysed in a fluorimeter (ext. 495 ems 515; Nakatsubo *et al*., 1998).

3.7.2. Histochemistry

H_2O_2 was histochemical, stainined with diaminobenzidine (DAB; Thordal-Christensen *et al*., 1997). The leaves were immersed in 1 mg/ml DAB (3,3'diaminobenzidine, Sigma) pH 3.8 for 12 h and in the dark at 25°C. After the incubation period, leaves were rinsed with water twice and

boiled in 95% ethanol (v/v) for 5 min. The production of H_2O_2 was visualized by dark brown precipitates. These precipitates were quantified from the images by calculating the intensity of the color using the Image J software.

$O_2^{\cdot -}$ was stained with NBT (Schraudner *et al*., 1998). The leaves were immersed in 0.1% NBT (w/v), in 50 mM potassium phosphate buffer, pH 6.4 and 10 mM sodium azide. Subsequently, the leaves were exposed to light until the appearance of dark spots, due to the formation of formazan.

3.7.3. Microscopy methods

H_2O_2, NO, $ONOO^-$, and $O_2^{\cdot -}$ were measured using specific fluorophores in root tissues. Details of the method are shown in 3.13 and Chapter 4.2.

3.8. Sample preparation for microscopy

3.8.1. Sample inclusion in agarose

Low melting point agarose 5% (w/v) were prepared in phosphate-buffer saline (PBS), placed in molds that contained 30-40 orderly seedling roots. After that, blocks were refrigerated at 4ºC at least 3 h. Then, a vibratome (Leica) was used to cut sample sections of 70 µm.

3.8.2. Sample preparation for electron microscopy

Leaves were cut, fixed, dehydrated and embedded in Spurr resin. After that, semithing and ultrathing sections were obtained in a microtome for light and electron microscopy analyses. The details of the method are shown in materials and methods in Chapter 4.3. Part of this process was carried out at the Center of Scientific Instrumentation of the University of Granada.

3.9. Peroxisomes imaging by confocal microscopy

To characterize peroxisome dynamic which mutants *px-ck* crosses with NO mutans described above, where used, to imaged peroxisome (blue color) that allows to observed blue fluorescent peroxisomes by confocal microscopy (Rodríguez-Serrano *et al*., 2016). To better characterize these Arabidopsis mutant and stablishe the role of NO in peroxisome phenotype were stained with DAB to identify peroxisome by light and electron microscopy. The process in detail was included in Materials and methods section in Chapter 4.3.

3.10. Other assays

3.10.1. Cadmium detection and location

Cd was located in the tissues of the seedlings by dithizone staining method as described in Chapter 4.3 (Seregin and Ivanov, 1997; Clabeaux *et al*., 2011)

3.10.2. Cellulose determination

Acid extracts and histological staining were carried out to detect cellulose content in different mutants as described in Chapter 4.4.

3.10.3. pH determination

For whole-plant alkalinization assays, two-week-old Arabidopsis seedlings, inoculated or not with *F. oxysporum*, were placed on 0.8% water noble-agar plates and incubated vertically in a growth chamber, as described above. After 1 day, the pH indicator bromocresol purple 0.833 µM was uniformly distributed on the plate surface. Subsequently, plates were incubated for two additional days and the changes of color was imaged.

3.10.4. Quantification of fluorescent phenolic compounds in root exudates.

The amount of phenolic compounds in the root exudates was quantified under UV light (365 nm; Berendsen *et al*., 2012; Stringlis *et al*., 2018). 100 µL of water with root exudates from plant inoculated or not with *F. oxyporum* were transferred into a 96 well microplate and, fluorescence emitted (ext at 360 nm; ems at 528 nm) was measured with a Varioskan LUX Multimode Microplate.

3.11. Bioinformatic analysis

Chapter 4.1, 4.2, 4.3, 4.4 and 4.5 showed the bioinformatics studies, which were performed by different tools specified in each chapter.

3.12. Statistical analysis

Mean values for all experiments were obtained from at least three independent experiments with at least three independent replicates. Statistical analyses were performed using one or two-way ANOVA test when necessary. Mean values for the different treatments were compared using Tukey's or Student-Newman-Keuls multiple comparison tests ($P<0.05$) after the two-way ANOVA analysis or T-Student test after one-way ANOVA analysis, which are specified in each figure, using IBM SPPS Statistic 24 and GraphPad Prism 6. Error bars representing standard error (SEM).

3.13. Detection of Reactive Oxygen and Nitrogen Species (ROS/RNS) During Hypersensitive Cell Death

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Highlights

In response to heavy metal stress exogenous NO prevents oxidative damage alleviating plant fitness-loss while endogenous NO should be fine-tune regulated and NO-dependent signalling pathways are involved in plant resistance.

ABSTRACT

Reactive oxygen and nitrogen species (ROS/RNS) are signalling molecules involved in a plethora of physiological processes in plants. Especially, ROS and nitric oxide (NO) are key players that are required for programmed cell death (PCD). The PCD associated with the hypersensitive response (HR) has been well characterized and the role of H_2O_2 and NO as key signalling molecules inducing HR has been established. Localization of ROS and NO production in plant tissues in response to pathogens can be imaged by confocal laser microscopy by using specific fluorescent probes. Deciphering the time and spatial regulation of ROS and NO is very important to establish the cellular response of plants to adverse conditions. This chapter is mainly focused on the imaging of ROS and RNS accumulation in vivo in plant tissues undergoing PCD.

Key words: Arabidopsis; Hypersensitive response; Nitric oxide; Peroxynitrite; *Pseudomonas syringae*; Reactive oxygen species

Published in De Gara L., Locato V. (eds) Plant Programmed Cell Death. Methods in Molecular Biology, vol 1743. (2018) Humana Press, New York, NY
https://doi.org/10.1007/978-1-4939-7668-3_9

3.13.1. Introduction

Plants interact constantly with a wide range of microorganisms and they have mechanisms to differentiate beneficial from pathogens (Vadassery and Oelmüller, 2009). Therefore, an attempted microbial infection activates a quick production of reactive oxygen species (ROS), mainly superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Levine *et al.*, 1994; Lamb and Dixon, 1997), and nitric oxide (NO) and reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) (Vandelle and Delledonne, 2011). Actually, the resistance response begins with the specific recognition of the pathogen (due to the pathogen-associated molecular patterns, PAMPs), by plant receptors leading to the hypersensitive response (HR; Romero-Puertas *et al*., 2004). The main sources of ROS during most biotic stress responses appear to be plasma membrane-localized NADPH oxidases that generate superoxide, or cell wall localized peroxidases that produce superoxide and hydrogen peroxide (Torres *et al*., 2002; Bolwell and Dauidi, 2009). Actually, it has been shown that peroxidase-dependent oxidative burst plays an important role in Arabidopsis basal resistance mediated by the recognition of PAMPs (Daudi *et al*., 2012). Additionally, NADPH oxidases (or RBOH for respiratory burst oxidases homolog) have been involved in ROS production leading to the HR in different plant species and have been characterized in detail in *Arabidopsis thaliana* (Torres and Dangl, 2005). In particular, RBOH D and RBOH F were found to be necessary for the production of the oxidative burst and PCD in response to avirulent strains of the bacterial pathogen *Pseudomonas syringae* (Torres *et al*., 2002). It appears that the main role of NADPH oxidases may be to edge the spread of cell death in cells surrounding the infection site (Torres and Dangl, 2005). On the other hand, there are a variety of mechanisms producing NO in higher plants, and both oxidative (arginine or hydroxylaminedependent) and reductive (nitrate-dependent) pathways have been related with NO production during the HR (Zaninotto *et al*., 2006; Mur *et al*., 2013).

HR is characterized by the programmed cell death (PCD) at the infection site that restricts pathogen spread and infection (Lamb and Dixon, 1997). Before the accomplishment of PCD a rapid synthesis of ROS and NO are necessary and the effective induction of HR requires a balance between ROS and NO production that should be finely tuned (Delledonne *et al*., 2001). Actually, NO and ROS function depends on the rate and location of its production and localization of these molecules in plant tissues is crucial. This chapter focuses on imaging of ROS and RNS accumulation in vivo in tissues undergoing HR-dependent PCD by histochemical reaction with DAB (3,3′-diaminobenzidine) and confocal laser microscopy using specific fluorescent probes.

3.13.2. Materials

3.13.2.1. Biological Material

1. *Arabidopsis thaliana* seeds (Col-0 ecotype): WT and *rbohD*.
2. *Pseudomonas syringae* pv. tomato DC3000 strain carrying *Avr*Rpm1 gene (*Pst Avr*Rpm1).

3.13.2.2. Chemicals

1. Fluorescent probes: 4,5-diaminofluorescein diacetate (DAF-2 DA), 2′-7′ dichlorodihydro-fluorescein (DCF-DA), and HK Green (Sun *et al*., 2009; see Note 1). Reaction for each probe is described in Fig. 3.13.1.
2. Rifampicin (50 mg/mL).
3. Kanamycin (50 mg/mL).
4. 3,3′-diaminobenzidine tetrahydrochloride (DAB).

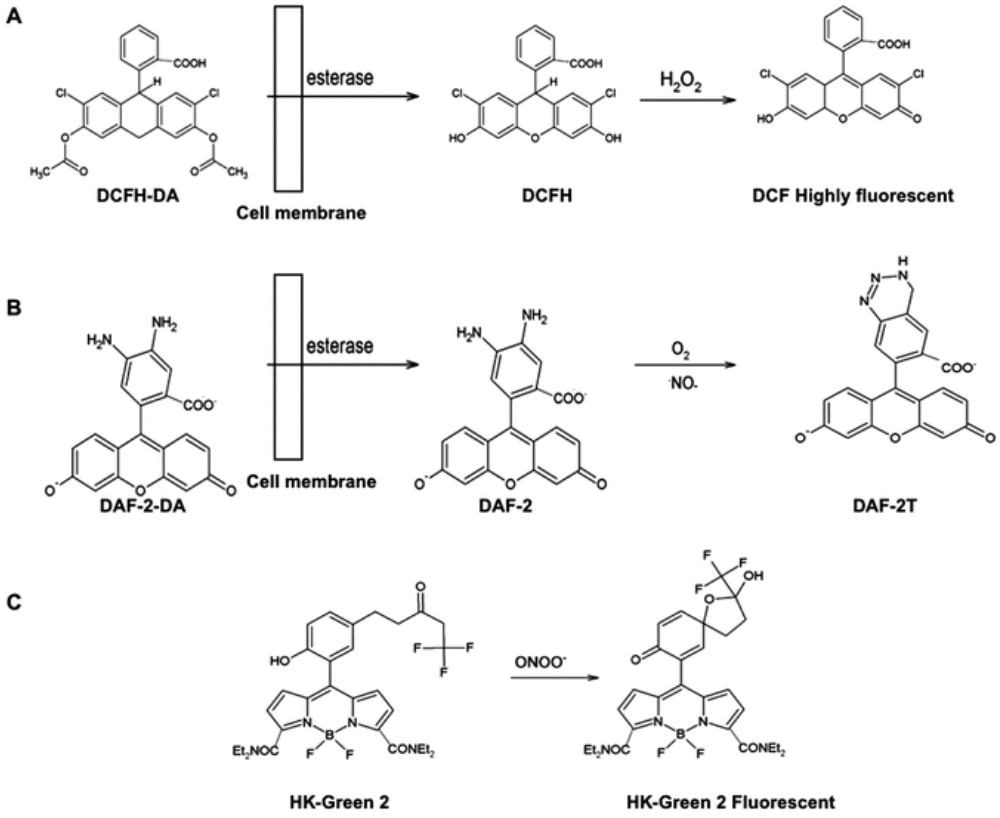

Fig. 3.13.1. Scheme of fluorophores reactions with ROS and RNS. Scheme of reactions for the detection of peroxides (**a**), nitric oxide (NO; **b**), and peroxynitrite (**c**) by DCFH-DA, DAF-2-DA, and HK-Green2, respectively. ACD/ChemSketch software was used.

3.13.2.3. Buffers and Media

1. 10 mM Tris–HCl adjusted to a pH of 7.4.
2. King's B medium: 2% (w/v) Proteose Peptone, 8.6 mM K_2HPO_4, 6.1 mM $MgSO_4$, and 1% (v/v) glycerol at pH 7.2.
3. 10 mM $MgCl_2$.

4. TBS buffer: 50 mM Tris 150 mM NaCl, pH 7.4.

3.13.3. Methods

3.13.3.1. Arabidopsis Infection

1. Vernalize *Arabidopsis thaliana* seeds for 24–48 h.
2. Sow seeds on sterile soil and transfer them into a growth chamber with short day conditions: 8/16 h (22/20 °C) day/night, with light at 120 μE and 60% relative humidity.
3. Grow the pathogen (Pst *Avr*Rpm1) in King's B medium (Containing Rifamicin and Kanamycin; see Note 2), at 28 °C overnight.
4. Precipitate bacteria and resuspend them in $MgCl_2$ at 1×10^7 cfu/mL.
5. Infiltrate bacteria solution into the abaxial surface of *A. thaliana* leaves using a hypodermic syringe without needle (see Note 3).
6. Collect *A. thaliana* leaves from plants 4 h after infiltration.

3.13.3.2 Plant Tissue Preparation and Fluorescent Staining

1. Cut *Arabidopsis thaliana* leave segments of approximately 1 mm avoiding main vascular tissue.
2. Incubate immediately sections in darkness with the fluorescent probes as indicated in Table 7.2.1.
3. Wash the pieces three times (10 min each) in the same buffer.
4. Check specificity of the reaction by using specific sequesters or inhibitors of ROS and RNS as negative controls, as indicated in Table 3.13.2. (see Note 4).

Table 3.13.1. Fluorescent probes and incubation conditions used.

ROS/RNS	Probes	μM	Buffer	Time (min)	Temp (ºC)
H_2O_2	DCF-DA	25	10 mM Tris–HCl (pH 7,4)	30	37
NO	DAF-2DA	10		60	25
$ONOO^-$	HK-green	10		60	25

Table 3.13.2. Control used to achieve specificity of fluorescent probes.

Concentration	mM	Buffer	Time (min)	Temp (ºC)	Function
Ascorbate (Asc)	1	10 mM Tris–HCl (pH 7,4)	60	25	Peroxide scavenger
Aminoguanidine(AG)	1		60	25	NO substrate competitor
Ephicatechin	1		60	25	Peroxinitrite scavenger

3.13.3.3 Detecting Fluorescence

1. Put samples in a slide using glycerol 6% and TBS buffer mix (1:1) in darkness and cover it with a coverslip.

2. Visualize samples in a Confocal Laser Scanning Microscope (CLSM). Conditions used to image each probe by CLMS are described in Table 3.13.3.
3. Use chlorophyll autofluorescence (excitation: 633 nm/emission: 680 nm) as a reference of cell structures.
4. Get maximum projection (3D images) with at least 25 frames 5 μm of thickness each. Representative sample images of the different fluorescent probes are shown in Figs. 3.13.2, 3.13.3., and, 3.13.4. A scheme of the complete process is presented in Fig. 3.13.5.

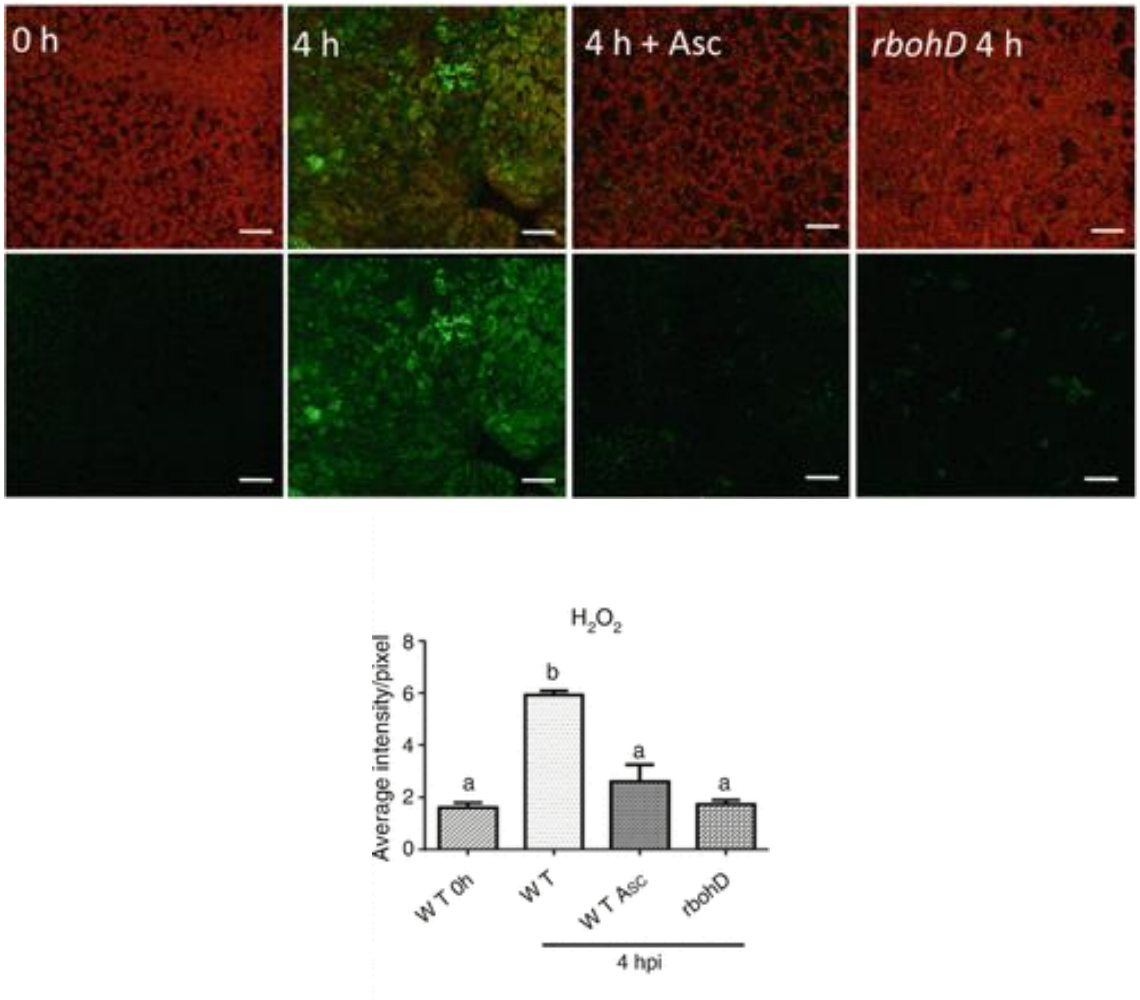

Fig. 3.13.2. Imaging and cuantification of H_2O_2 in leaf. Imaging of peroxide-dependent DCF-DA fluorescence (green) and chlorophyll auto-fluorescence (red) in Arabidopsis leaves after 4 h infiltration with *Pst Avr*Rpm1 (4 h), in non-treated samples (0 h) and leaves incubated with ascorbate before the dye staining (4 h + Asc). *rboh D* plants that do not produce oxidative burst during hypersensitive cell death (Torres *et al*., 2002) were also used as control. The graph shows fluorescence across the section tissue quantified in arbitrary units using Image J software. Different letters stand for significant values (T-Student $p > 0.05$). Scale bar 100 μM; hpi: h postinfection.

Table 3.13.3. CLSM conditions: excitation (exc.) and emission (ems.) nm of each probe used

Objetive	Plan Apo 10×
Average	4
DCF exc./ems.	480/530
DAF-2 exc./ems.	495/515
HK-Green 2 exc/ems.	520/539

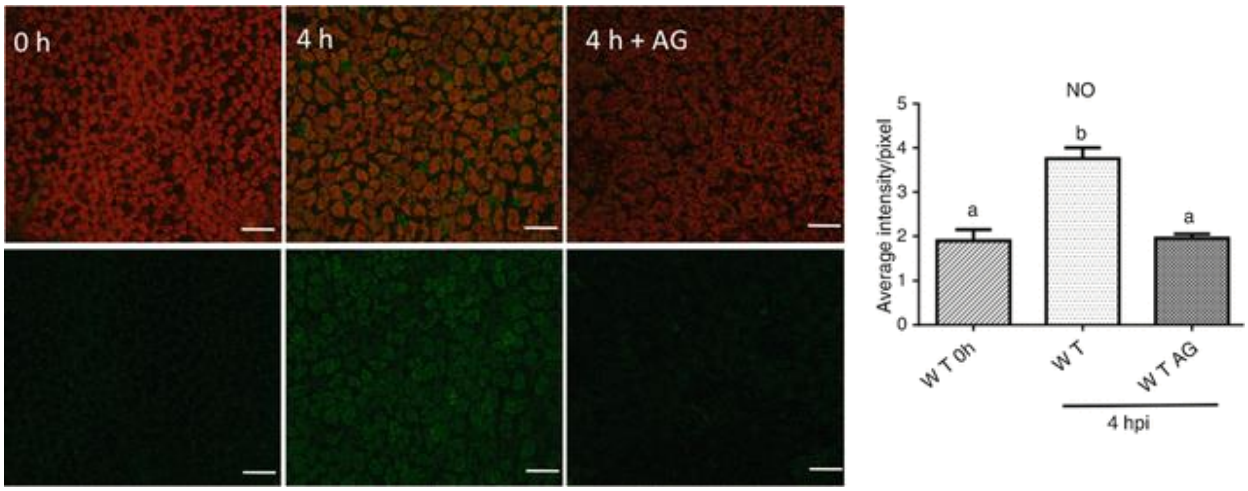

Fig. 3.13.3. Imaging of NO-dependent DAF-2-DA fluorescence (green) and chlorophyll auto-fluorescence (red) in Arabidopsis leaves after 4 h infiltration with *Pst Avr*Rpm1 (4 h), in non-treated samples (0 h) and leaves incubated with aminoguanidine before the dye staining (4 h + AG). The graph shows fluorescence across the section quantified in arbitrary units using Image J software. Different letters stand for significant values (T Student $p < 0.05$). Scale bar 100 μM; hpi: h post-infection.

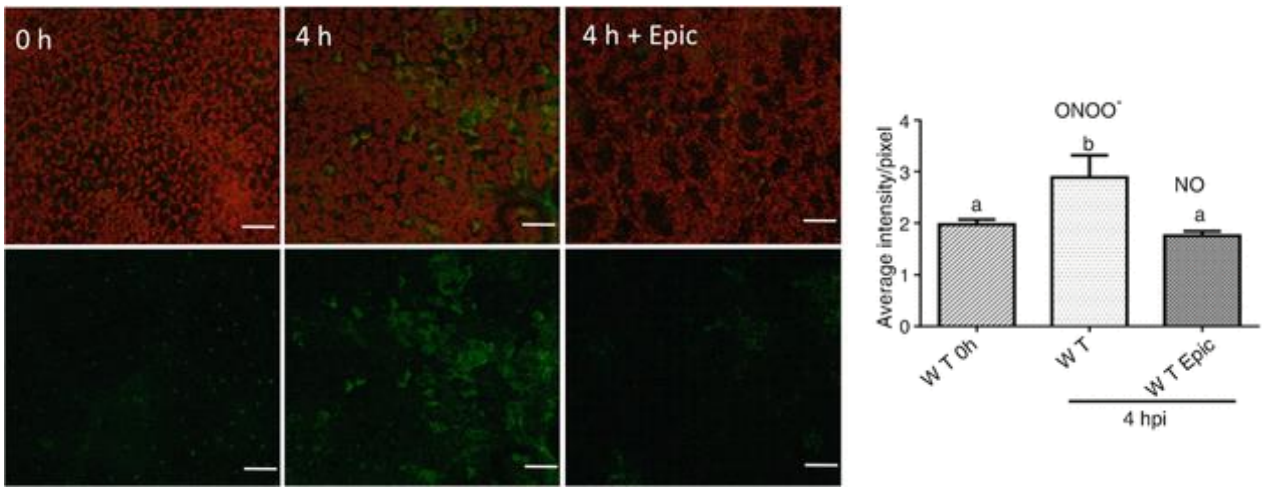

Fig. 3.13.4. Imaging of $ONOO^-$-dependent HK-Green fluorescence (green) and chlorophyll autofluorescence (red) in Arabidopsis leaves after 4 h infiltration with *Pst Avr*Rpm1 (4 h), in nontreated samples (0 h) and leaves incubated with ephicatechin before the dye staining (4 h + Epic). The graph shows fluorescence across the section quantified in arbitrary units using Image J software. Different letters stand for significant values (T-Student $p < 0.05$). Scale bar 100 μM; hpi: h post-infection.

A scheme of the complete process is presented in Fig. 3.13.5.

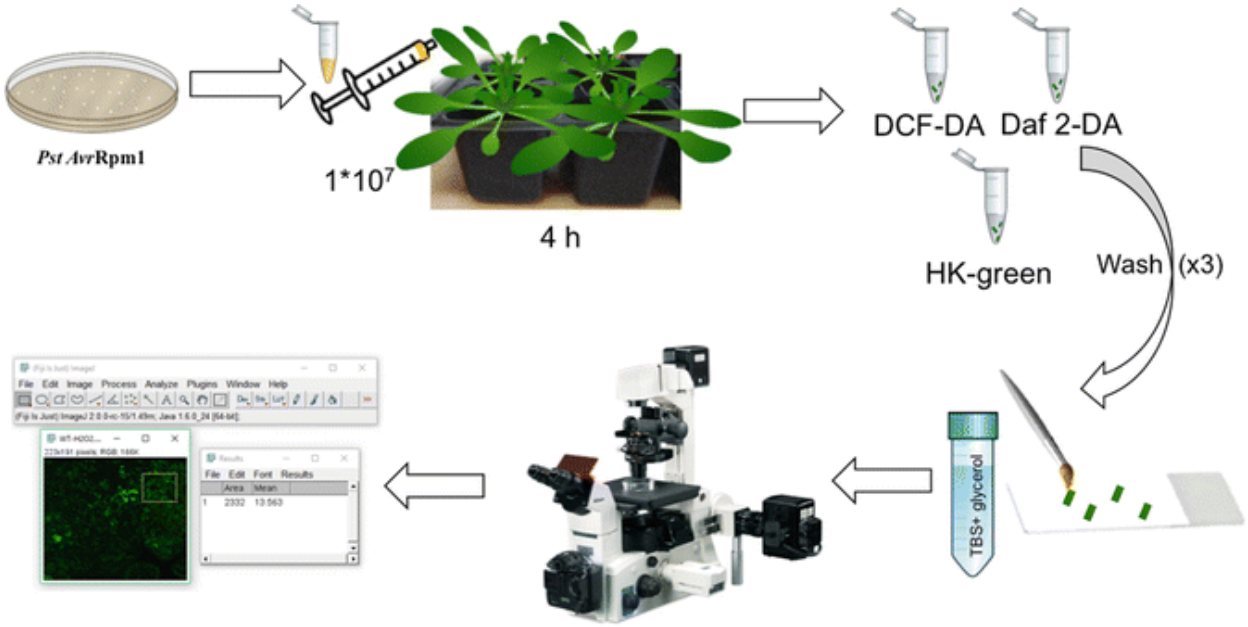

Fig. 3.13.5. Scheme of the complete microscopy process: Plants were infiltrated with the avirulent pathogen (*Pst Avr*Rpm1), cut and incubated with the dyes and then observed with the CLSM.

3.13.3.4 DAB Staining

Alternatively, H_2O_2 production can also be visualized by DAB staining:

1. Incubate whole leaves in darkness with 1 mg/mL DAB at pH 3.8 (adjusted with NaOH) overnight.
2. Wash the leaves three times in water.
3. Remove chlorophyll by boiling leaves in ethanol 100%.
4. Scan leaves to quantify staining. Representative sample images of the DAB staining are shown in Fig. 3.13.6.

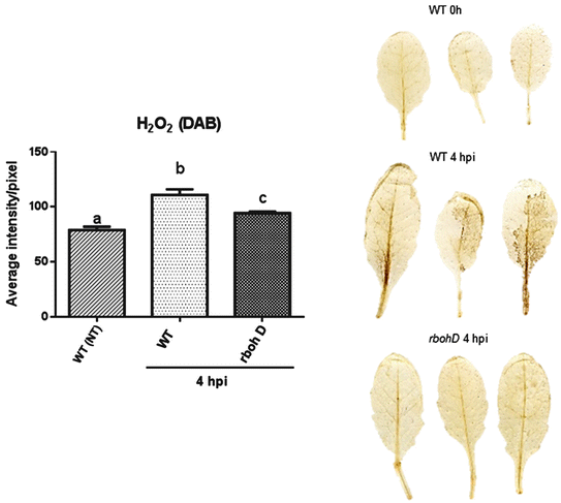

Fig. 3.13.6. DAB staining of Arabidopsis leaves to assess H_2O_2 accumulation during the oxidative burst. PstavrRpm1 were infiltrated into Arabidopsis leaves, and DAB staining was initiated 4 h after infection. rboh D plants were also used as control. The graph shows quantification of DAB staining. The average of pixels stained inside the infiltration space was assessed by Image J software. Different letters stand for significant values (T-Student $p > 0.05$). hpi: h postinfection.

3.13.3.5. Quantification of the Staining

1. Use any imaging program, such as Image J or Adobe Photoshop to quantify staining of the leaves.

2. Measure average intensity by pixel in each image and make an average of 3–5 independent squares per image with the same size avoiding the edge of the leaf.

3. Follow the steps as indicated:

- In File: open or drag images.
- In Image type: select 8 bits.
- In Select rectangular tools: select make a rectangle in each image.

Then select: Analyze; then select: tool; then select: ROI manager and finally select: ADD.

- In Analyze: select Set measurement; then Select Mean grey value and press OK.
- In Analyze: select Measure.
- Copy data in a spreadsheet and perform a basic statistical analysis (mean, SD, and T-Student; see Note 5).

Representative quantification of the images of the different fluorescent probes and DAB staining are shown in Figs. 3.13.2, 3.13.3, 3.13.4, and 3.13.6.

3.13.4. Notes

1. Fluorescent dyes are solubilized in dimethyl sulfoxide.

2. Filter Kanamycin before adding to the medium.

3. Use leaves infiltrated with 10 mM $MgCl_2$ or not treated (NT) as controls.

4. Prepare samples incubated with buffer without fluorescent probes in order to eliminate background.

5. Follow the steps below with the imaging software such as image J for analyze and quantify images: Install plugins → LOCI → Bio-Formants Importer. File → import → image sequence. View stack with Standard ImageJ and split channels and select green Channel. Image → stacks → Z projects max intensity.

3.13.5. References

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4. Results and Discussion

Chapter 4.1

4.1. Role of nitric oxide in plant responses to heavy metal stress: exogenous application vs. endogenous production

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Highlights

In response to heavy metal stress exogenous NO prevents oxidative damage alleviating plant fitness-loss while endogenous NO should be fine-tune regulated and NO-dependent signalling pathways are involved in plant resistance.

ABSTRACT

Anthropogenic activities, such as industrial processes, mining and agriculture, lead to an increase in heavy metal concentrations in soil, water and air. Given their stability in the environment, heavy metals are difficult to eliminate and can even constitute a human health risk by entering the food chain through uptake by crop plants. An excess of heavy metals is toxic for plants, which have different mechanisms to prevent their accumulation. However, once metals enter the plant, oxidative damage sometimes occurs, which can lead to plant death. Initial nitric oxide (NO) production, which may play a role in plant perception, signalling and stress acclimation, has been shown to protect against heavy metals. Very little is known about NO-dependent mechanisms downstream from signalling pathways in plant responses to heavy metal stress. In this review, using bioinformatic techniques, we analyse studies of the involvement of NO in responses to heavy metal stress, its possible role as a cyto-protective molecule and its relationship with reactive oxygen species (ROS). Some conclusions are drawn and future research perspectives are outlined in order to further elucidate the signalling mechanisms underlying the role of NO in plant responses to heavy metal stress.

Key words: arsenic; heavy metals; cadmium; nitric oxide; reactive oxygen species; reactive nitrogen species; signalling

Published in Journal of Experimental Botany, erz184. (2019) Online access https://doi.org/10.1093/jxb/erz184

4.1.1. Introduction

Heavy metals are metallic elements with relatively high density compared to water (Tchounwou *et al*., 2012) and, as recommended by Appenroth (2010), can be defined on the basis of the periodic table of the following elements: 1) transition elements; 2) rare earth elements, (lanthanides and actinides); and 3) lead group, which is a heterogeneous group which includes elements that form amphoteric oxides (Al, Ga, In, Tl, Sn, Pb, Sb and Po), the metal Bi and the metalloids Ge, As and Te. Some of these metals are non-essential and may be highly toxic even at low concentrations, such as cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As; Emsley, 2011; Mustafa and Komatsu, 2016). Many other elements, which have different functions in metabolisms can be toxic when concentrations in plants exceed requirements, are essential for life (Viehweger, 2014; Andresen *et al.*, 2018). Approximately 70 metallic chemical elements are classified as heavy metals, whose concentrations in the earth's crust range from less than 0.1% to less than 0.01% (Appenroth, 2010; Tchounwou *et al.*, 2012; Hurdebise *et al.*, 2015); nevertheless, some of these metals are among the most dangerous pollutants according to the United States Environmental Protection Agency (Chen *et al.*, 2006).

Naturally accumulated metals are insignificant compared to those caused by anthropogenic activity. Agricultural activities (irrigation, limestone amendments, as well as inorganic fertilizers, pesticides and sewage sludge), electricity generated from coal and oil, industrial activities (iron and steel smelting and chemical products), mining (Jaishankar *et al.*, 2014) and houlsehod waste are the main causes of heavy metal contamination. Their accumulation leads to a decrease in soil quality and contaminates plants, giving rise to vegetal cover loss and erosion and to the transport of pollutants to subterranean and superficial water and to the trophic food chain (Clemens and Ma, 2016). Plant roots also upload heavy metals, which are translocated to other organs and consequently enter the food chain (Shahid *et al.*, 2016). A deeper understanding of plant responses to these plant-toxic heavy metals should contribute to the development of more heavy metal-tolerant plants with phytoremediation properties (Clemens and Ma, 2016; Sanz-Fernández *et al.*, 2017).

Nitric oxide (NO), which is a gaseous free radical capable of diffusing through membranes, has, over the last twenty years, been found to be involved in regulating numerous physiological and patho-physiological processes in plants including responses to heavy metals (He *et al.*, 2014; Domingos *et al.*, 2015; Sahay and Gupta, 2017). In this review, we discuss these plant responses, with a particular emphasis on the entry and translocation of metals, as well as the sources and role of NO. The dual function of NO, when exogenously supplied and endogenously produced, will also be discussed. In addition, we have carried out a bioinformatic analysis of several articles

published in the last ten years to draw certain conclusions and to highlight future research perspectives to better understand the role played by NO in plant responses to heavy metals.

4.1.2. Entry of heavy metals into plants and their toxicity

Plants use specific transporters to take up nutrient metals, which are used by metals with no known function (Clemens *et al.*, 2013). Thus, it has been suggested, for example, that Cd enters plant cells via cation transporters of minerals such as Fe, Ca and Zn (Thomine *et al.*, 2000; Aravind and Prasad, 2005). High affinity-Ca channels in tobacco have also been shown to interact with Pb and Ni (Maestri and Marmiroli, 2012). The transporter low-affinity cation transporter (LCT1) has the ability to regulate not only the transport of Ca but also of Cd in *Triticum aestivum* (Perfus-Barbeoch *et al.*, 2002; Antosiewicz and Hennig, 2004). In addition, Zn transporter family members, (Zn regulated transporter/iron regulated transporter, ZIP, ZRT/IRT-related protein) are involved in the entry of Fe^{2+} and Zn^{2+} into plants (Fox and Guerinot, 1998), being ZIP2 and ZIP4 Cu-specific (Guerinot, 2000; Wintz *et al.*, 2003). Furthermore, ZRT1, ZRT2, ZRT3 and ZRT4, which have been identified in *Saccharomyces*, *Thlaspi* and *Arabidopsis* plants, are mainly involved in transporting Zn (Zhao and Eide, 1996; Grotz *et al.*, 1998; Pence *et al.*, 2000) and are also able to transport other metals such as Fe (Eide, 1996). These transporters are also involved in the entry of Zn into *Saccharomyces cerevisiae* vacuoles where they are immobilised (MacDiarmid *et al.*, 2000). Members of the copper cation transporter (COPT) and Yellow stripe-like (YSL) transporter families also transport Cu/nicotianamine conjugates (Curie *et al.*, 2009). Although IRT1 is the main entry vehicle for Fe^{2+} in *Arabidopsis* roots (Guerinot, 2000; Vert *et al.*, 2002), it can also transport other metals such as Cu, Mn, Zn and Cd (Eide *et al.*, 1996; Korshunova *et al.*, 1999; Komal *et al.*, 2015). In addition, nodulin-26-like intrinsic protein (NIP) aquaporins are involved in AsIII absorption and translocation (Bienert *et al.*, 2008; Xu *et al.*, 2015; Chen *et al.*, 2016; Farooq *et al.*, 2016; Souri *et al.*, 2017), while high-affinity Pi transport systems have the capacity to absorb AsV (Finnegan and Chen, 2012; Souri *et al.*, 2017). The transcription factor WRKY6 has also been observed to regulate the arsenate-induced expression of phosphate transporter PHT1 (Catarecha *et al.*, 2007; Castrillo *et al.*, 2013; Sánchez-Bermejo *et al.*, 2014).

After entering root cells, metals are immobilized in the vacuole or translocated to the upper side of the xylem through the apoplast and/or symplastic complexes. Most of the transporters involved in Mn translocation are broadly specific to divalent cations such as Fe, Zn, Cu, Cd, Ca, Co and Ni. These transporters include natural resistance associated macrophage protein (NRAMP), YSL, ZIP, cation exchanger (CAX), cationic diffusion facilitator/metal tolerance protein (CDF/MTP), P-type ATPase and vacuolar iron transporters (VITs; Socha and Guerinot, 2014). While VIT1 is involved in transporting Fe, Cd and Mn into the vacuole, NRAMP3 and NRAMP4 are mainly involved in their removal (Thomine *et al.*, 2000; Lanquar *et al.*, 2005). On

the other hand, the P-type ATPase superfamily plays a role in the transport of a wide range of cations across cell membranes (Axelsen and Palmgren, 2001; Mills *et al.*, 2012). ATP-binding cassette (ABC) transporters are a superfamily of transmembrane proteins involved in a wide variety of transport functions (Kang *et al.*, 2011; Theodoulou and Kerr, 2015). In plants, 13 subfamilies of this superfamily have been identified, including multidrug resistance-associated protein, peroxisomal membrane protein, pleiotropic drug resistance, and multiple drug resistance (MRP, PMP, PDR and MDR; Kang *et al.*, 2011).

Once accumulated in plant cells, it has been suggested that heavy metal toxicity is manifested in four main ways: a) similarity to nutrient cations, resulting in competition for absorption at the root; b) direct interaction with the sulfhydryl protein group (-SH), which disrupts their structure and function; c) displacement of essential cations from specific binding sites which inhibits protein function; and d) generation of reactive oxygen species (ROS), which damage macromolecules (Luo *et al.*, 2016; Singh *et al.*, 2016). Thus, the functions of non-essential metals may replace those of essential metals due to their affinity for different ligands, which leads to protein function loss (Sharma and Dubey, 2005; Schiavon *et al.*, 2012). Therefore, while Mn, Zn, Fe, Ni, Cd, Pb and Cu have a preference for S- or N-bond ligands (SH, –SS–, $–NH_2$, =NH, etc.), K, Ca, Na, Mg, Al, and Cs favour O-donor ligands, all of which bind through oxygen (COOH, $–H_2PO_4$, –OH, –CHO, among others; Gupta and Sandalio, 2012). All these mechanisms lead, in most cases, to a macro- and micro-nutrient imbalance, inactivation of enzymes involved in the Calvin cycle, carbohydrate and phosphorus metabolism and CO_2 fixation; this lead to an inhibition of seed germination, plant growth and yields and sometimes to genotoxicity and plant death (Kalaivanan and Ganeshamurthy, 2016; Mustafa and Komatsu, 2016; Ayangbenro and Babalola, 2017; Tiwari and Lata, 2018).

One of the principal effects of heavy metal toxicity in plants is oxidative stress due to an excess of ROS and changes in the antioxidant system (Cuypers *et al.*, 2016; Romero-Puertas *et al.*, 2018). ROS is produced by Fenton and Haber-Weiss reactions, in which $O_2^{\cdot-}$ and H_2O_2 require the presence of metals, such as Cu and Fe, whose redox state can change, resulting in the production of $HO^{\cdot}$, one of the most powerful known oxidants (Halliwell and Gutteridge, 2015). However, although certain metals such as Cd and Pb, which do not undergo oxide reduction, are not directly involved in ROS generation, they can act as pro-oxidants and decrease antioxidant availability (Rodríguez-Serrano *et al.*, 2009; Singh and Shah, 2015; Gupta *et al.*, 2017; Loix *et al.*, 2017). These metals may also boost ROS generation by diverting electrons from electronic transport mainly in mitochondria and chloroplast (Heyno *et al.*, 2008; Keunen *et al.*, 2011; Souri *et al.*, 2017).

4.1.3. Mechanisms enabling plants to cope with heavy metal toxicity

Plants respond to heavy metal toxicity through a variety of mechanisms. To prevent metals from entering cells, as a first line of defence, plants alter the permeability of the plasma membrane to restrict the transport of metal ions to the apoplast (Manara, 2012) and induce metal ion transporters involved in the exit flux such as NRAMPs, ATPases, ZIPs and CDFs (Clemens *et al.*, 2013). The generation of cellular exudates, resulting in an external coating around the root, also favours the formation of complexes with heavy metals including Cd, Cu and Pb (Clemens, 2006; Shah *et al.*, 2010).

Plants can modulate the absorption of specific metals by using, for example, citrate and histidine to prevent Ni uptake (Mustafa and Komatsu, 2016). In addition, they can bind metals to the cell wall through electrostatic interactions to keep them within the cellulose and lignin structures (Van Belleghem *et al.*, 2007; Memon and Schröder, 2009; Loix *et al.*, 2017). Recently, the *Arabidopsis* mutant MRC-32 has been shown to arrest development in the Cd-affected zone and to induce a number of lateral roots located in the Cd-free zone (Watanabe *et al.*, 2010). Interestingly, mycorrhized plants are more resistant to heavy metals, as fungi can retain metals in roots and thus prevent their translocation to aerial parts, although microbial antioxidant defences may also be responsible for their higher tolerance to heavy metals (Göhre and Paszkowski, 2006; Azcón *et al.*, 2010; Ferrol *et al.*, 2016). Furthermore, plant-microorganism interactions with bacterial strains and mycorrhizal fungi present in the rhizosphere of the hyperaccumulator plant *A. halleri* are able to reduce Cd concentrations in shoots (Farinati *et al.*, 2009).

When these options are exhausted, metals already in the cell are countered through a range of storage and detoxification strategies, including immobilization, synthesis of specific heavy metal transporters, chelation, trafficking and heavy metal sequestration by particular ligands. Therefore, the best characterized plant response to Cd and, to a lesser extent, to Ag, As, and Hg, is the synthesis of phytochelatins (PCs), a family of Cys-rich peptides produced for purposes of heavy metal chelation and sequestration in the vacuole (Cobbett and Meagher, 2002; Mendoza-Cózatl *et al.*, 2010; Mustafa and Komatsu, 2016). Metallothioneins, which are ubiquitous Cys-rich proteins with unique structural characteristics that facilitate metal binding, play an essential role as Zn donors for several essential metalloproteins comprising matrix metalloproteinases and zinc fingers (Ryvolova *et al.*, 2011). On the other hand, amino acids and organic acids can react with metal ions through S, N and O atoms, thereby enabling oxalate and malate to transport metals through the xylem and to keep them in the vacuole (Kumar *et al.*, 2015). Biological transformations can also reduce heavy metal damage by converting toxic compounds into more polar components through the addition of polar groups such as amino, hydroxyl, and carboxyl groups which are easily removed (Yin *et al.*, 2019). When all the strategies described above fail,

plants activate defence responses consisting of heat shock proteins, proline, polyamines, antioxidant systems, signalling molecules and hormones (Kalaivanan and Ganeshamurthy, 2016; Romero-Puertas *et al.*, 2018). MicroRNAs (miRNAs), which regulate different transcription factors (TFs), as well as stress response-related genes at the post-transcriptional level have also recently been reported to be involved in heavy metal plant tolerance mechanisms.

4.1.4. Nitric oxide metabolism in plant responses to heavy metals

The highly reactive molecule nitric oxide (NO) is a gaseous free radical, which is involved in virtually all plant physiological and patho-pysiological processes such as development, senescence and responses to environmental cues (Sanz *et al.*, 2015; Astier *et al.*, 2017; Umbreen *et al.*, 2018; Costa-Broseta *et al.*, 2018). However, more research into the complex NO metabolism in plants is required, especially in areas such as the sources of NO, as well as the proteins/molecules that regulate NO in the cell (Baudouin and Hancock, 2013).

Thus, unlike animals, in which NO production is mainly dependent on the nitric oxide synthase (NOS) family of enzymes (Forstermann and Sessa, 2012), up to eleven different mechanisms are capable of producing NO in plants (Mur *et al.*, 2013; Astier *et al.*, 2017). Two main pathways are broadly involved in all these mechanisms: a reductive nitrate-dependent pathway and an oxidative arginine-dependent and hydroxylamine-dependent pathway (Fröhlich and Durner, 2011; Gupta *et al.*, 2011; Mur *et al.*, 2013). In the reductive pathway, nitrate reductase (NR), with its nitrite:NO reductase activity, is able to produce NO in plants, although specific environments, such as a*nox*ic and acidic conditions, are necessary for this reaction to take place (Rockel, 2002; Meyer *et al.*, 2005; Chamizo-Ampudia *et al.*, 2017; Astier *et al.*, 2017). Non-enzymatic reduction of nitrite, xanthine oxidoreductase, mitochondrial nitrite reduction via cytochrome c oxidase/reductase and nitrite:NO reductase in root apoplasts are also involved in reductive pathways (Godber *et al.*, 2000; Stöhr *et al.*, 2001; Stoimenova *et al.*, 2007; Wang *et al.*, 2010). More recently, NO-forming nitrite reductase (NOFNiR) in *Chlamydomonas reinhardtii* algae has been reported to reduce nitrite to NO via NR-dependent electrons (Chamizo-Ampudia *et al.*, 2017). Apart from reductive NO-production pathways, several studies have demonstrated the existence of an oxidative route, similar to that in animals, although no homologous NOS sequences have been found in approximately 1000 transcriptomes in the land plants analysed, with similar sequences only found in green algae (Jeandroz *et al.*, 2016). However, biochemical and molecular data show that a link exists between arginine and NO in plants (Astier *et al.*, 2017).

We sourced studies of NO and heavy metals in plants and carried out a deep web search of the relevant literature with the aid of databases (https://www.ncbi.nlm.nih.gov/pubmed/; https://www.scopus.com/search/; https://apps.webofknowledge.com/UA). The search was

narrowed down to the last ten years. The articles retrieved were divided into two groups: one dealing with the analysis of endogenous NO production (produced by the plant) as a response to applications of metal (Suppl. Table S1) and the other devoted to supply of exogenous NO before and/or during metal exposure (Suppl. Table S2). We data-mined both groups of papers and then created a database of the plant fitness, oxidative stress damage and general plant state parameters measured in each article. After eliminating studies with less than four variables measured, the databases were curated. 14 of the 68 papers in the endogenous NO group (Suppl. Table S1), and 28 of the 98 papers in the NO exogenous group (Suppl. Table S2) were eliminated. In addition, all variables were categorised in order to avoid the very different measurements used in the studies. All figures were then categorised under the following headings: increase, decrease, no change and not data.

In order to pinpoint groups of papers dealing with similar plant behaviours in response to heavy metals, we performed a hierarchical clustering analysis of the two databases generated. This enabled us to objectively search for groups of articles in an unsupervised way without specifying the number of clusters to be created. We used H-clustering in the R software package, the k-means and Ward's parameters (Mitchell, 1995; Jain *et al*., 1999) and the previously estimated data clusterability index. We found 10 clusters of papers in the endogenous NO group (Fig. 1) and 2 cluster in the exogenous NO group (Fig. 2). We used a similar approach for studies dealing with As and Cd alone, which were the most numerous studies (Suppl. Fig. S1-S4).

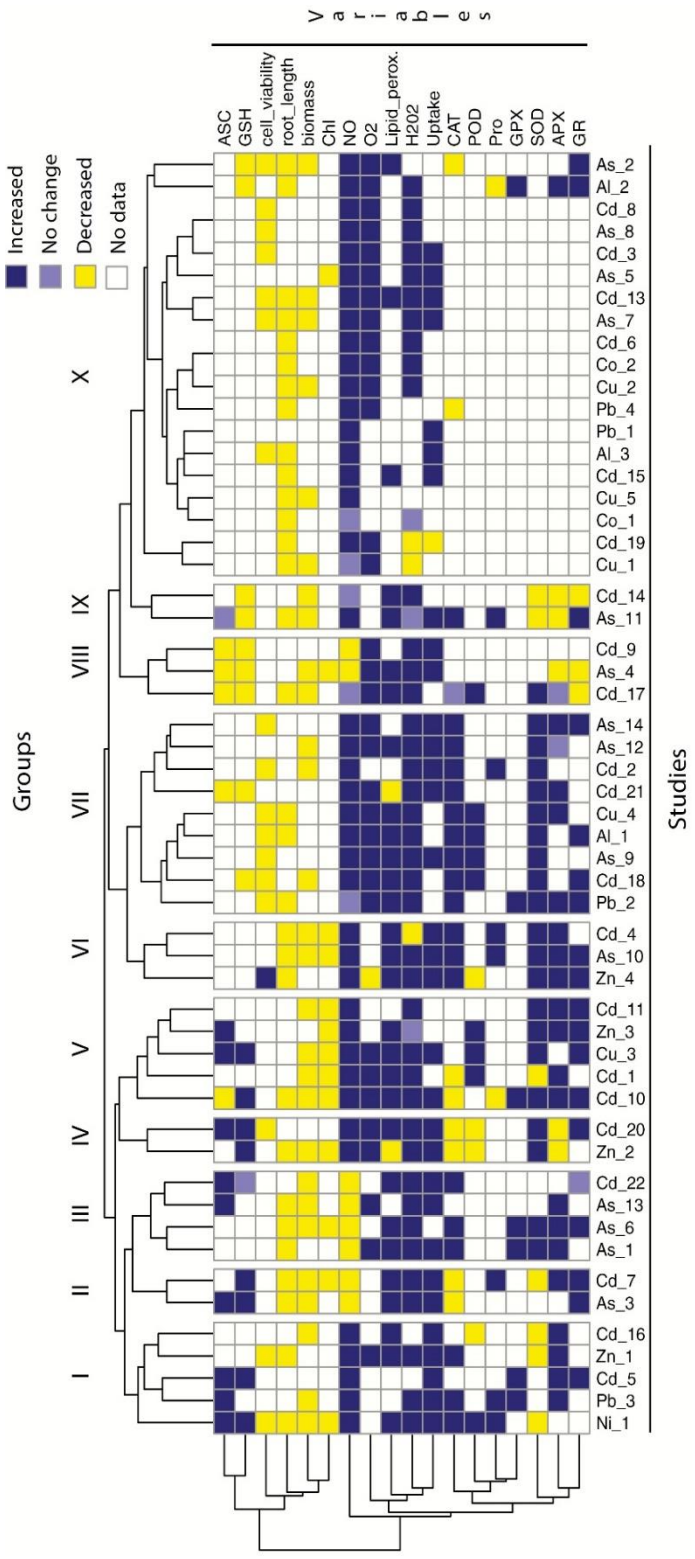


Fig. 4.1.1. Bioinformatics analysis of studies in which endogenous NO production in response to heavy metal stress was shown. Analysis of the data summarized in Supplementary Table S7.1.1 related to endogenous NO production as a response to metal application, and the main variables measured in each study. Categorization of all variables converted all quantitative numbers to the categories increased (blue), decreased (yellow), no change (light blue), or no data (white), according to the corresponding study. The hierarchical clustering in an unbiased form showed 10 groups (I to X). The code for each study is represented by the metal used and the number on the associated table.

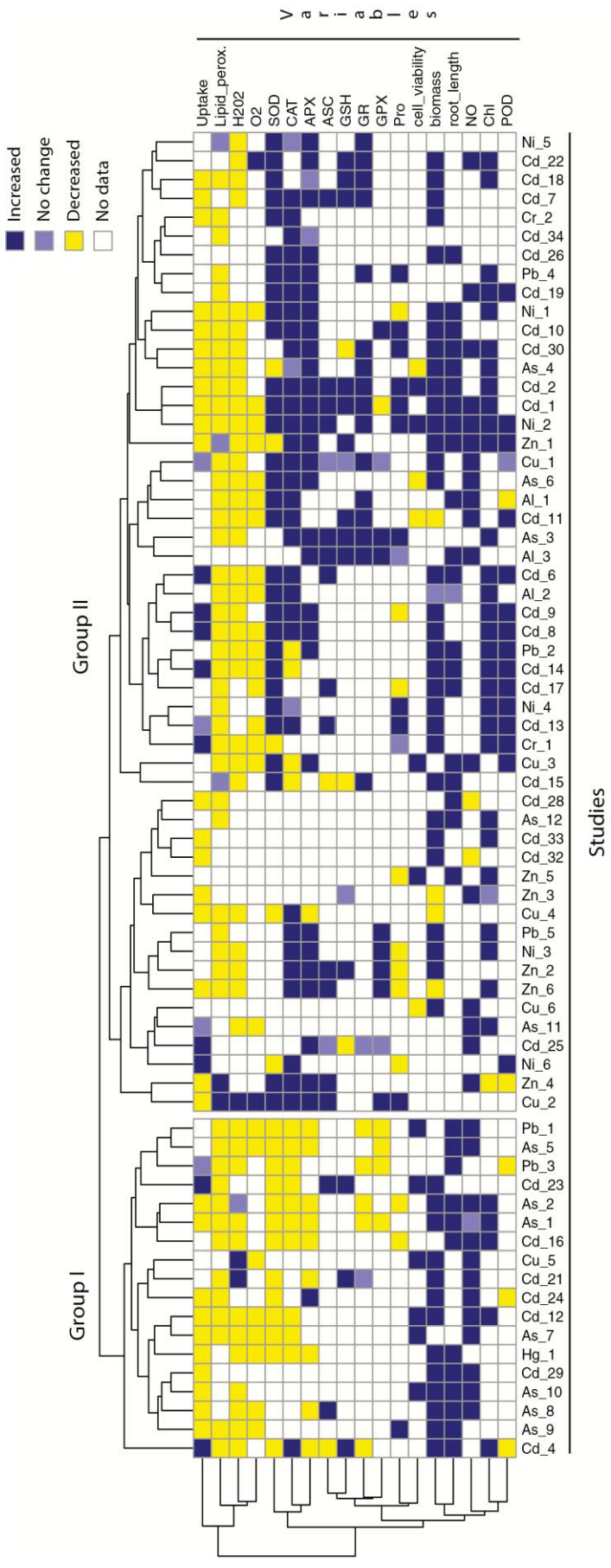
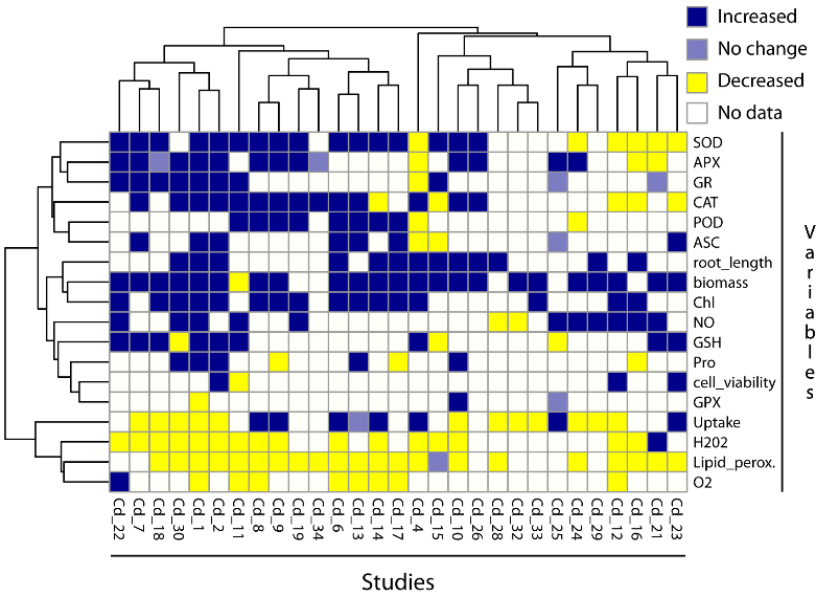
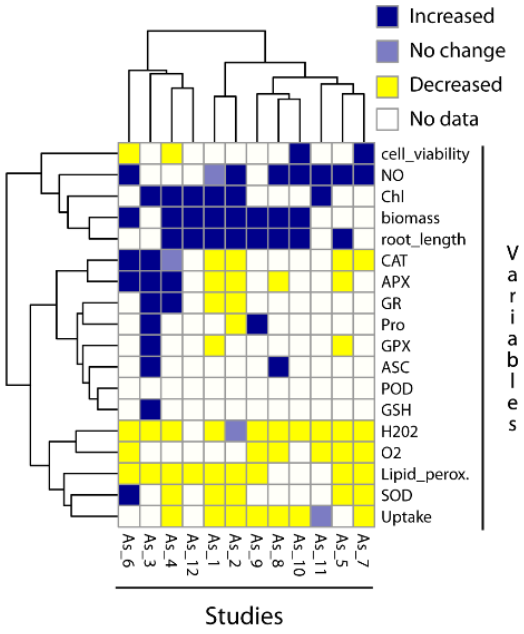


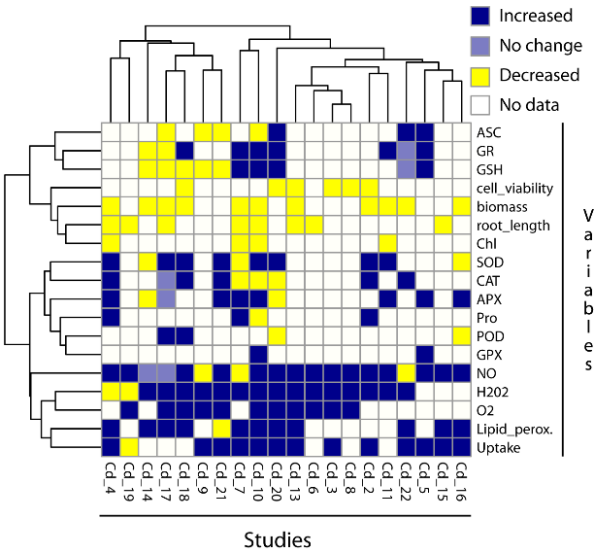
Fig.4.1.2. Bioinformatics analysis of studies in which exogenous NO application was applied in plants exposed to heavy metal stress. Analysis of the data summarized in Supplementary Table S7.1.2 related to exogenous NO supply before and/or during the metal application, and the main variables measured in each study. Categorization of all variables converted all quantitative numbers to the categories increased (blue), decreased (yellow), no change (light blue), or no data (white), according to the corresponding study. The hierarchical clustering in an unbiased form showed two groups (I and II). The code for each study is represented by the metal used and the number on the associated table.



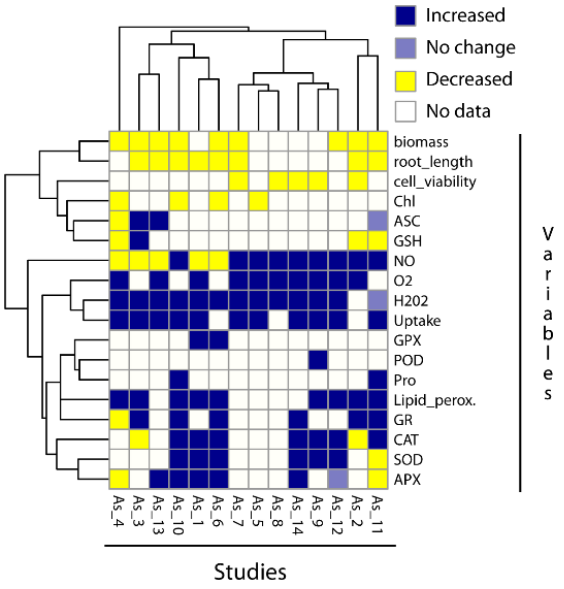
Suppl. Figure S4.1.1. Analysis by bioinformatics when exogenous NO application is used in response to Cd stress. Bioinformatics analysis of the data summarized in Suppl. Table S2 (studies) related to exogenous NO supply previously and/or during Cd application and the main variables measured in each article. Categorization of all variables converted all quantitative numbers to the categories: increased (blue), decreased (yellow), no change (light blue) or no data (white), according to the corresponding paper. The hierarchical clustering in an unbiased form showed only one group. Code for each study is represented by the metal used and the number on the table associated.



Suppl. Figure S4.1.2. Analysis by bioinformatics when exogenous NO application is used in response to As stress. Bioinformatics analysis of the data summarized in Suppl. Table S2 (studies) related to exogenous NO supply previously and/or during As application and the main variables measured in each article. Categorization of all variables converted all quantitative numbers to the categories: increased (blue), decreased (yellow), no change (light blue) or no data (white), according to the corresponding paper. The hierarchical clustering in an unbiased form showed only one group. Code for each study is represented by the metal used and the number on the table associated.



Suppl. Figure S4.1.3. Analysis by bioinformatics when endogenous NO production in response to Cd stress is showed. Bioinformatics analysis of the data summarized in Suppl. Table S1 (studies) related to endogenous NO production as a response to Cd application and the main variables measured in each article. Categorization of all variables converted all quantitative numbers to the categories: increased (blue), decreased (yellow), no change (light blue) or no data (white), according to the corresponding paper. The hierarchical clustering in an unbiased form showed one group. Code for each study is represented by the metal used and the number on the table associated.



Suppl. Figure S4.1.4. Analysis by bioinformatics when endogenous NO production in response to As stress is showed. Bioinformatics analysis of the data summarized in Suppl. Table S1 (studies) related to endogenous NO production as a response to As application and the main variables measured in each article. Categorization of all variables converted all quantitative numbers to the categories: increased (blue), decreased (yellow), no change (light blue) or no data (white), according to the corresponding paper. The hierarchical clustering in an unbiased form showed one group. Code for each study is represented by the metal used and the number on the table associated.

After this analysis, an overall increase in endogenous NO production was observed in plant responses to heavy metals particularly after short-term treatments (Fig. 6.1.1). For instance, after Cd treatments more than 80% of the studies showed an increase of NO production after short treatments while less than 4% showed a decrease. For long treatments (over a one-week period) however, 66% of the papers showed a decrease of NO production, clustered in groups II, III and

VIII (Suppl. Table S6.1.1; Fig. 6.1.1). Long-term treatments with heavy metals can induce early senescence, excess ROS and ethylene, which may affect NO production (McCarthy *et al.*, 2001; Rodríguez-Serrano *et al.*, 2009; Romero-Puertas *et al.*, 2012). Groups VII and IX, mainly involving long-term treatments, are also affected in both enzymatic and non-enzymatic antioxidants (Fig. 6.1.1). However, in groups V to VII, involving different species except for *Arabidopsis*, enzymatic antioxidants are induced.

Most of these studies suggest that NOS-l activity is the main source of NO production in response to heavy metals and focus, in particular, on the use of NOS inhibitors in this type of pathway (Suppl. Table S6.1.1; Fig.6.1.1). Following long-term treatment with heavy metals, Ca deficiency may also affect NOS-l activity and consequently NO production (Rodríguez-Serrano *et al.*, 2009). Some reports however, point to a NR-dependent NO burst in response to heavy metals such as Cu, Al and As (Kolbert *et al.*, 2012; Sun *et al.*, 2014; Xue and Yi, 2017; Supp. Table S1). On the other hand, LeSPL-CNR (an SPL-type transcription factor) has been shown to be negatively involved in NO production by modulating NR expression and activity, which contributes to Cd tolerance suggesting a possible balance and/or crosstalk between the different NO sources, which contributes to NO level in plant response to heavy metals (Chen *et al.*, 2018). Recently, NO homeostasis in respiratory burst oxidase homologes (*rboh*) mutants has been shown to differ from that in WT plants in response to Cd, suggesting also that an unknown ROS-dependent NO regulation mechanism is present under these conditions (Gupta *et al.*, 2017).

NO needs to be highly regulated and/or metabolized in order to control its impact and functioning, with only non-symbiotic haemoglobin1 (nsHb1) and so-called phytoglobin1 from different species being capable of metabolising NO-producing nitrate (Perazzolli *et al.*, 2006; Gupta *et al.*, 2011). In the green algae *Chlamydomonas*, the truncated haemoglobin (THB1) showed NO-dioxygenase activity associated with NR (Sanz-Luque *et al.*, 2015; Chamizo-Ampudia *et al.*, 2017), although the functioning of this activity in plants has not yet been studied. In addition, nsHb1 regulates NO production under hypoxic and low oxygen stress conditions when an increase in NO caused by NR is assumed to protect plants against these stresses (Perazzolli *et al.*, 2004; Gupta *et al.*, 2011). In addition, non-symbiotic haemoglobins appear to play a role in the establishment of symbiosis, as the over-expression of *LjnHb1* and *AfnHb1* in *L. japonicus* induces an increase in the number of nodules with a concomitant decrease in NO production (Shimoda *et al.*, 2009; Hichri *et al.*, 2015; Fukudome *et al.*, 2016). Overexpression of *Nicotiana tabacum NtHb1* in both tobacco and *Arabidopsis* plants has recently been shown to downregulate NO levels in response to Cd and to induce greater Cd tolerance due, in part, to lower metal accumulation (Lee and Hwang, 2015; Bahmani *et al.*, 2019). On the other hand, NO can react with glutahione (GSH) and produce *S*-nitrosogluthatione (GSNO), which is considered to be a reservoir

of NO and less reactive than this molecule (Liu *et al.*, 2001; Sakamoto *et al.*, 2002). The GSNO reductase (GSNOR) enzyme, which metabolizes nitrosothiols and indirectly regulates NO levels, is possibly involved in plant responses to heavy metal stress (Feechan *et al.*, 2005; Frungillo *et al.*, 2014). The *gsnor1-3* mutant, has been used in *Arabidopsis* plants under Cu stress conditions to show that excess NO due to a lack of GSNOR activity, whose role depends on a finely tuned balance between NO and ROS, can increase metal sensitivity or tolerance depending on the mildness or severity of the stress (Peto *et al.*, 2013). Several studies demonstrate that GSNOR activity increases or decreases in response to Cd (Wang *et al.*, 2015; Hu *et al.*, 2019) and As stress (Leterrier *et al.*, 2012; Rodríguez-Ruiz *et al.*, 2018) in different species depending on the strength of the treatment and the level of NO in the cell (Wang *et al.*, 2015; Hu *et al.*, 2019).

Given its half life of just a few seconds, NO can react rapidly with oxygen to produce nitrogen dioxide (NO_2), which leads to the production of nitrite and nitrate in aqueous solutions (Neill *et al.*, 2008). The reaction of NO with $O_2^{\cdot-}$ also produces $ONOO^-$, a highly pro-oxidant species (Ischiropoulos and al-Mehdi, 1995; Radi, 2018). As will be seen below, the relationship between NO and ROS plays a very important role in the regulation of NO and in NO-dependent functions.

4.1.5. Nitric oxide role in plant responses to heavy metals

Given that the highly reactive nature of NO molecules facilitates their regulatory role, a key feature of their mode of action is an ability to directly alter proteins through covalent post-translational modifications (PTMs), which enable biological processes in the cell to be regulated (Martínez-Ruiz *et al.*, 2011). NO-dependent PTMs facilitate gene regulation, interactions with most phytohormone-dependent signalling pathways and ROS regulation (Simontacchi *et al.*, 2013; Gibbs *et al.*, 2014; Albertos *et al.*, 2015; Romero-Puertas and Sandalio, 2016; Castillo *et al.*, 2018; Cui *et al.*, 2018). The role played by endogenous (produced by the plant) and exogenous NO (a cyto-protective molecule) in response to heavy metals is also analysed.

4.1.5.1. Protection by exogenous nitric oxide against heavy metals stress

Numerous studies have shown that exogenous applications of NO donors (mainly sodium nitroprusside: SNP) protect plants against heavy metal-induced damage (summarized in Suppl. Table S2). Hierarchical clustering analysis described above produced two groups of profiles, which mainly differ in terms of the antioxidant system response. Exogenous NO correlated negatively with the antioxidants in group I, but correlated positively with antioxidants in group II (Fig. 3.1.2). Group I is mainly associated with high metal concentrations and/or long-term treatment with the metal (over a one-week period) and/or high concentrations of a NO donor, suggesting that the plant's antioxidant system is affected by intense/long-term treatments (Suppl.

Fig. S1). Although it is difficult to detect a pattern in either group, all AsIII treatments are localized in group I, while group II contains AsV, Ni, Zn, Cu and Al (not heavy metals as such), which we have been also analysed (Fig. 3.1.2; Suppl. Table S3.1.2).

In general, NO donors applied prior to or at the same time as the treatment with heavy metal, regardless of the metal and species used, showed a positive correlation with biomass, root length and chlorophyl. On the other hand, the presence of the donor negatively correlated with ROS ($O_2^{\cdot -}$ and H_2O_2) production and oxidative damage mainly caused by lipid peroxidation (Suppl. Table S2; Fig. 3.1.2). Thus, exogenous NO protects against heavy metal stress and alleviates oxidative stress (Fig. 3.1.2). While high concentrations of NO have been shown to have cytotoxic properties (Beligni and Lamattina, 2001), the response of antioxidant system to As in rice appears to be more sensitive to NO donors (SNP), as five out of eight studies show that the antioxidant system is negatively co-regulated (Suppl. Fig. S3.1.2).

Interestingly, NO donors correlated negatively with heavy metal uptake except for Cd which showed a positive correlation in over 40% of the studies analysed and in two Cr and Ni studies (Suppl. Fig. S3.1.1). One of the problems associated with the use of NO donors could be the induction of IRT1, which has been shown to be NO-dependent in different species and is repressed by plants under Cd stress conditions to prevent Cd accumulation (Connolly, 2002; Connolly *et al.*, 2003; Graziano and Lamattina, 2007; Jin *et al.*, 2009). This repressive mechanism is enhanced in *Arabidopsis* roots treated with Cd and in the presence of a nitric oxide synthase inhibitor, thus suggesting that NO may promote Cd accumulation (Besson-Bard *et al.*, 2009). NO donors may have a stronger effect on IRT1 during Cd uptake, as this metal, whose main entrance to the cell appears to be IRT1, has no specific transporter. Recently, NO has been shown to modulate metal transporters such as NIP, NRAMP and ABC in rice plants treated with AsIII, as well as proton pumps and antiporters (CAX) in *Trifolium*, *Arabidopsis* and tobacco under Cd stress conditions (Singh *et al.*, 2017b; Liu *et al.*, 2015; Lee and Hwang, 2015; Bahmani *et al.*, 2019). The negative correlation between heavy metal uptake and NO donors observed also points to NO-dependent regulation of not only uptake transporters but probably also of heavy metal translocation from roots to shoots, a topic that needs future research (Fig. 3.1.3).

Most of the studies analysed focus on the clear relationship between NO and ROS metabolism. However, some analyses focus on other protective mechanisms used by NO, such as increases in pectin and hemicellulose content in the root cell wall to prevent Cd accumulation in the soluble fraction of the cell in rice leaves (Xiong *et al.*, 2009) and to maintain the auxin equilibrium (Xu *et al.*, 2010). Hormone levels are also regulated by NO donors in plant responses to heavy metals; gibberellins, indol acetic acid, abscisic acid, cytokinins (GA3, IAA, ABA and CKs) increase in *Vicia faba* plants treated with As (Mohamed *et al.*, 2016); jasmonic acid (JA)

increases in *Trifolium* plants subjected to Cd stress, while salicylic acid (SA) and ET were shown to decrease (Liu *et al.*, 2015).

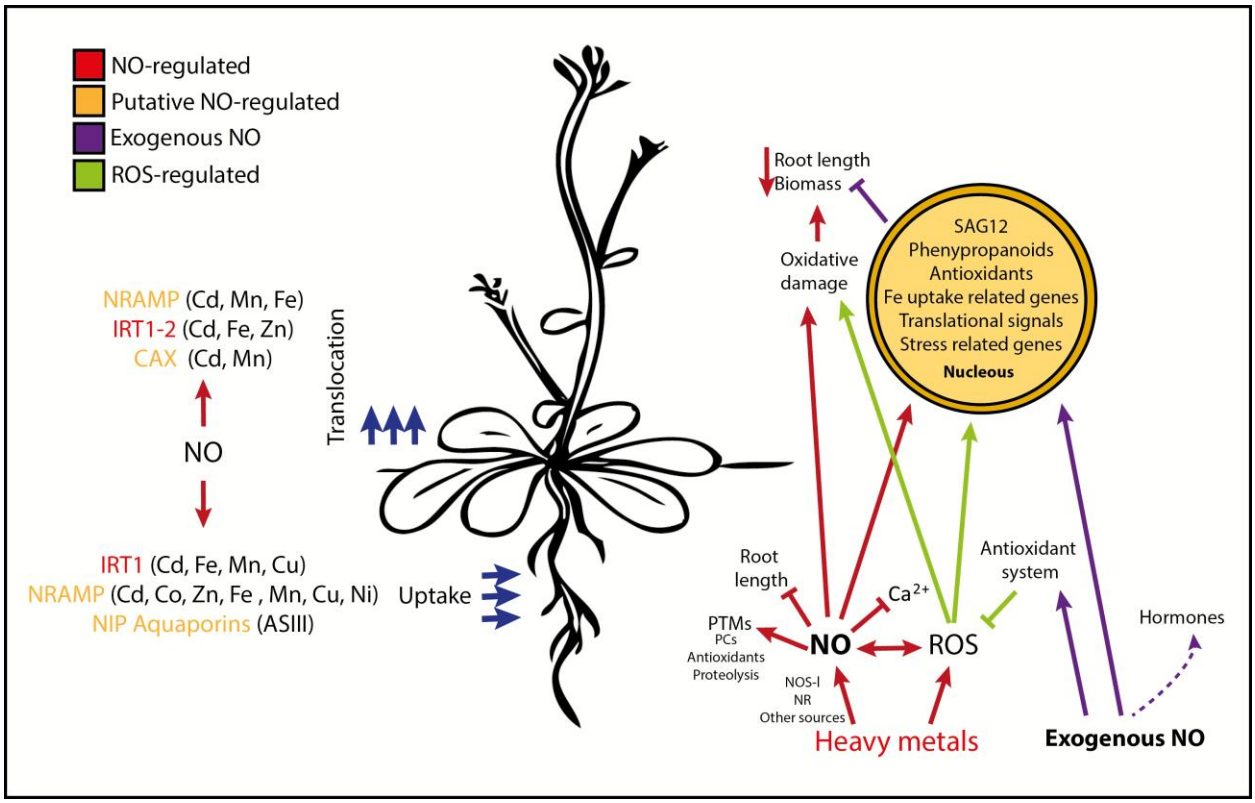


Fig. 4.1.3. Main functions of NO in plant responses to heavy metal stress. Exogenous NO, when supplied in appropriate concentrations, induces antioxidant systems, preventing excess ROS and oxidative damage, which, together with a transcriptomic modulation, alleviates loss of plant fitness in response to heavy metal stress. Endogenous NO production in response to heavy metal stress has been involved in regulating ROS metabolism, phytochelatins (PCs), and proteolysis through post-transcriptional modifications (PTMs). Transcriptomic analyses have revealed the NO-dependent signalling pathways involving Ca, hormones, MAPKs and transcription factors, among others. An excess of endogenous NO, however, appears to promote Cd-dependent root growth inhibition and Cd uptake. The main transporters associated with the uptake and translocation of heavy metals in the plant that might be regulated by NO are also shown.

NO indirectly contributes to plant protection against Cd toxicity, with *Bacillus amyloliquefaciens* acting as a signalling molecule downstream of auxins (Zhou *et al.*, 2017). Recently, NO has been shown to reduce AsIII toxicity by modulating JA biosynthesis (Singh *et al.*, 2017*b*) and to increase Pb resistance by increasing IAA, CKs and GA3 levels and by decreasing ABA content (Sadeghipour, 2017). In rice plants treated with SA and As, NO production and NR activity were found to increase, with a concomitant reduction in oxidative damage (Singh *et al.*, 2017a) and GA alleviates Cd toxicity by reducing NO accumulation (Zhu *et al.*, 2012), thus suggesting the presence of a feedback loop between NO and hormones. However, further research is needed to unravel the mechanisms underlying other NO-dependent signalling pathways, involved in plant responses to heavy metals.

4.1.5.2. Role of endogenous nitric oxide in plant responses to heavy metals

Several studies have analysed NO production in response to heavy metal stress as described in the section on metabolism (Fig. 3.1.1; Suppl. Table S1). As mentioned above, length of treatment appears to affect NO production (Fig. 3.1.1), and a clear relationship exists between oxidative stress and heavy metal toxicity. Information concerning the function of endogenous NO produced in response to heavy metal is still limited, although initial studies have established that NO-dependent PTMs are involved in ROS regulation and cellular metabolic pathways such as photorespiration. In plant responses to Cd stress, CAT *S*-nitrosylation in pea peroxisomes decreases (Ortega-Galisteo *et al.*, 2012), which may account for the increase in activity described in a previous study (Romero-Puertas *et al.*, 1999), with H_2O_2-producing glycolate oxidase (GOX) *S*-nitrosylation also changing in response to Cd (Ortega-Galisteo *et al.*, 2012). Furthermore, the *S*-nitrosylation of PCs increases in *Arabidopsis* culture cells under Cd stress conditions which affects their capacity to chelate the metal (de Michele *et al.*, 2009). Nitration was also observed in soybean responses to Cd stress, and, although no significant changes were detected in the pattern of nitrated proteins, Cd may induce finely tuned regulation rather than major modifications (Pérez-Chaca *et al.*, 2014). A later study showed that half of the nitrated proteins identified were related with proteolysis (Gzyl *et al.*, 2016), a highly important process in plant responses to heavy metal stress.

NO-dependent transcript changes in response to heavy metals have also been analysed, although the underlying mechanisms involved remain unclear. Thus, over forty genes were found be regulated by NO in *Arabidopsis* plants in response to Cd, which are associated with root growth, iron homeostasis, nitrogen assimilation and metabolic proteolysis (Besson-Bard *et al.*, 2009). Although exogenous NO prevents a decrease in root growth in response to heavy metals, various studies show that endogenous NO is involved in reducing root growth (Groppa *et al.*, 2008; Besson-Bard *et al.*, 2009; Valentovičová *et al.*, 2010); this points to a timely fine-tuned relationship between NO and root growth. Furthermore, NO represses auxin accumulation and signalling in *Arabidopsis* plants in response to Cd stress, which inhibits root meristem growth (Yuan and Huang, 2016). Similarly, metal uptake, especially in response to Cd, is regulated by endogenous NO. Biochemical and genetic techniques have shown that an increase in NO can increase metal uptake, while a decrease reduces uptake (Besson-Bard *et al.*, 2009; Lee and Hwang, 2015; Bahmani *et al*., 2019). Interactions between Ca and NO also appear to play an important role in plant responses to heavy metals, although the mechanisms involved are unclear. Therefore, Ca prevented the NO synthase-dependent NO production reduction in response to Cd in pea plants (Rodríguez-Serrano *et al*., 2009) suggesting a cross-talk between NO and Ca as it has been shown also in *Arabidopsis* plants where NO appears to favor Cd versus Ca uptake and/or Ca extrusion

through regulating Cd channels or transporters (Besson-Bard *et al*., 2009). However, Ca was not found to affect NO production in chamomile plants treated with CrIV (Kováčik *et al*., 2014).

Ethylene biosynthesis, mitogen-activated protein kinase2 (MAPKK2) signalling pathways and the induction of various TFs have been observed in response to Cd in soybeans (Chmielowska-Bak and Deckert, 2013). However, further research is required to better understand the role played by NO in plant responses to heavy metals.

4.1.6. Conclusion and future challenges

In recent years, numerous studies have shown how NO is involved in plant responses to different heavy metal stresses, although the underlying mechanisms remain unclear. The use of NO donors and the cytoprotective role of NO have been extensively analysed. NO, induces antioxidant systems when present in appropriate concentrations, prevents excess ROS and oxidative damage and thus alleviates plant fitness loss. Most of the parameters analysed in these studies relate to ROS metabolism, while information on hormones and other signalling molecule is limited (Fig. 3.1.3). Future research therefore needs to be carried out on the protective role of NO in plant responses to heavy metal stress. The mechanisms underlying NO-dependent induction of antioxidant systems also need to be studied in more depth, although some initial data are available on NO-dependent PTMs which regulate ROS production-related enzymes and antioxidant systems. As problems such as the ferricyanide release are associated with SNP, which is by far the most commonly used donor, special care needs to be taken in relation to NO donors and their concentrations, adequate controls are required (Kováčik *et al*., 2014), and if possible, more than one NO donor should be used.

The production of endogenous NO during plant responses to heavy metal stress has also been extensively analysed, with NO generally being observed to increase except in the case of long-term Cd/As treatments and the presence of metals in high concentrations. Studies also show that NO-dependent PTMs regulate ROS metabolism, phytochelatins and proteolysis in plant responses to heavy metal stress. Transcriptomic analyses have revealed the presence of signalling pathways such as Ca, hormones, MAPKs and TFs (Fig. 3.1.3). Timing and concentrations of endogenous NO however, need to be finely tuned during plant responses to Cd, with further research on other metals possibly producing similar results, as an excess of endogenous NO appears to promote Cd-dependent root-growth inhibition, Cd uptake and disturbances in the antioxidant system. These functions, regardless of their metal specificity, need to be studied further, although our bioinformatic analysis shows that the entry of Cd into the plant appears to be the main one affected positively by NO. Negative correlation between NO and the entry of other

metals also needs to be studied in more depth in order to clarify its association in heavy metal uptake and translocation.

Although the involvement of NO in plant responses to heavy metal stress has been extensively studied, the mechanisms underlying crosstalk between NO and ROS and other signalling components, such as hormones and TFs, are just beginning to be investigated and further research in these areas is needed. Research into the specific functions and general role of NO, such as its involvement in the entry and distribution of heavy metals in different plant species, could facilitate the production of crops with lower heavy metal uptake and enhanced phytoremediation properties in soils normally contaminated by more than one metal.

4.1.7. References

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Chapter 4.2

4.2. Low endogenous NO levels in roots and antioxidant systems are determinants for the resistance of Arabidopsis seedlings grown in Cd

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Highlights

- **Oxidative stress and loss of fitness in 7 days-seedlings growing in Cd is directed by the increase of ROS/RNS in the roots.**
- ***Atnoa1* mutants showed oxidative stress analysed by protein oxidation and nitration, even under control conditions.**
- **An excess of endogenous NO under Cd stress induces oxidative damaged being the antioxidant system crucial for avoided it.**

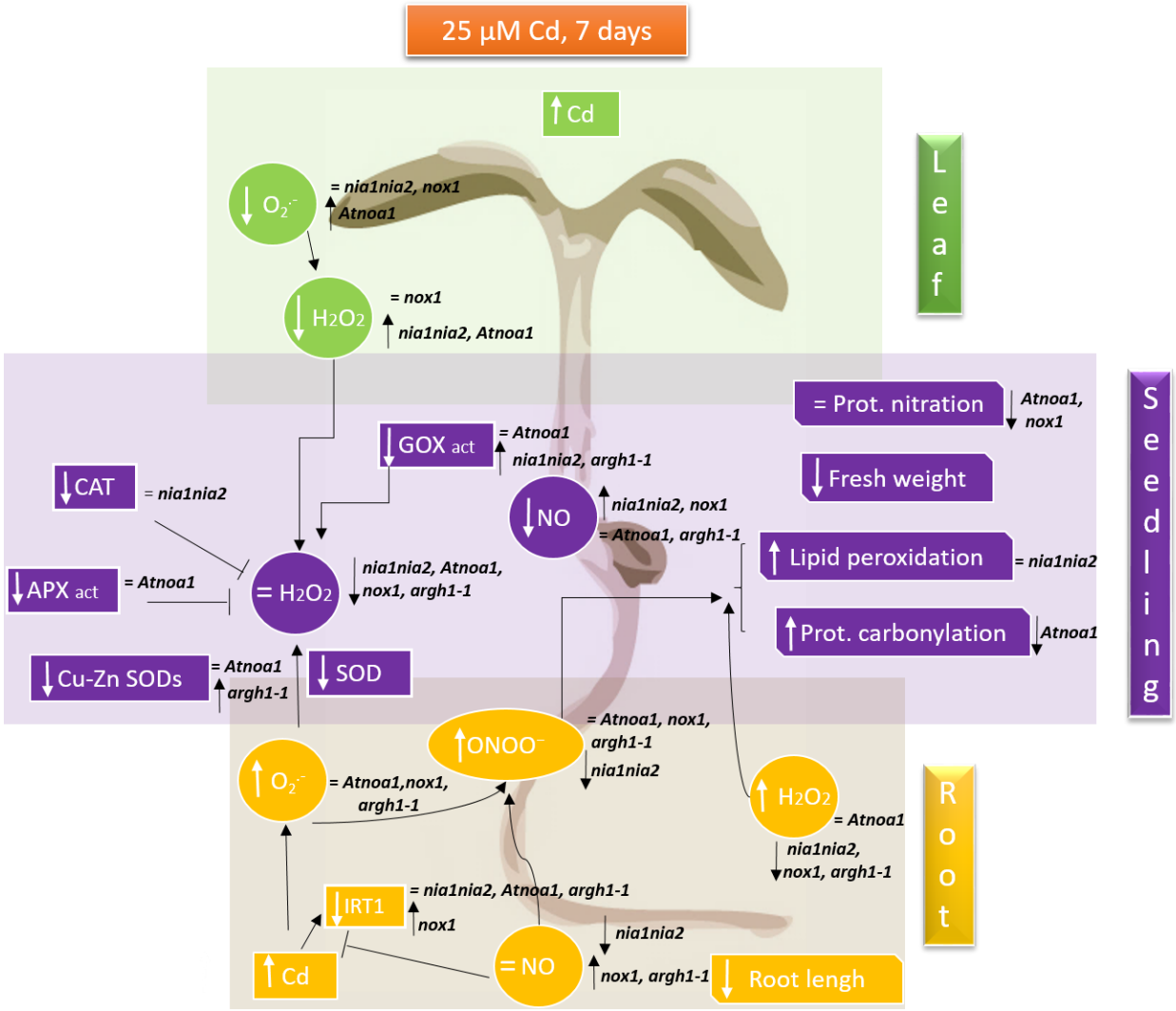
ABSTRACT

Cadmium (Cd), which is a toxic non-essential heavy metal capable of entering plants and thus the food chain, constitutes a major environmental and health concern worldwide. An understanding of the tools used by plants to overcome Cd stress could lead to the production of food crops with lower Cd uptake capacity and of plants with greater Cd uptake potential for phytoremediation purposes in order to restore soil efficiency in self-sustaining ecosystems. The signalling molecule nitric oxide (NO), whose function remains unclear, has recently been involved in responses to Cd stress. Using different mutants, such as *nia1 nia2*, *nox1*, *argh1-1* and *Atnoa1*, which were altered in NO metabolism, we analysed various parameters related to reactive oxygen and nitrogen species (ROS/RNS) metabolism and seedling fitness following germination and growth under Cd treatment conditions for seven days. Seedling roots were the most affected, with an increase in ROS and RNS observed in wild type (WT) seedling roots, leading to increased oxidative damage and fitness loss. Mutants that showed lower NO levels in seedling roots under Cd stress were more resistant than WT seedlings due to the maintenance of antioxidant systems which protect against oxidative damage.

Keywords: Nitric oxide, Cadmium, *Arabidopsis thaliana*, Nitrate reductase, NOS-l

Published in Environmental Pollution, (2020) https://doi.org/10.1016/j.envpol.2019.113411

GRAPHICAL ABSTRACT



4.2.1. Introduction

The heavy metal cadmium (Cd) enters the environment essentially due to mining and industrial activity and phosphate fertilizers used in agriculture (Clemens *et al*., 2013). Cd first enters roots through the cortical tissue and is translocated to above-ground tissues under most environmental conditions. The main problem with regard to its accumulation in plant tissues is its rapid entry into the food chain, which constitutes both an environmental and health hazard worldwide (Clemens *et al*., 2013). In recent years, much evidence points to an excess of reactive oxygen species (ROS) and oxidative stress as one of the main causes of long-term Cd-related toxicity (Romero-Puertas *et al*., 2002; Rodríguez-Serrano *et al*., 2006; Bi *et al*., 2009). However, ROS, whose toxicity and signalling role are affected by the finely tuned balance between production and detoxification, can act as signalling molecules (Mittler *et al*., 2011). The free radical nitric oxide (NO), which acts as a ubiquitous inter- and intra-cellular signalling molecule in the regulation of plant development processes, plant defences and responses to stress (Yu *et al*., 2014; Sanz *et al*., 2015; León *et al*., 2016), has recently been found to be involved in plant Cd responses (Besson-Bard *et al*., 2009; Romero-Puertas *et al*., 2019). However, contradictory evidence has shown that NO production following exposure to Cd may be time- and dose-dependent (Besson-Bard *et al*., 2009; Arasimowicz-Jelonek *et al*., 2011; Romero-Puertas *et al*., 2012). NO levels appear to peak under short term treatment (Besson-Bard *et al*., 2008; De Michele *et al*., 2009; Pérez-Chaca *et al*., 2014), while NO production was found to decrease under long term treatment in different plant species (Rodríguez-Serrano *et al*., 2006; Rodríguez-Serrano *et al*., 2009; Xiong *et al*., 2009; Pérez-Chaca *et al*., 2014). On the other hand, it is well established that exogenous pre-treatment with NO protects against Cd toxicity by increasing antioxidant capacity and heavy metal stress tolerance in plants (Kopyra *et al*., 2006; Noriega *et al*., 2007; Terrón-Camero *et al*., 2019).

NO production and the contribution of the different NO sources at any point in the treatment remain unclear and are the subject of much debate in plant biology. A variety of NO production mechanisms in plants, including arginine and hydroxylamine-dependent oxidative pathways, as well as nitrate-dependent reductive pathways, have been described (Mur *et al*., 2013; Santolini *et al*., 2017). Thus, apart from non-enzymatic production mechanisms, NO may be generated by a putative nitric oxide synthase-like (NOS-l) protein, which occurs in mammals and generates NO from L-arginine (Astier *et al*., 2017), and by nitrate reductase (NR) which catalyses the reduction of nitrite to NO (Gupta *et al*., 2011).

Although some NO sources, such as NOS-l and NR, may be involved in NO production in response to Cd (Besson-Bard *et al*., 2008; Arasimowicz-Jelonek *et al*., 2011; Pérez-Chaca *et al*., 2014), their contribution to NO production and their role in plant responses to Cd remain unclear.

This paper aims to assess the relationship between antioxidant mechanisms, ROS and RNS production and nitrosative stress in Arabidopsis seedlings, on the one hand, and changes in endogenous NO levels during germination and growth in media caused by Cd on the other. Four pre-characterised mutants were used in this study: two mutants, one impaired in the *Atnoa1* (*Atnoa1*) protein and the other in nitrate reductases (NR1/NIA1 and NR2/NIA2; *nia1 nia2*), previously reported to play a role in NO biosynthesis (Yamasaki and Sakihama, 2000; Desikan *et al*., 2002; Rockel *et al*., 2002; Modolo *et al*., 2005; Moreau *et al*., 2008); and two NO overproducers, one impaired in the arginase structural gene ARGH1 (*argh1-1*; Flores *et al*., 2008) and the other in a chloroplast phosphoenolpyruvate/phosphate translocator (*nox1/cue1*; Streatfield *et al*., 1999; He *et al*., 2004). These four mutants enabled us to characterize endogenous NO-related responses and resistance to Cd stress.

4.2.2. Materials and methods

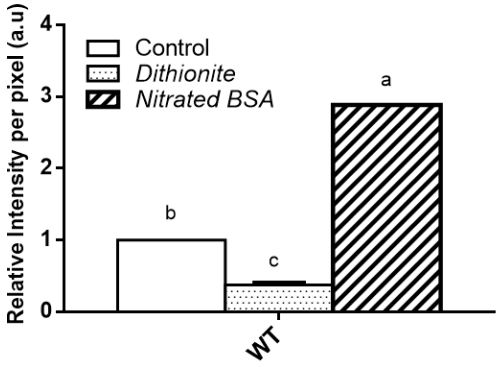
4.2.2.1. Plant material and growth conditions

Arabidopsis thaliana (Col-0) seeds (WT, *nia1 nia2*, *Atnoa1*, *nox1* and *argh1.1*) were surface-sterilized and stratified for 48 h at 4 °C. The seedlings were then grown vertically in Hoagland solid medium (0,5x), pH 5.6 (Hoagland and Arnon, 1950) with and without (control) 25 μM cadmium (Cd) in a growth chamber at 22 °C under 100 μE irradiance, 60–65% relative humidity and 16/8 light/dark conditions for 7 days. When indicated, seedlings were supplemented with nitric oxide (NO) donor S-nitroso-N-acetyl-DL-penicillamine (SNAP, 1 mM; WT, *nia1 nia2*, *Atnoa1*) and NO production inhibitor aminoguanidine (AG, 1 mM; WT, *nox1*, *argh1.1*) for 4 days. The plates were scanned to analyse seedling root length. The whole seedlings were then harvested, weighed and processed, and frozen when necessary, for the purposes of enzymatic and non-enzymatic, Western blot and gene expression analysis. Only seedling leaves were used to analyse hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) with the aid of 3,3′-diaminobenzidine (DAB) and 4-nitro blue tetrazolium chloride (NBT), respectively. The seedling roots were observed under a confocal laser scanning microscope to analyse reactive oxygen and nitrogen species (ROS and RNS) production.

4.2.2.2. Enzymatic and Western blot analyses

Whole seedlings were homogenized as described elsewhere (Pérez-Chaca *et al*., 2014). Supernatants were used to measure superoxide dismutase (SOD; EC 1.15.1.1; Beauchamp and Fridovich, 1971), catalase (CAT; EC 1.11.1.6; Aebi, 1984), glycolate oxidase (GOX; EC 1.1.3.1; Kerr and Groves, 1975) and ascorbate peroxidase (APX; EC 1.11.1.11; Jimenez *et al*., 1997). Ascorbate (1 mM) was added to the extraction buffer to analyse APX activity.

To detect carbonyl groups and nitrated proteins, anti-dinitrophenyl (anti-DNP; 1:40000 dilution; Sigma) and anti-nitrotyrosine (N-Tyr; 1:2000 dilution; Sigma) were used as described elsewhere (Romero-Puertas *et al*., 2002 and Pérez-Chaca *et al*., 2014, respectively). Protein extracts were incubated with 5 mM sodium dithionite to ensure anti-nitrotyrosine specificity and nitrated BSA was used as positive control (Suppl. Fig. S4.2.1). Anti-catalase (1:5000; Agrisera) was used to quantify CAT.



Suppl. Fig. S4.2.1. Anti-nitro-Tyr antibody controls. Quantification of the chemiluminescent signal in WT seedlings protein extract under control conditions and treated with sodium dithionite, and commercial nitrated BSA (Sigma). Data represent mean ± standard error (error bars) of two independent experiments. Different letters denote significant differences determined by the T-test ($P<0.05$).

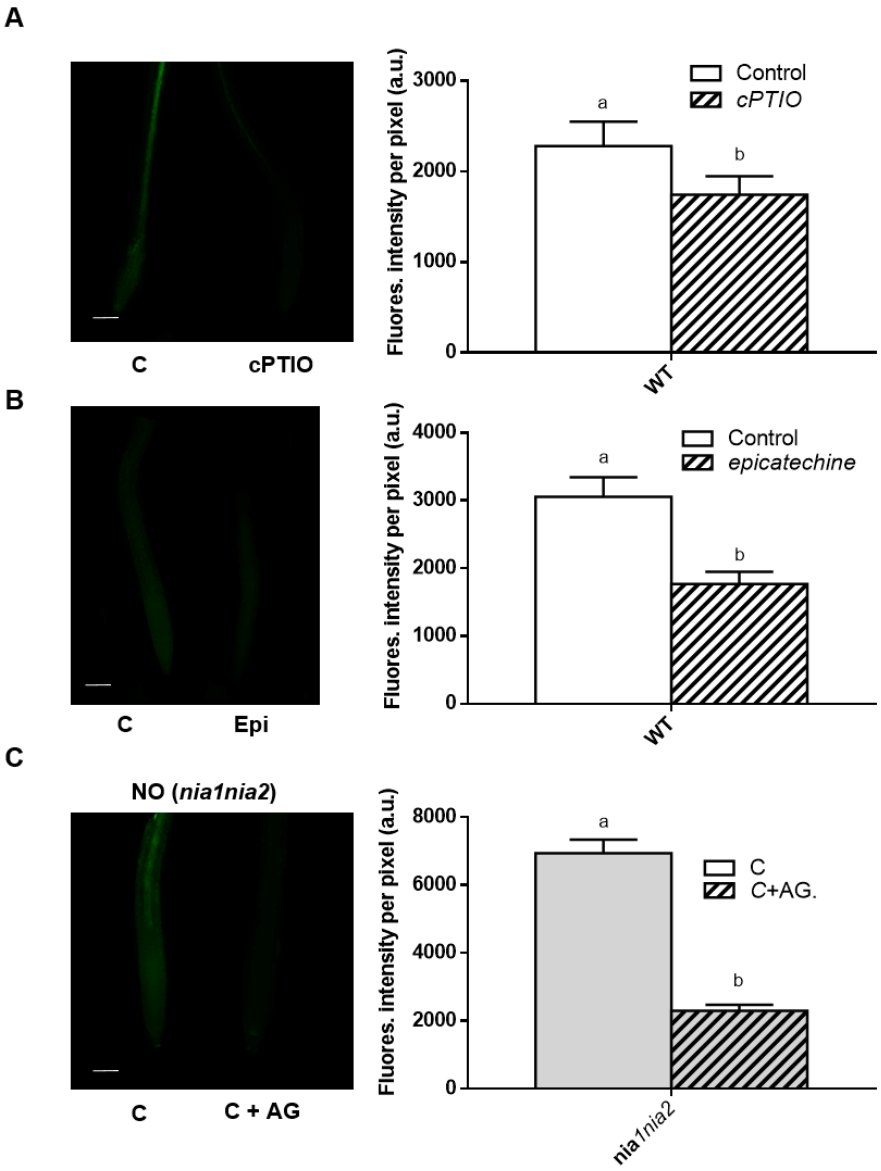
4.2.2.3. Non-enzymatic assays

A lipid peroxidation assay procedure, as described by Buege and Aust (1978), was used. Cd was localized in plant tissues using diphenylthiocarbazone (650 μM; dithizone; Clabeaux *et al*., 2011; Seregin and Ivanov, 1997) prepared in acetone (33%) and acetic acid (55%). The samples were stained for 1 h and then rinsed 3 times with ethanol. Cd-dependent deposits in seedling roots were analysed using a Leica M165FC stereomicroscope.

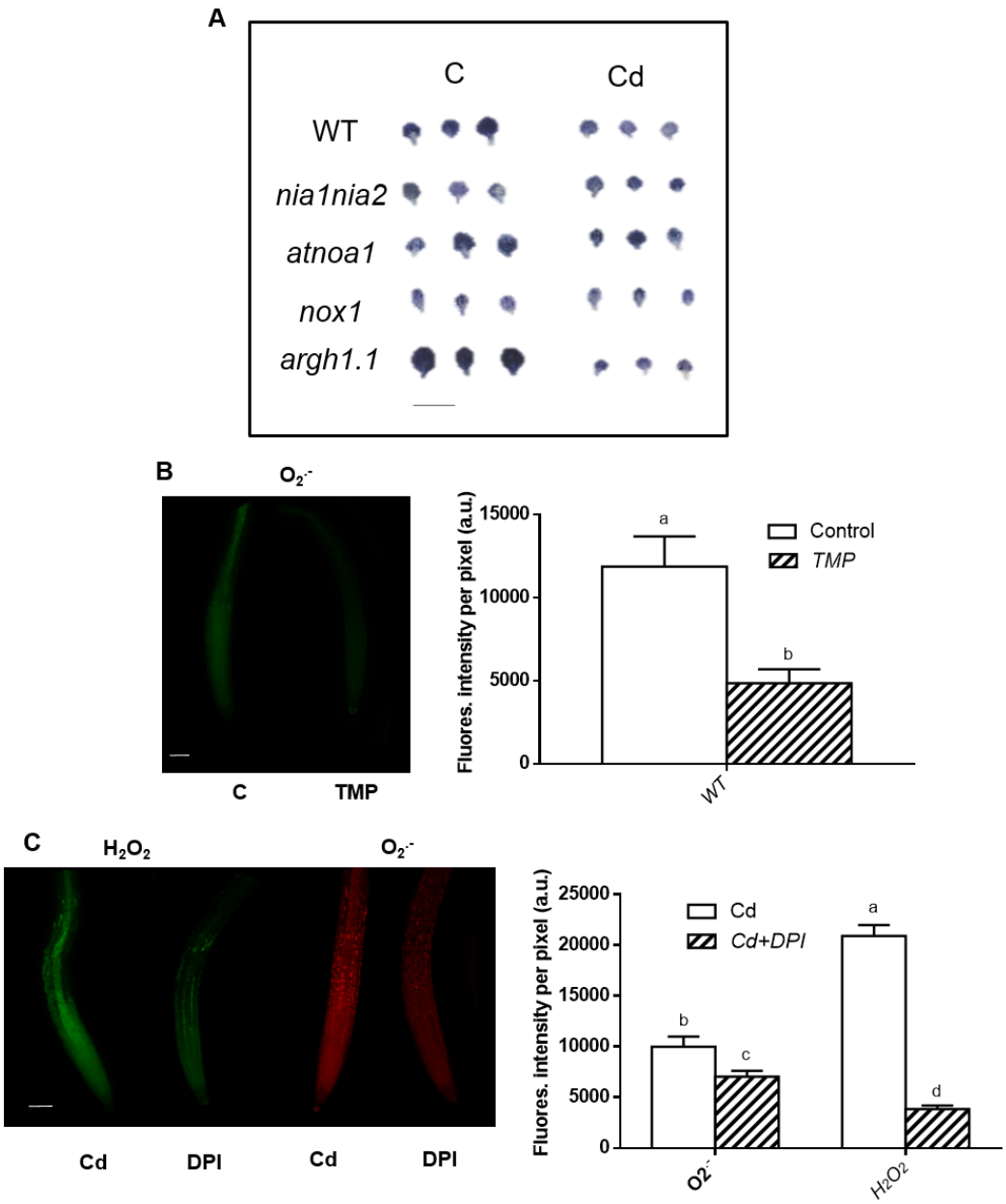
4.2.2.4. Reactive oxygen species, nitric oxide and peroxynitrite detection

To detect NO, $O_2^{\cdot-}$, H_2O_2 and peroxynitrite ($ONOO^-$), the seedling roots were incubated with 10 μM 4,5-diaminofluorescein diacetate (DAF-2DA), 10 μM dihydroethidium (DHE), 25 μM 2′7′-dichlorofluorescein diacetate (DCF-DA) and 25 μM HK2 Green, respectively, as described elsewhere (Rodríguez-Serrano *et al*., 2006; Sun *et al*., 2009; Terrón-Camero *et al*., 2018). Controls were made by pre-incubating samples with 500 μM carboxy-2-phenyl-4,4,5,5-tetramethy-limidaziline-1-oxyl-3-oxide (cPTIO; Suppl. Fig. S4.2.2A), a NO scavenger; 1 mM epicatechin (Suppl. Fig. S4.2.2B), an $ONOO^-$-specific scavenger (Pannala *et al*., 1997); 1 mM tetramethyl piperidinooxy (TMP), an $O_2^{\cdot-}$ scavenger (Suppl. Fig. S4.2.3B), and 1 mM ascorbate (ASC; Suppl. Fig. S4.2.4B), a H_2O_2 scavenger. Root fluorescence was examined under a confocal laser scanning microscope (Leica TCS). Diphenyliodonium chloride (DPI; 1 mM; Suppl. Fig. S4.2.3C) and

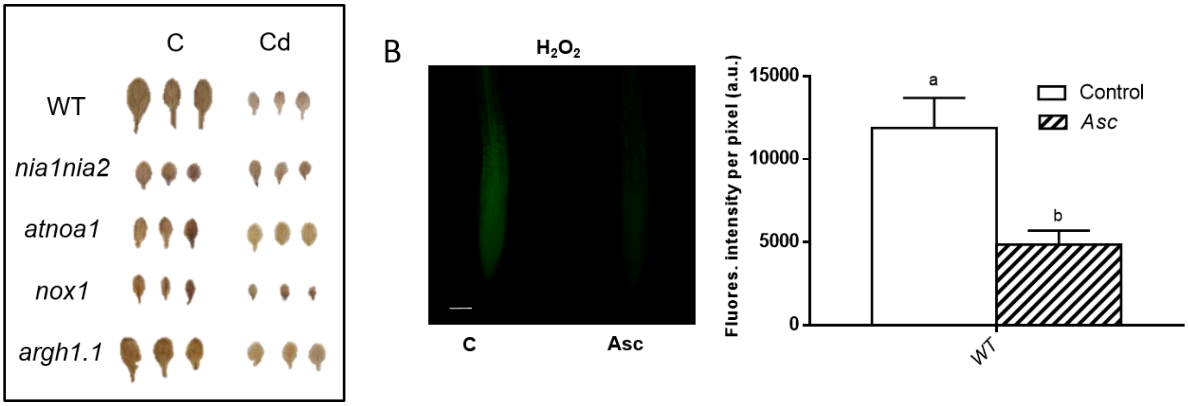
aminoguanidine (AG; 1 mM; Suppl. Fig. S4.2.2C) were used as NADPH oxidase and NOS-like activity inhibitors, respectively. Fluorimetry was used to determine H_2O_2 in whole seedlings as described elsewhere (Romero-Puertas *et al*., 2004). To detect NO in the whole seedling, extracts were incubated with 20 µM DAF-2 for 2 h at 37 °C (excitation: 495 nm, emission: 515 nm; Nakatsubo *et al*., 1998).



Suppl. Fig. S4.2.2. NO and $ONOO^-$ detection by CLMS controls and sources. Specificity of DAF2-DA fluorescence for NO is verified by root incubation with the scavenger cPTIO (0.5 mM; A) and specificity of HK2-Green for ONOO- is verified by root incubation with the scavenger epicatechine (1 mM; B). *nia1 nia2* roots were pre-incubated with the NOS inhibitor aminoguianidine (AG; 1 mM; C) under control conditions. Data represent mean ± standard error (error bars) of three independent experiments. Different letters denote significant differences determined by the T-test ($P<0.05$).



Suppl. Fig. S4.2.3. NBT staining in leaves and $O_2^{\cdot-}$ detection by CLMS controls and sources. Representative image of $O_2^{\cdot-}$-dependent precipitates in leaves by NBT staining (A). Specificity of DHE fluorescence for $O_2^{\cdot-}$ is verified by roots incubation with the scavenger TMP (1 mM; B). To look for possible source of $O_2^{\cdot-}$ and H_2O_2, roots were pre-incubated with the NADPH oxidase inhibitor, DPI (C). Data represent mean ± standard error (error bars) of three independent experiments. Different letters denote significant differences determined by the T-test ($P<0.05$).



Suppl. Fig. S4.2.4. DAB staining in leaves and H_2O_2 detection by CLMS controls. Representative image of H_2O_2-dependent precipitates in leaves by DAB staining (A). Specificity of DCF-DA fluorescence for H_2O_2 is verified by root incubation with ascorbate (1 mM) (B, C). Data represent mean ± standard error (error bars) of two independent experiments. Different letters denote significant differences determined by the T-test (P<0.05).

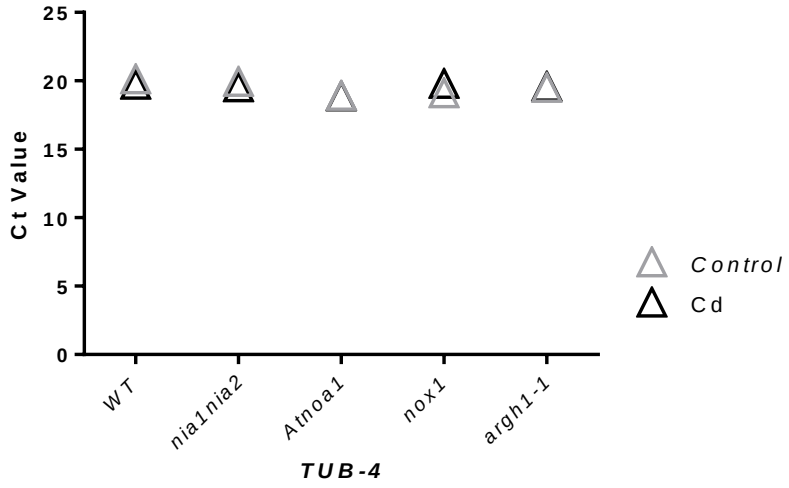
4.2.2.5. Histochemistry and images quantification

A histochemical analysis was carried out to detect $O_2^{\cdot-}$ and H_2O_2 in seedling leaves using NBT (0,1%; w/v) and DAB (1 mg/ml), pH 3.8, respectively, as described elsewhere (Romero-Puertas *et al*., 2004). Primary seedling root elongation, DAB and NBT staining and fluorescence in seedling root tissue were quantified with the aid of Image J Fiji software (Terrón-Camero *et al*., 2018). Briefly, average intensity per pixel was analysed in each image with three independent squares of the same size.

4.2.2.6. Reverse transcription quantitative PCR analysis of gene expression (RT-qPCR)

RNA was isolated from 0.1 g frozen and homogenized seedlings using Trizol reagent (Invitrogen) and deoxyribonuclease (DNase) according to the manufacturer's protocol (www.invitrogen.com/content/sfs/manuals/15596026.pdf) and Ambion DNA-free kit, respectively. RNA quantification and integrity were verified with the aid of a NanoDrop® ND-1000 spectrophotometer and 1% agarose electrophoresis gel. 1 µg RNA was used as a template for the reverse transcriptase (RT) reaction, and cDNA synthesis was obtained by using a PrimerScript RT reagent Kit (Takara). cDNA was two-fold diluted and stored at −20 °C for future use. RT-qPCR was performed with the aid of the iCycler iQ Real-Time PCR Detection System (Bio-Rad) using SYBR Premix Ex Taq™ II (https://www.takarabio.com% 20Manual /RR820L_e.v1611Da.pdf.). Primers efficiency was determined by a standard curve using two-fold serial dilution of pooled samples. Primer melting curves with 90–105% efficiency were performed to validate amplification specificity (Suppl. Table S1). Five candidate reference genes, TUB4 (AT5G44340), ACT2_1 (AT3G18780_1), ACT2_2 (AT3G18780_2), UBI10 (AT4G05320) and GAPC1 (AT3G04120), were selected from the literature (Calero-Muñoz *et al*., 2019; Cuypers *et al*., 2011; Czechowski *et al*., 2005), and the best combination for normalization was selected using the GrayNorm algorithm (Remans *et al*., 2014) with expression data from an in-house microarray

containing Cd-treated seedlings. Only the TUB4 gene, whose stability under our conditions in all backgrounds was tested, was selected by the algorithm (Suppl. Fig. S4.2.5; Suppl. Table S2). The relative expression of each gene was then normalized using the reference gene selected (TUB4), and the results were analysed with the aid of the comparative critical threshold method (Pfaffl, 2001).



Suppl. Fig. S4.2.5: qRT-PCR Ct values of the final candidate reference genes in our condition. Mean values.

4.2.2.7. Bioinformatic analysis

The dataset containing 26 variables was analysed using hierarchical clustering (Jain *et al*., 1999; Mitchell, 1997). As a first step, data clusterability was estimated using the Hopkins statistic (Lawson and Jurs, 1990). Hierarchical clustering was performed using Ward distance and the complete linkage method (Jain, 2010; Kaufman *et al*., 1990; Mitchell, 1997; Taiyun-Wei *et al*., 2017).

We used the silhouette method (Kaufman *et al*., 1990) to estimate the number of clusters. The potentially optimal number of clusters was then chosen in order to maximize the average distance between silhouette means (ADSM). We also studied dependencies between the variables as a result of Cd stress in the different mutants. The correlation coefficient between the variables was calculated as Pearson's product-moment coefficient from previous standard normalization of the variables using the corrplot R package (Taiyun-Wei *et al*., 2017).

Differences in the quantitative experiments were compared using two-way ANOVA. Mean values for the different treatments were compared using the S-N-K (Student-Newman-Keuls) multiple comparison test ($P < 0.05$; Salkind, 2010), by IBM SPPS Statistic 24 and GraphPad Prism 6. Error bars representing standard error median (SEM) are shown in the figures, and the data represented are the mean of at least three independent experiments.

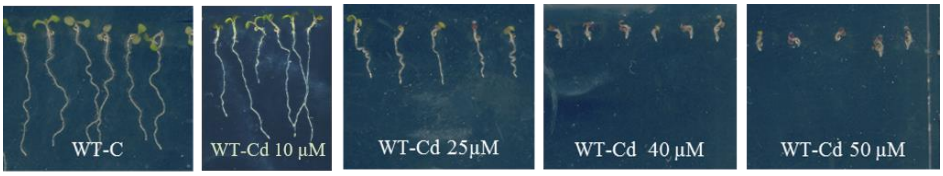
4.2.3. Results and discussion

4.2.3.1. Damage to cadmium-treated seedlings

Recent studies of plants, mostly adult when treated, have linked nitric oxide (NO) to the plant's response to cadmium (Cd), although the mechanisms involved remain unclear. However, the phenotype observed in plants following Cd treatment has common signatures such as growth inhibition (fresh weight and root length) and a redox status imbalance causing oxidative stress (Kolbert, 2016; Romero-Puertas *et al*., 2019; Terrón-Camero *et al*., 2019). In this study, we analysed how seedlings respond to Cd-related stress during germination and growth, as well as the effect of the different levels of NO present in four pre-characterised mutants and the role played by NO metabolism. Both reductive and oxidative NO-producing pathways in plants are involved in plant responses to Cd stress (Romero-Puertas *et al*., 2019). Thus, two of the mutants used in this study are directly associated with NO production: *nia1 nia2*, which is affected in nitrate reductase (NR) activity (reduced to less than 1%), mainly localized in the cytosol (Yamasaki and Sakihama, 2000; Rockel *et al*., 2002; Desikan *et al*., 2002; Guo *et al*., 2003; Modolo *et al*., 2005; Moreau *et al*., 2008) and *argh1-1*, which lacks the arginase gene (mainly localized in the chloroplast and mitochondria), leading to an increase in the nitric oxide synthase (NOS-like) substrate (L-arginine; Flores *et al*., 2008). In addition, we used two other mutants in which, though not directly involved in NO production, its levels are modified in the plant: the *Atnoa1* mutant, which lacks the *Atnoa1* gene, a cGTPase involved in mRNA translation to proteins in chloroplasts and mitochondria, which shows lower levels of NO under certain conditions such as abcisic acid (ABA)-dependent stomatal closure and flowering (Moreau *et al*., 2008); and the *nox1*/cue1 mutant, which is altered in a chloroplast phosphoenolpyruvate/phosphate translocator, which shows generally higher levels of NO (Streatfield *et al*., 1999; He *et al*., 2004). The contribution of each direct and indirect biosynthetic pathway may fluctuate in different organs in this developmental stage, as the gene expression patterns affected vary according to plant topology as demonstrated by open-access databases (BAR; http://www.bar.utoronto.ca/).

Having tested different Cd concentrations (10–50 μM), we selected a Cd concentration of 25 μM. Even though this concentration had visible effects on seedling roots such as decreased root growth, the seedlings were suitable for morphological and biochemical analysis (Suppl. Fig. S4.2.6). The effects of lower concentrations of Cd were difficult to quantify, while those caused by higher concentrations were excessively strong in seedlings which barely developed (Suppl. Fig. S4.2.6). Three of these mutants: *nia1 nia2*, *Atnoa1* and *nox1*, showed lower fresh weight than that for WT seedlings under control conditions, while *Atnoa1* and *nox1* also recorded lower rates of seedling root growth (Fig. 4.2.1). These results are in line with previous characterizations of these mutants. NO-deficient seedlings (*Atnoa1* and *nia1 nia2*) showed a decrease in shoot and root

growth under normal conditions (Kolbert *et al*., 2015), and most of which phenotype is reversed when the mutants are supplemented with NO (Lozano-Juste and Leon, 2010). Similarly, the NO overproducer *nox1*/*cue1* showed physiological defects including diminished biomass and root growth (He *et al*., 2004; Frungillo *et al*., 2014). This appears to show an absence of correlation between NO levels and root elongation, while the level of NO, which needs to be optimised for normal growth, needs to be strictly regulated (Kolbert, 2016). Mutations in the *ARGH1* gene in *argh1-1* mutants, led to an increase in the formation of lateral and adventitious roots whose length appeared to be unaffected (Flores *et al*., 2008).



Suppl. Fig. S4.2.6: Arabidopsis seedlings phenotype growing 7d in different concentrations of Cd (0-50 µM).

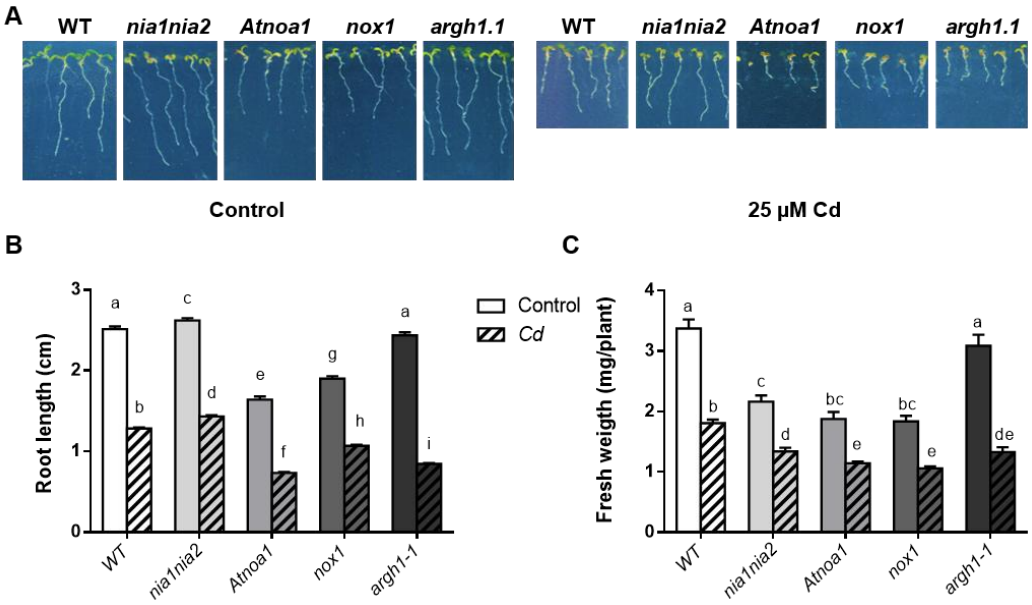


Fig. 4.2.1. Effect of cadmium on Arabidopsis seedlings phenotype and fitness. WT seedlings, as well as *nia1 nia2*, *Atnoa1*, *nox1* and *argh1-1* mutants, were germinated and grown in Hoagland medium, with and without (control) 25 µM Cd. Pictures of seedlings (A), seedling root length (B) and fresh weight (C) after 7 days are shown. Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P < 0.05$).

Given these results, we compared seedling root length in Cd-treated 7-day-old seedlings with those under control background conditions, as root growth inhibition is one of the earliest marker symptoms of Cd toxicity (Sandalio *et al*., 2001; Kolbert, 2016). A significant decrease in seedling root length was observed in WT plants (49%) and NO-related mutants, with *argh1-1* and *Atnoa1* being more affected (65% and 55%, respectively) and *nia1 nia2* and *nox1* less affected (45% and 44%, respectively) than WT plants (Fig. 4.2.1A and B). Similar decreases in the root length of 7- and 8-day-old Arabidopsis seedlings treated with 30 and 15 µM Cd respect to the non-

treated ones (35–55% and of roughly 50%) have been observed in previous studies (Kim *et al*., 2006; Carrió-Seguí *et al*., 2015). Fresh weight also decreased under Cd stress conditions in WT plants (46%), being *argh1-1* mutants more affected (57%) and *nia1 nia2*, *Atnoa1* and *nox1* less affected (38%, 39% and 42%, respectively; Fig. 4.2.1A and C). Although NO levels in mutants and growth inhibition in response to Cd do not appear to correlate, the *nia1 nia2* mutant was the least affected while *argh1-1* was the most affected. Under similar conditions, excessive Cu has been shown to inhibit seedling growth in *nox1* mutants to a degree similar to that in WT seedlings, while fresh weight loss was less pronounced in WT seedlings than in *nia1 nia2* mutants (Pető *et al*., 2013). Given the aforementioned complexity of NO metabolism, NO needs to be strictly regulated even under control conditions (Kolbert, 2016). In addition, other signalling molecules such as hormones are involved in seedling responses to Cd stress, which might interact with NO. Different levels of NO may also lead to different patterns of NO-dependent post-translational modifications of proteins, such as glyceraldehyde-3-phosphate dehydrogenase, involved in root growth previously (Wang *et al*., 2017).

Oxidative stress is one of the primary effects of Cd treatment in both adult plants and seedlings (Ortega-Galisteo *et al*., 2012; Chen *et al*., 2017; Gupta *et al*., 2017). We have used the whole seedlings to analyse oxidative markers, as roots and leaves at the seedling stage are still too small to be analysed separately. Thus, we observed a Cd-dependent increase in carbonyl group content, a marker of oxidative damage to proteins, in WT seedlings and all the mutants analysed, although *Atnoa1*, which had shown high levels of protein oxidation under control conditions, was unaffected (Fig. 4.2.2B). It has been hypothesised that the changes in the levels of NO in *Atnoa1* are caused by defective organelle function. This gives rise to ROS production that may scavenge NO and therefore lower its bioavailability (Sudhamsu *et al*., 2008; Moreau *et al*., 2010). An increase in carbonyl content in response to Cd, which depends on its concentration and exposure time, has been observed in Arabidopsis seedlings (Calero-Muñoz *et al*., 2019) and other species in both root and leaves. In soybean roots, carbonyl content increases at the beginning of treatment with Cd and then declines following longer exposure (Pérez-Chaca *et al*., 2014). Carbonyl content also increases in wheat, tomato, pea and maize leaves (Sandalio *et al*., 2001; Pena *et al*., 2007; Djebali *et al*., 2008; Paradiso *et al*., 2008) when Cd concentrations are increased. Roots sometimes behave differentially to leaves showing an opposite pattern of oxidation (Djebali *et al*., 2008) and others, in the same way to leaves (Paradiso *et al*., 2008). These controversial results probably depend on the developmental stage of the plant, as well as treatment length and dosage (Romero-Puertas *et al*., 2019).

An increase in lipid damage was also observed in WT seedlings and NO-dependent mutants except for *nia1 nia2*, whose lipid integrity appeared to be unaffected in response to Cd

(Fig. 4.2.2A). *nia1 nia2* phenotype is reversed by adding a NO donor *S*-Nitroso-N-acetyl-DL-penicillamine (SNAP) to the seedlings, giving rise to a statistically significant increase in NO and lipid peroxidation in this mutant during Cd treatment (Suppl. Figs. S4.2.7 and S4.2.8). This result suggests that NR-dependent NO is involved, at least in part, in the increase in oxidative damage observed in response to Cd. Actually, lipid peroxidation in *nia1 nia2* under control conditions is slightly higher than in WT, probably due to the increased level of NO observed in the seedling roots of the mutant. On the other hand, the increase in lipid peroxidation in response to Cd is four times higher in *argh1-1* than in WT and similar to that in WT in *nox1* seedlings (Fig. 4.2.2A). The increase in lipid peroxidation caused by Cd is reduced by 15% in *argh1-1* and by 40% in *nox1* (Suppl. Fig. S4.2.8), in line with the decrease observed in NO in the mutants, by adding the NO production inhibitor AG during Cd treatment (Suppl. Fig. S4.2.7). These findings further corroborate the involvement of NO in the increase in oxidative damage observed in response to Cd. Most data currently available on lipid peroxidation in response to Cd stress concern adult plants (Romero-Puertas *et al*., 2019); less is known about seedlings germinated and grown under Cd treatment. Thus, lipid peroxidation has been observed to increase in Cd-treated 6-day-old Arabidopsis seedlings (Chen *et al*., 2017), which is in line with our findings.

Protein nitration is a major post-translational modification initially associated with nitrosative stress (Radi, 2004), about which little is known in Cd-treated plants. No differences in the total content of nitro-tyrosine (N-Tyr) residues have been found in soybean roots following Cd treatment (Pérez-Chaca *et al*., 2014), suggesting that Cd induces a finely tuned regulation of protein nitration rather than any major changes. A later study identified nitrated proteins in soybean under Cd treatment; five of the ten Tyr-nitrated proteins identified were down-regulated during both moderate and intense exposure to Cd, while five were up-regulated only during intense exposure to Cd, with the majority of the identified proteins being associated with proteolysis (Gzyl *et al*., 2016). While we did not find any significant differences in the pattern of nitrated proteins in seedlings grown under Cd treatment conditions, *Atnoa1* and *nox1* mutants did show a decrease in nitration, although basal levels were higher than those in WT seedlings (Fig. 4.2.2C). This result suggests that, as mentioned elsewhere, *Atnoa1* and *nox1* mutants alter RNS-dependent damage even under control conditions (Moreau *et al*., 2010; Hu *et al*., 2014).

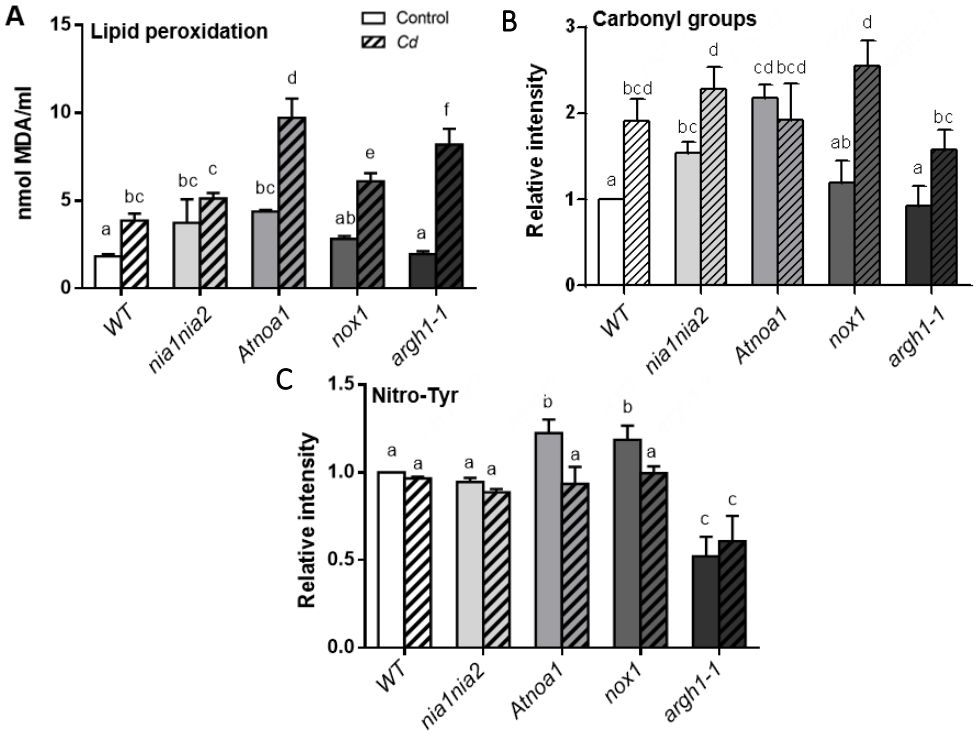
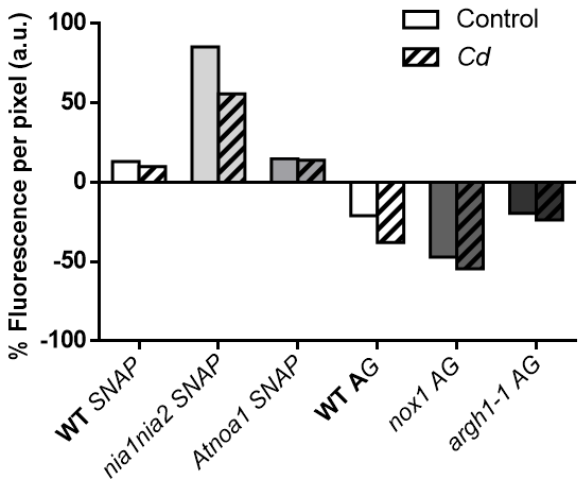
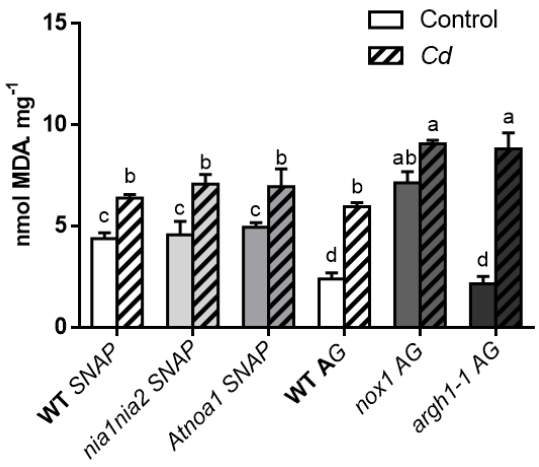


Fig. 4.2.2. Effect of cadmium on lipids and proteins of Arabidopsis seedlings. (A) Lipid peroxidation measured as malondyaldehyde (MDA) content; (B) protein oxidation measured as carbonyl group content and (C) nitration level determined by Western blot. Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P < 0.05$).



Suppl. Fig. S4.2.7: Percentage of NO production in roots treatment with 1 mM SNAP and 1 mM aminoguanidine (AG) supplementation during 4 days respect to control condition (Control and Cd treatment).



Suppl. Fig. S4.2.8. Lipid peroxidation in seedlings supplemented with 1 mM SNAP (NO donor) and 1 mM aminoguanidine (AG) during 4 days in control and Cd conditions. Different letters denote significant differences determined by Student-Newman-Keuls test ($P<0.05$).

4.2.3.2. NO mutants showed altered ROS and RNS metabolism under Cd stress

When analysing the whole seedling, no changes in total H_2O_2 (Fig. 4.2.3D) and a decrease in total NO levels were observed in WT seedlings (Fig. 4.2.5C). We then analysed the different parts of the seedling and observed a decrease in ROS (H_2O_2 and $O_2^{\cdot-}$) production with the aid of DAB and NBT histochemistry in WT seedling leaves (Fig. 4.2.3, Fig. 4.2.4A and Suppl. Figs. S4.2.3A and S4.2.4A). Using confocal laser scanning microscope (CLSM), we observed an increase in ROS/RNS (H_2O_2, $O_2^{\cdot-}$ and $ONOO^-$) in WT seedling roots (Fig. 4.2.3B and C; Fig. 4.2.4B and C; Fig. 4.2.5D and E). Thus, under the conditions studied, oxidative stress and fitness loss in the seedling appears to have been caused by an increase in ROS/RNS production in seedling roots and, to a lesser extent, in seedling leaves. At this stage, the small size of seedling leaves may affect seedling responses. Consequently, the damage observed in WT seedlings grown under Cd treatment occurred concurrently with the increase in H_2O_2, $O_2^{\cdot-}$ and $ONOO^-$ in 7-days-old WT seedling roots (Fig. 4.2.3B and C; Fig. 4.2.4B and C; Fig. 4.2.5D and E). ROS production has previously been shown to increase in different species including Arabidopsis in response to Cd, especially under long-term treatment conditions, both in adult plants (Ortega-Villasante *et al*., 2007; Rodríguez-Serrano *et al*., 2006; Pérez-Chaca *et al*., 2014; Gupta *et al*., 2017) and in seedlings (Kulik *et al*., 2012). H_2O_2 production has been shown to increase in the roots of 3-week-old Arabidopsis plants treated with 50 µM Cd (Bahmani *et al*., 2019), while H_2S prevented this increase in 2-week-old seedling roots (Jia *et al*., 2016). However, in adult plants growing for three weeks under normal conditions and then treated with low concentrations of Cd, an increase in H_2O_2 was observed in both leaves and roots (Cuypers *et al*., 2011).

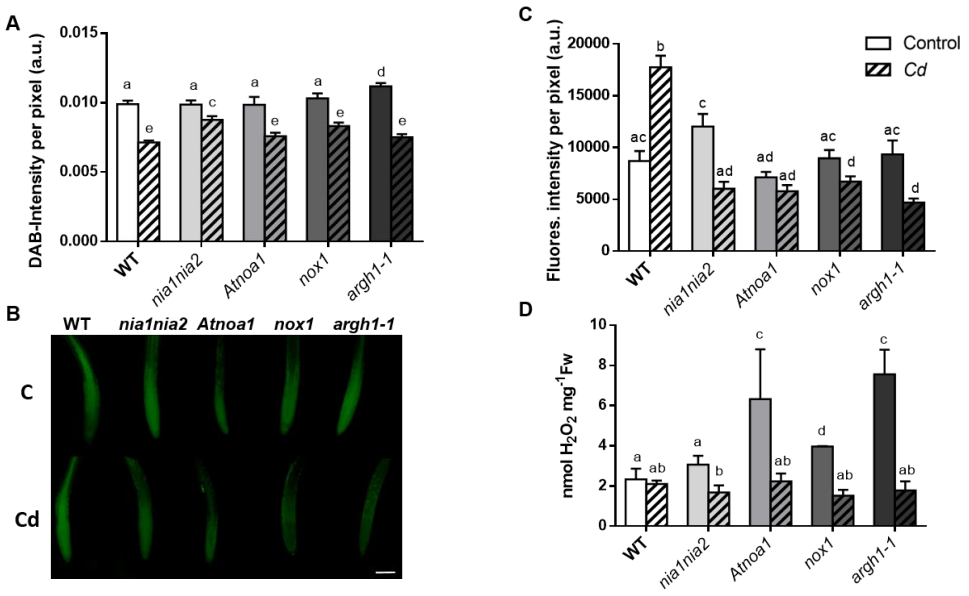


Fig. 4.2. Effect of cadmium on H_2O_2 accumulation in Arabidopsis seedlings. Quantification of images (Suppl. Fig. S4.2.4A) with H_2O_2-dependent precipitates in seedling leaves by DAB staining (A); (B) representative images of H_2O_2-dependent fluorescence in seedling roots and quantification (C); total H_2O_2 content (D) in the whole seedlings grown or not under Cd conditions for 7 days. Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P < 0.05$).

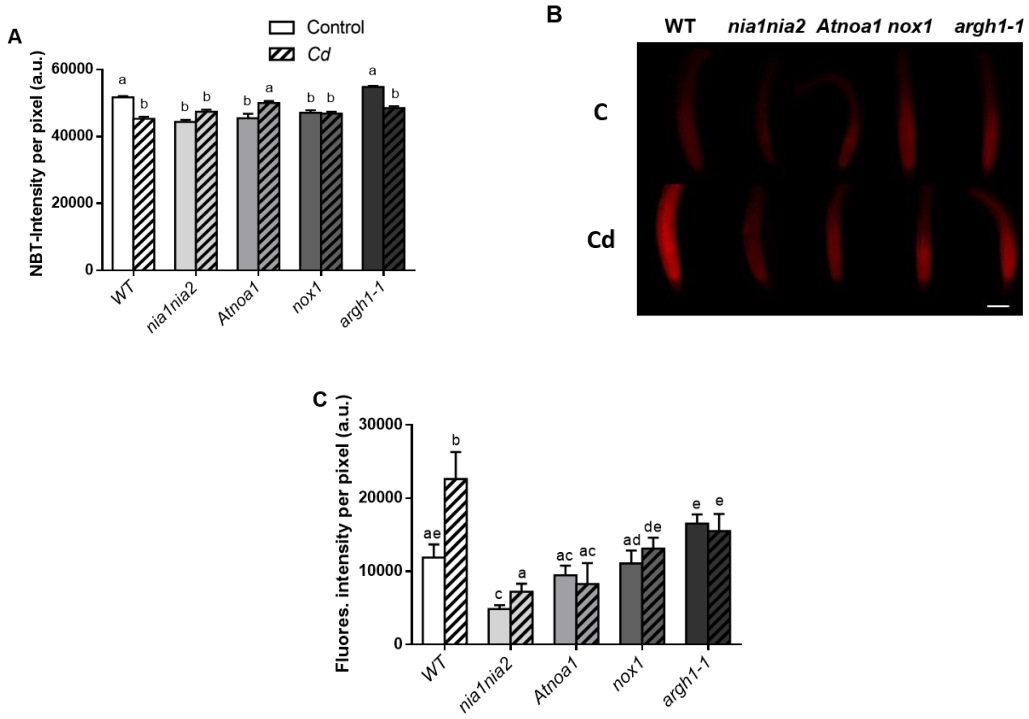


Fig. 4.2.4. Effect of cadmium on $O_2^{\cdot-}$ accumulation in Arabidopsis seedlings. Quantification of images (Suppl. Fig. S4.2.3A) with $O_2^{\cdot-}$-dependent precipitates in seedling leaves by NBT staining (A); (B) representative images of $O_2^{\cdot-}$-dependent fluorescence in seedling roots grown or not (control) under Cd conditions for 7-days and quantification of the images (C). Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P < 0.05$).

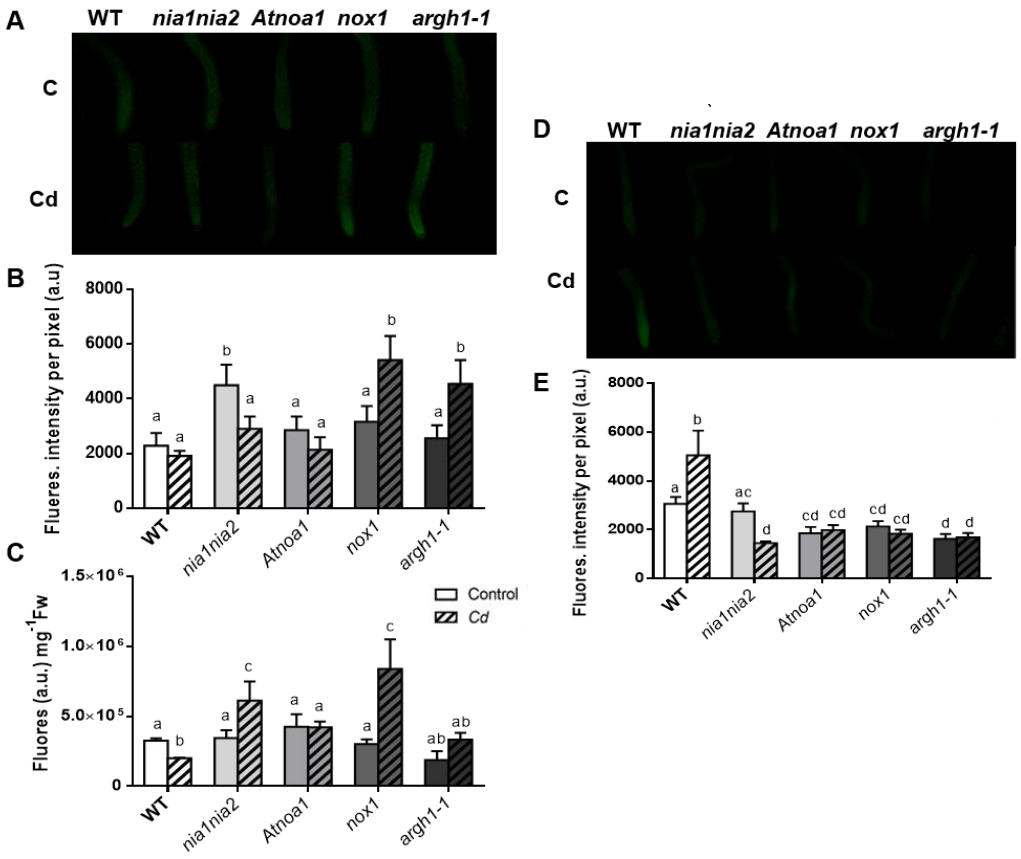


Fig. 4.2.5. Effect of cadmium on NO and ONOO$^-$ accumulation in Arabidopsis seedlings. (A) Representative images of NO-dependent fluorescence in seedling roots and image quantification (B); total NO content determined by fluorimetry using DAF2-DA (C) in seedlings grown or not (control) under Cd conditions for 7-days. (D) Representative images of $ONOO^-$-dependent HKGreen-2 fluorescence in seedling roots and image quantification (E). Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P < 0.05$).

We observed a decrease in total NO in WT seedlings in response to Cd (Fig. 4.2.5C), with no changes being observed in seedling roots (Fig. 4.2.5A and B). This suggests that, although an initial increase in NO is usually observed in plant responses to Cd stress (Bartha *et al*., 2005; Groppa *et al*., 2008; Balestrazzi *et al*., 2009; Mahmood *et al*., 2009; Valentovičová *et al*., 2010; Pérez-Chaca *et al*., 2014;), probably associated with signalling, NO levels are brought under control at a later stage (Terrón-Camero *et al*., 2019). Thus, NO has been shown to be involved in reducing root growth in plant responses to Cd in different species (Groppa *et al*., 2008; Besson-Bard *et al*., 2009; Valentovičová *et al*., 2010) by repressing auxin accumulation and signalling in Arabidopsis plants (Yuan and Huang, 2016). Longer treatments might also affect NOS-l activity due to a calcium (Ca) deficiency and decreasing NO levels (Rodríguez-Serrano *et al*., 2009), and to an induced early senescence (McCarthy *et al*., 2001; Rodríguez-Serrano *et al*., 2009).

The increase in ROS and RNS production observed in WT seedling roots was concomitant with a decrease in antioxidant enzymes, such as ascorbate peroxidase (APX) and catalase (CAT), which maintain ROS homeostasis (Fig. 4.2.6). CAT activity, which may be responsible for the bulk removal of excess H_2O_2 produced under stress conditions (Mittler, 2002), is affected in Cd-

treated seedlings grown, decreasing its activity by more than 50% (Fig. 4.2.6B). *CAT2* gene expression also fell by over 50% and the protein almost disappeared (Fig. 4.2.6C and D). Several studies have analysed APX activity under Cd stress, mainly in adult plants, which generally respond via increased APX activity in roots and leaves, especially under short-term treatment with Cd (Pérez-Chaca *et al*., 2014; Cuypers *et al*., 2016, 2011; Terrón-Camero *et al*., 2019). As with APX, a number of studies have shown that CAT activity and/or gene expression under Cd stress conditions are dependent on tissue, as well as species and length of the treatment, without, at times, any direct relationship between CAT activity and gene expression (Cuypers *et al*., 2016; Romero-Puertas *et al*., 2019; Terrón-Camero *et al*., 2019). For example, in 3-week-old Arabidopsis plants, changes in CAT activity under Cd stress conditions depend on the plant tissue and concentrations of the metal (Cuypers *et al*., 2011), while, in 5-weeks-old plants, CAT activity increases in leaves after 1- and 5-days treatments (Gupta *et al*., 2017). However, in pea leaves, as in our study, following a long high-dose treatment, this activity was found to decrease (Sandalio *et al*., 2001).

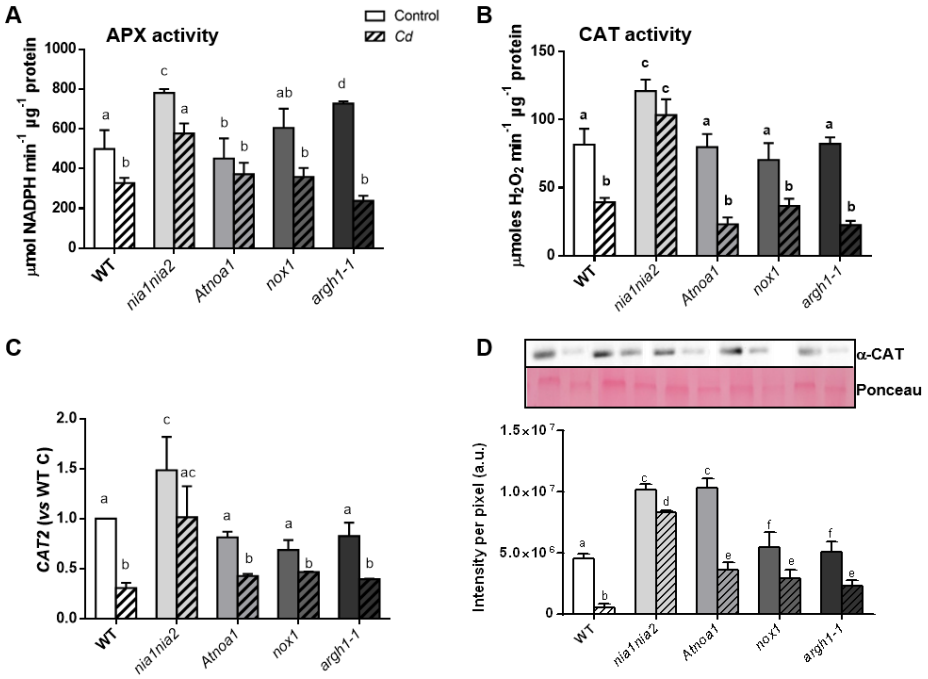


Fig. 4.2.6. Effect of cadmium on superoxide dismutase in Arabidopsis seedlings. APX (A) and CAT (B) activities; CAT2 gene expression (C) and catalase content (D) by Western blot in seedlings grown or not (control) under Cd conditions. Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P < 0.05$)

In our study, ROS and NO production in NO mutants differed with respect to responses to Cd and, at times, under control conditions, in relation to one another and with respect to WT plants, suggesting, as described elsewhere, that there is intense crosstalk between NO and ROS (Lindermayr and Durner, 2015; Romero-Puertas and Sandalio, 2016). Thus, overproduction of NO in seedling roots (Fig. 4.2.5A and B) in response to Cd, a decrease in H_2O_2 (Fig. 4.2.3B and C) and no changes in $O_2^{\cdot-}$ and $ONOO^-$ (Fig. 4.2.4B and C and Fig. 4.2.5D and E, respectively) were

observed in *argh1-1* and *nox1* mutants. Both these mutants have been found to increase the arginine substrate for NOS-l activity (He *et al*., 2004; Flores *et al*., 2008), suggesting that, as described elsewhere, the arginine-dependent pathway is involved in increasing seedling root NO production under Cd stress (Rodríguez-Serrano *et al*., 2009; Besson-Bard *et al*., 2009). Increased NO content in seedling roots may also directly or indirectly affect other antioxidant systems lowering H_2O_2 levels in these mutants. However, the mutants *argh1-1* and *nox1* behave in very similar way to WT plants in relation to oxidative damage and down-regulation of antioxidant activity. These results suggest that the excess of NO observed in *argh1-1* and *nox1* mutants and disturbances in ROS homeostasis caused by the down-regulation of antioxidant enzymes induce oxidative damage. The two antioxidants analysed in our study are regulated by NO-dependent post-transcriptional modifications (PTMs) in different species (Correa-Aragunde *et al*., 2013; de Pinto *et al*., 2013; Begara-Morales *et al*., 2014;), while CAT is specifically regulated by oxidation and *S*-nitrosylation under Cd stress conditions in pea leaves (Romero-Puertas *et al*., 2002; Ortega-Galisteo *et al*., 2012). Although low levels of NO may boost the antioxidant system, especially under Cd stress conditions, excessive NO has the potential to become toxic to plants (Romero-Puertas *et al*., 2019).

Surprisingly, *nia1 nia2* mutants showed higher levels of NO in seedling roots under control conditions (Fig. 4.2.5A and B), which decreased after treatment with the NOS inhibitor aminoguanidin (AG; Suppl. Fig. S4.2.2C). This suggests that NOS-l activity may be involved in increasing the level of NO in *nia1 nia2* mutants, indicating that there is crosstalk between the different sources of NO in plants. Other studies have suggested that the different NO production pathways interact with one another and that nitrate reductase (NR) regulation in response to high levels of CO_2 is NOS-l-dependent (Du *et al*., 2016; Romero-Puertas and Sandalio, 2016). We found that Cd treatment reduced the level of NO in *nia1 nia2* seedling roots to that observed in WT seedling roots under control conditions, while an opposite pattern was observed in the NOS-l related mutants *argh1-1* and *nox1* (Fig. 4.2.5A and B). Although the level of $O_2^{\cdot-}$ was found to decrease in *nia1 nia2* mutants under control conditions and to increase following Cd treatment, it was similar to that recorded in WT seedlings under control conditions (Fig. 4.2.4B and C). The decrease in NO production following Cd treatment in *nia1 nia2* mutants was found to coincide with a decrease in $ONOO^-$, caused by the reaction of $O_2^{\cdot-}$ and NO (Fig. 4.2.5D and E; Koppenol *et al*., 1992). In addition, H_2O_2 was observed to decrease in *nia1 nia2* after treatment with Cd (Fig. 4.2.3B and C), probably due to the maintenance and apparent protection of CAT activity (Fig. 4.2.6B and C). This result suggests that NR plays a role in preventing the down-regulation of CAT activity under Cd stress conditions, although the mechanisms involved, apart from the above-mentioned NO-dependent PTMs, need further investigation.

Despite the apparent effect of stress on *Atnoa1* mutants in our study and its impact on seedling development described elsewhere (Moreau *et al*., 2008; Sudhamsu *et al*., 2008), we did not find any differences in ROS/RNS production in WT seedlings which was not found to differ under Cd stress as compared to control conditions (Fig. 4.2.3, Fig. 4.2.5). As mentioned previously, an imbalance in ROS metabolism has been described in these mutants, and, while the NOA1 protein may play a role in ROS/RNS production under Cd stress conditions, the mechanism involved needs to be studied in greater depth. Interestingly, APX activity in the *Atnoa1* mutant was unaffected by Cd treatment (Fig. 4.2.6A), suggesting that NOA1 is involved in decreasing this activity.

As previously shown in different species, incubation with diphenyliodonium (DPI) suggests that NADPH oxidases and possibly peroxidases (Rodríguez-Serrano *et al*., 2006) were involved in $O_2^{\cdot-}$ production (Suppl. Fig. S4.2.3C) under Cd stress conditions (Ros-Barceló, 1999; Rodríguez-Serrano *et al*., 2006; Pérez-Chaca *et al*., 2014; Gupta *et al*., 2017). It is interesting to note that treatment with DPI also decreased H_2O_2 production after Cd treatment (Suppl. Fig. S4.2.3C), suggesting that this was mostly due to the spontaneous dismutation of the $O_2^{\cdot-}$ radical, as SOD activity and gene expression were found to decrease with Cd treatment in our experiments (Fig. 4.2.7). The Cu, Zn-SOD isoforms showed the highest levels of activity, accounting for 85–93% of the total activity depending on background and conditions (Suppl. Fig. S4.2.9B). We observed a decrease in Cu, Zn-SOD activity in response to Cd in all genotypes except for *argh1-1* and *Atnoa1* mutants (Fig. 4.2.7A and Suppl. Fig. S4.2.9B). On the other hand, the plastidic gene Cu, Zn-SOD2 was down-regulated in all genotypes (Fig. 4.2.7) under Cd stress conditions, suggesting that NO was not involved in regulating this gene. Similarly, the Cu, Zn-SOD2 gene was down-regulated in 3-week-old Arabidopsis leaves under Cd stress conditions for 24 h (Smeets *et al*., 2009). Interestingly, a down-regulation of the peroxisomal Cu, Zn-SOD3 gene was also observed in *nia1 nia2* mutants (Fig. 4.2.7D), suggesting that NR may regulate this isoenzyme under Cd toxicity conditions. However, to the best of our knowledge, the molecular mechanism involved in this process has not been elucidated and requires further analysis. Mn- and Fe-SOD activities account for 7–15% of the total activity in our conditions suggesting that these activities may have played a less important role than Cu, Zn-SOD. Though difficult to detect in gel, we quantified a decrease in the percentage of Mn- and Fe-SOD activities under Cd treatment conditions (Suppl. Fig. S4.2.9C). To our knowledge, no data exist on SOD activity in seedlings germinated and grown under Cd treatment conditions, while the various outcomes of plants and seedlings grown under normal conditions, which were then treated with Cd, have been the subject of considerable study. Total SOD activity has been observed to increase in wheat and sunflower leaves, as well as in Arabidopsis seedlings (Laspina *et al*., 2005; DalCorso *et al*., 2008; Song *et*

al., 2012) and to decrease in wheat and bean (Cardinaels *et al*., 1984 ;Milone *et al*., 2003), with no significant effect found in Arabidopsis seedlings (Smeets *et al*., 2009; Cuypers *et al*., 2011). Different SOD isoforms have also been observed to behave differentially, with Cu,Zn-SOD activity decreasing under Cd stress conditions in pea leaves and roots and Arabidopsis leaves (Sandalio *et al*., 2001; Rodríguez-Serrano *et al*., 2006; Gupta *et al*., 2017), and increasing in Arabidopsis seedlings and soybean roots (Pérez-Chaca *et al*., 2014; Abozeid *et al*., 2017). Mn-SOD activity appears to be less affected by Cd in pea leaves (Sandalio *et al*., 2001), with no changes observed in pea roots or Arabidopsis seedlings (Rodríguez-Serrano *et al*., 2006; Abozeid *et al*., 2017). Fe-SOD activity, which also behaves differentially depending on the age, species and strength of the treatment, increases in Cd-treated Arabidopsis seedlings (Abozeid *et al*., 2017) while it decreases and remains unaffected in pea leaves and roots, respectively (Sandalio *et al*., 2001; Rodríguez-Serrano *et al*., 2006).

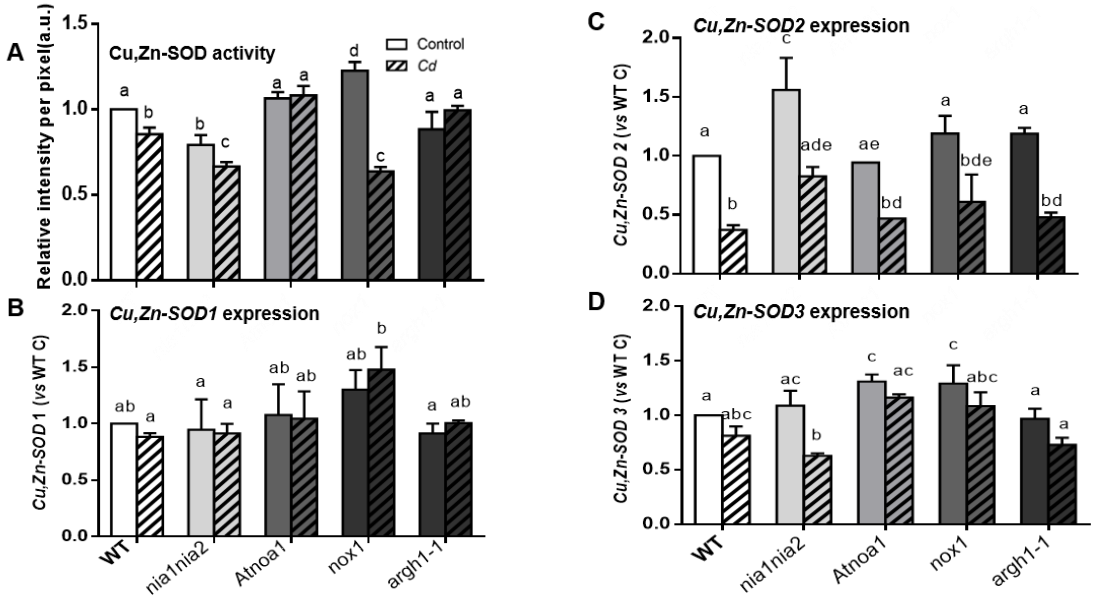
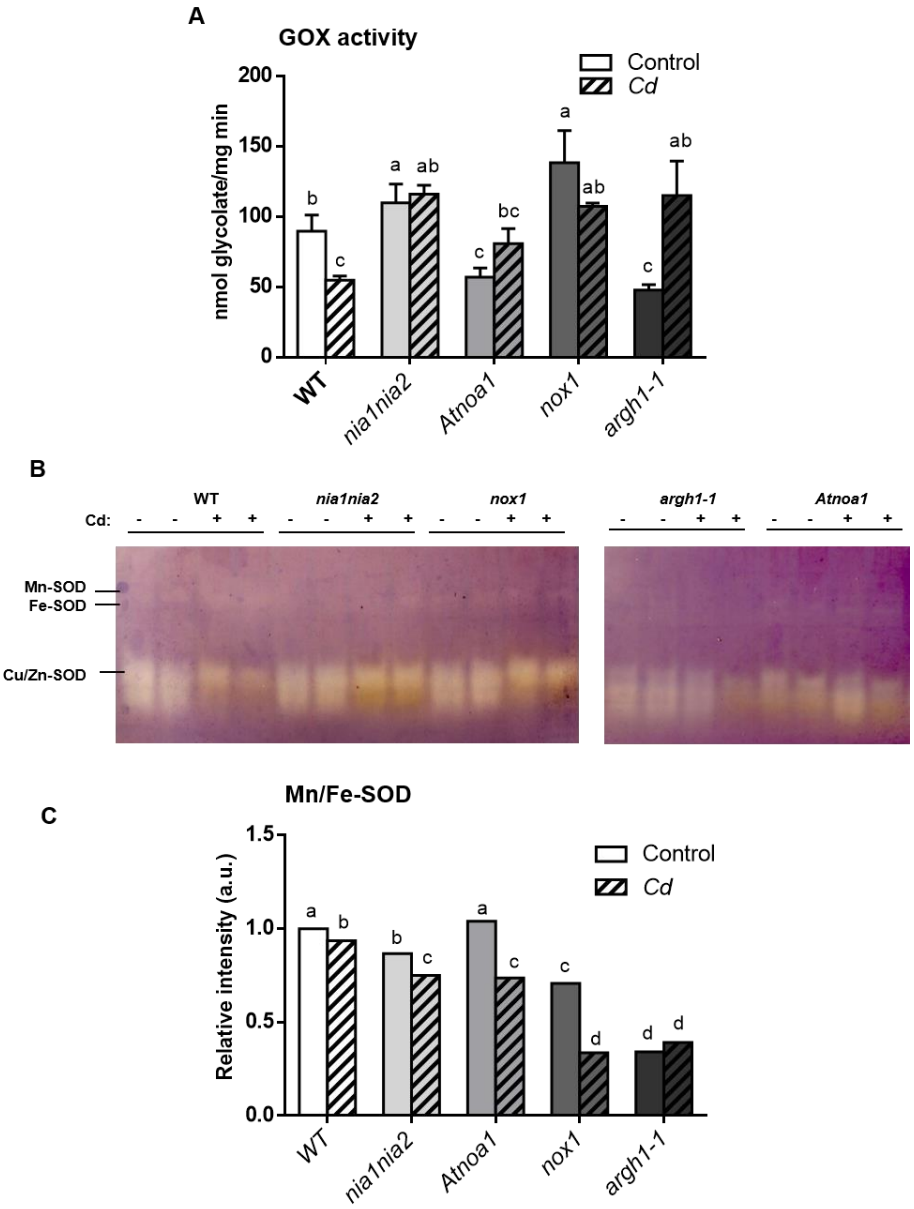


Fig. 4.2.7. Effect of cadmium on antioxidant enzymes in Arabidopsis seedlings. (A) Quantification by Image J software of Cu,Zn-SOD activity. Total SOD activity was carried out by native gels with seedlings grown or not (control) under Cd conditions (shown in Suppl. Fig. S4.2.9B). (B, C, D) Analysis of Cu,Zn-SOD1, Cu,Zn-SOD2 and Cu,Zn-SOD3 gene expression in seedlings under control and Cd treatment. Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P < 0.05$).

Glycolate oxidase (GOX), a key photorespiration enzyme associated with peroxisomes, has been reported to contribute to Cd-dependent ROS production in Arabidopsis and pea plants after long-term treatment (Romero-Puertas *et al*., 1999; Gupta *et al*., 2017) and also in soybean (Pérez-Chaca *et al*., 2014). We therefore analysed GOX activity, which was found to decrease in WT seedlings following Cd treatment. No significant changes were observed in NO-related mutants, except for *argh1-1*, which recorded an increase in GOX activity, suggesting that GOX is regulated differently in this mutant (Suppl. Fig. S4.2.9).



Suppl. Fig. S4.2.9. Effect of cadmium on GOX and Mn-SOD activities in Arabidopsis seedlings. GOX activity in seedlings grown or not (Control) in Cd conditions (A). (B) Representative image of superoxide dismutases (Mn-SOD, Fe SOD, Cu/Zn SOD). and quantification (C) in seedlings grown or not (Control) in Cd conditions by Image J. Proteins (45 µg) were subjected to native polyacrylamide gel electrophoresis (6% acrylamide) and isoenzymes were visualized by a photochemical method. Data represent mean ± standard error (error bars) of at least three independent experiments. Different letters denote significant differences determined by Student-Newman-Keuls test ($P<0.05$).

4.2.3.3. Cadmium uptake in NO mutants

Cadmium is known to be able to enter the food chain through plant roots and then to translocate to above-ground tissues (Shahid *et al*., 2016). After using dithizone to precipitate Cd, similar levels of the metal were accumulated in seedling leaves for all genotypes, except for *nia1 nia2*, which accumulated two-fold more than WT seedlings (Fig. 4.2.8A and B). Few Cd

precipitates were observed in *nia1 nia2* seedling roots as compared to WT seedlings (Fig. 4.2.8A and C), suggesting that translocation of Cd is induced in this mutant, a process that requires further investigation. Half of the Cd-dependent precipitates in the WT seedling roots were found in *nox1* mutant seedling roots, while in *argh1-1* accumulated more Cd (Fig. 4.2.8A and B). The entry of Cd into the root is well known to be partly due to the IRT1 iron transporter (Clemens *et al*., 2013) whose gene expression is considerably inhibited by Cd treatment, probably in order to prevent metal accumulation (Connolly, 2002; Connolly *et al*., 2003). Though down-regulated in Cd-treated WT seedlings, the IRT1 iron transporter was up-regulated in *nox1* mutants which had higher levels of NO (Fig. 4.2.8D). Although IRT1 was not upregulated in *argh1-1*, IRT1 gene expression was significantly higher in this mutant than in WT seedlings under control conditions, which is in line with the higher level of Cd accumulation observed in roots (Fig. 4.2.8C and D). Lower expression of IRT1 in *nox1* mutant is in accordance with the lower Cd accumulation found in *nox1* seedling roots. This result pointed to the possibility that other mechanism, beyond NO, are involved in the regulation of IRT1 in *nox1* mutants under control conditions. On the other hand, IRT1 was not repressed in *nia1 nia2* or *Atnoa1*, suggesting that NR and NOA1 play a role in its regulation, a mechanism which requires further investigation (Fig. 4.2.8D). It has been shown that down-regulation of IRT1 intensified in plants co-treated with Cd and a NOS inhibitor (Nω-Nitro-L-arginine methyl ester hydrochloride; L-NAME), suggesting that NO is involved in the up-regulation of IRT1 under Cd stress (Besson-Bard *et al*., 2009). Therefore, NO needs to be tightly controlled in plant responses to Cd, as endogenous NO may increase metal uptake. Thus, overexpression of *Nicotiana tabacum* haemoglobin (NtHb1) in both tobacco and Arabidopsis plants, which down-regulate NO in response to Cd, was recently found to increase Cd tolerance, partly due to lower metal accumulation (Lee and Hwang, 2015; Bahmani *et al*., 2019). Furthermore, HY1 has been shown to increase Cd tolerance in Arabidopsis plants by reducing NO production and enhancing Fe homeostasis (Han *et al*., 2014).

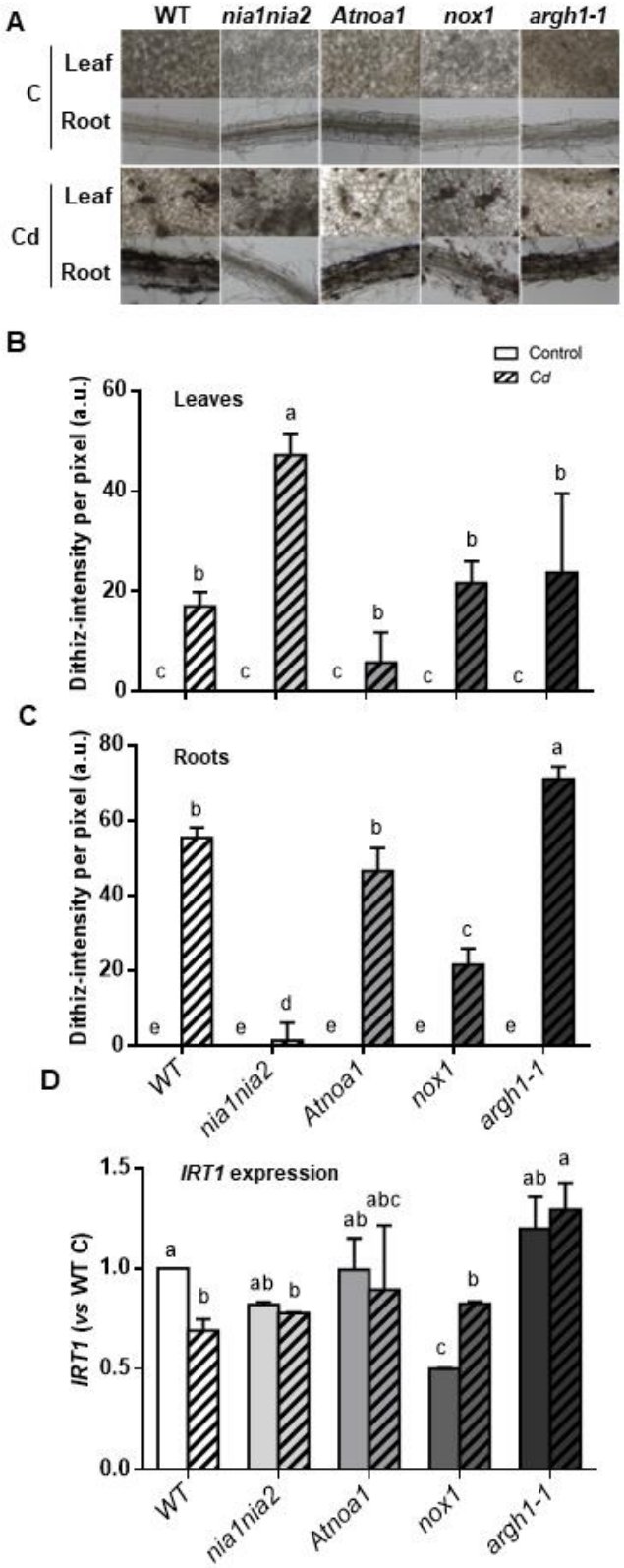
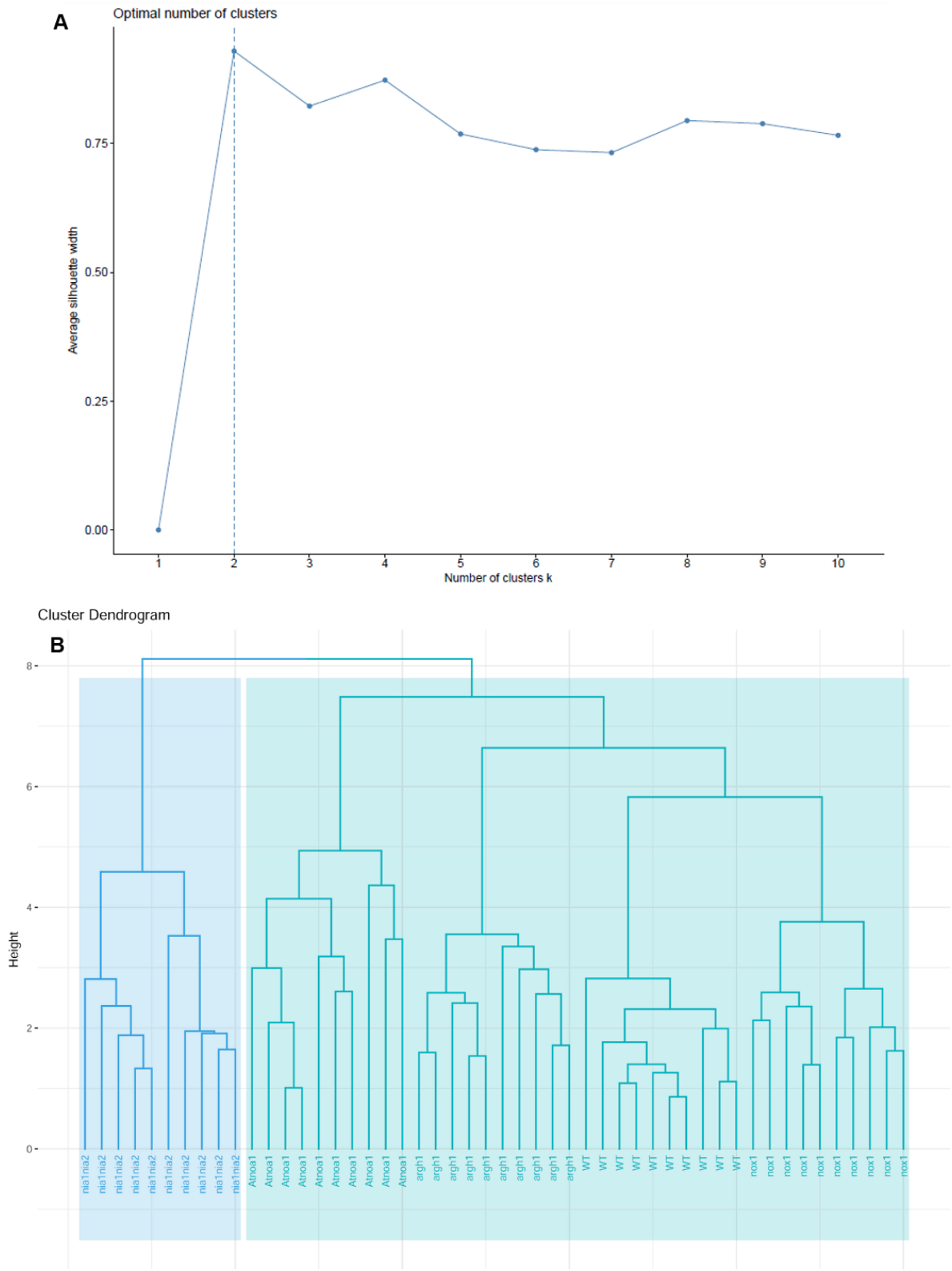


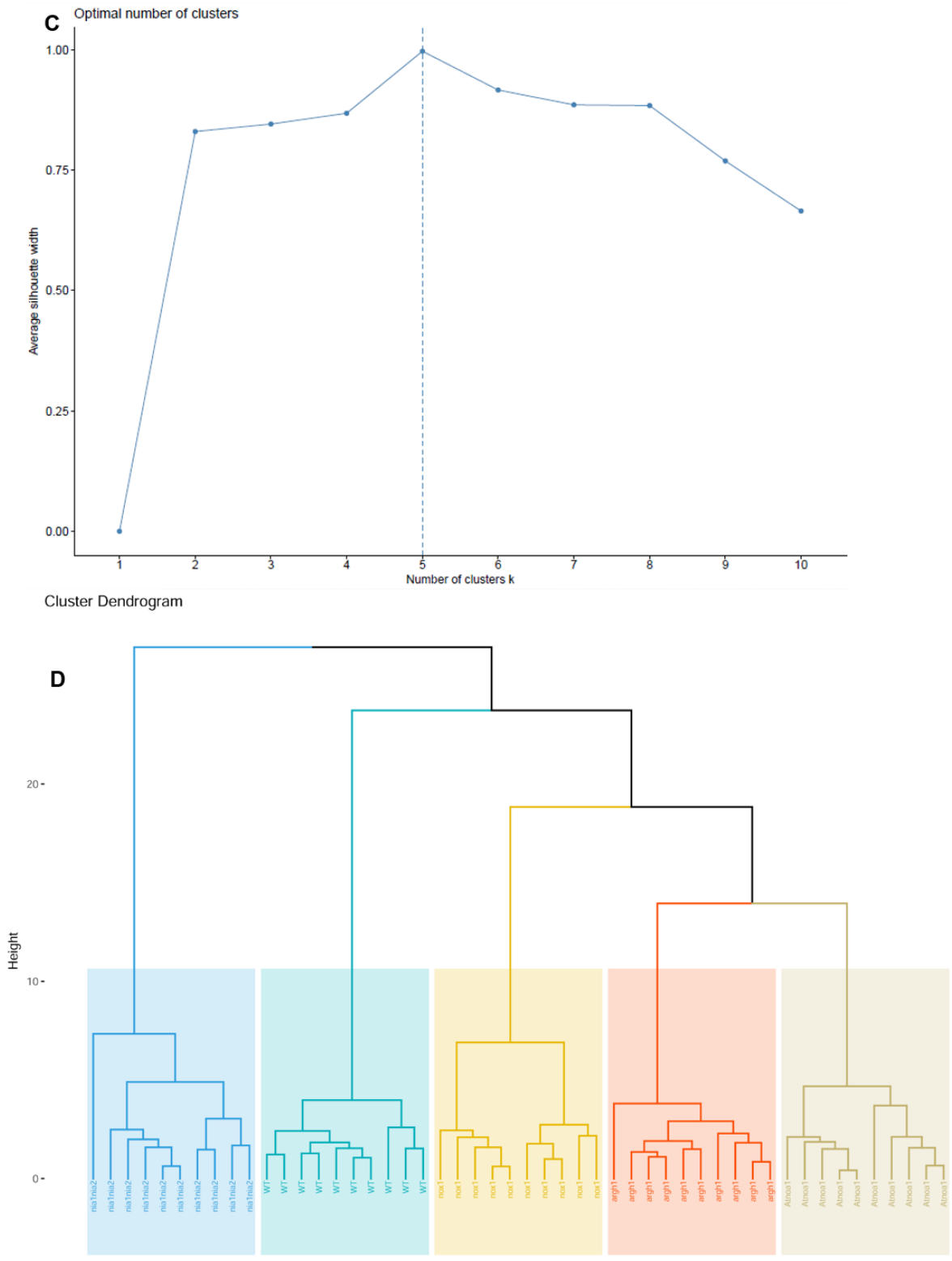
Fig.4.2.8. Cd accumulation in Arabidopsis seedlings. Representative image of Cd-dependent precipitates in seedling leaves and roots (A) and quantification of the images by the Image J software (B, C). *IRT1* gene expression (D) in seedlings grown or not (control) under Cd conditions. Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P < 0.05$).

4.2.3.4. Differential responses of nitric oxide-related mutants and WT seedlings to Cadmium stress

To gain more insight into WT seedling and NO-related mutant responses to Cd, we performed a clustering analysis of all the parameters analysed under control and Cd treatment conditions. We obtained two clusters under normal conditions: one cluster contained WT seedlings and NO-related mutants, except for *nia1 nia2*, which was in the other cluster (Suppl. Figs. S4.2.10A and S4.2.10B). In the first cluster obtained under control conditions, the *Atnoa1* mutant exhibited the most differential behaviour. However, under Cd treatment conditions, we obtained five clusters being each background in its own cluster. Comparing the clusters obtained under Cd conditions, *nia1 nia2* again exhibit the most differential behaviour and *nox1* behave more like WT seedlings (Suppl. Figs. S4.2.10C and S4.2.10D). We also performed a correlation analysis of all the parameters studied. We found that Cd treatment of WT seedlings closely correlated with oxidative damage to proteins and lipids, ROS and $ONOO^-$ production in seedling roots and an expected accumulation of Cd in seedling roots and leaves (Fig. 4.2.9A). By contrast, we observed a negative correlation of Cd with seedling biomass, ROS production in seedling leaves and in the whole seedlings. GOX and SOD activity, as well as CAT and APX, also correlated negatively with Cd. Finally, Cd exhibited a negative correlation with *IRT1* gene expression (Fig. 4.2.9A). The main difference between the *nia1 nia2* mutant and WT seedlings was the oxidative damage to lipids which were unaffected in the mutant and being this behaviour reversed by NO donors. GOX activity was positively correlated with Cd in *nia1 nia2* mutant. However, H_2O_2 and RNS production in seedling roots in *nia1 nia2* were characterised by a trend opposite to that in WT seedlings, in which the regulation of CAT activity in response to Cd was also altered (Fig. 4.2.9B). $O_2^{\cdot -}$ and RNS remained unchanged in *Atnoa1* mutants in response to Cd stress, resulting in a negative correlation between Cd and protein damage (Fig. 4.2.9C). ROS and RNS production was altered in *nox1* mutants in response to Cd as compared to WT seedlings, while nitration increased significantly. Unlike in WT seedlings, IRT1 expression correlated positively with Cd in *nox1* mutants (Fig. 4.2.9D). The *argh1-1* seedlings behaved differently from WT seedling roots with respect to ROS and RNS production, while SOD activity was not so affected (Fig. 4.2.9E).



Suppl. Fig. S4.2.10B. Analysis of the different parameters by bioinformatics. Cluster number estimation using silhouette method under control (A) and cadmium conditions (C). Hierarchical clustering using Ward distance in WT and nia1 nia2, Atnoa1, *nox*1 and argh1.1 mutants under control (B) and cadmium conditions (D).



Suppl. Fig. S4.2.9. **Analysis of the different parameters by bioinformatics.** Cluster number estimation using silhouette method under control (A) and cadmium conditions (C). Hierarchical clustering using Ward distance in WT and *nia1 nia2*, *Atnoa1*, *nox1* and *argh1.1* mutants under control (B) and cadmium conditions (D).

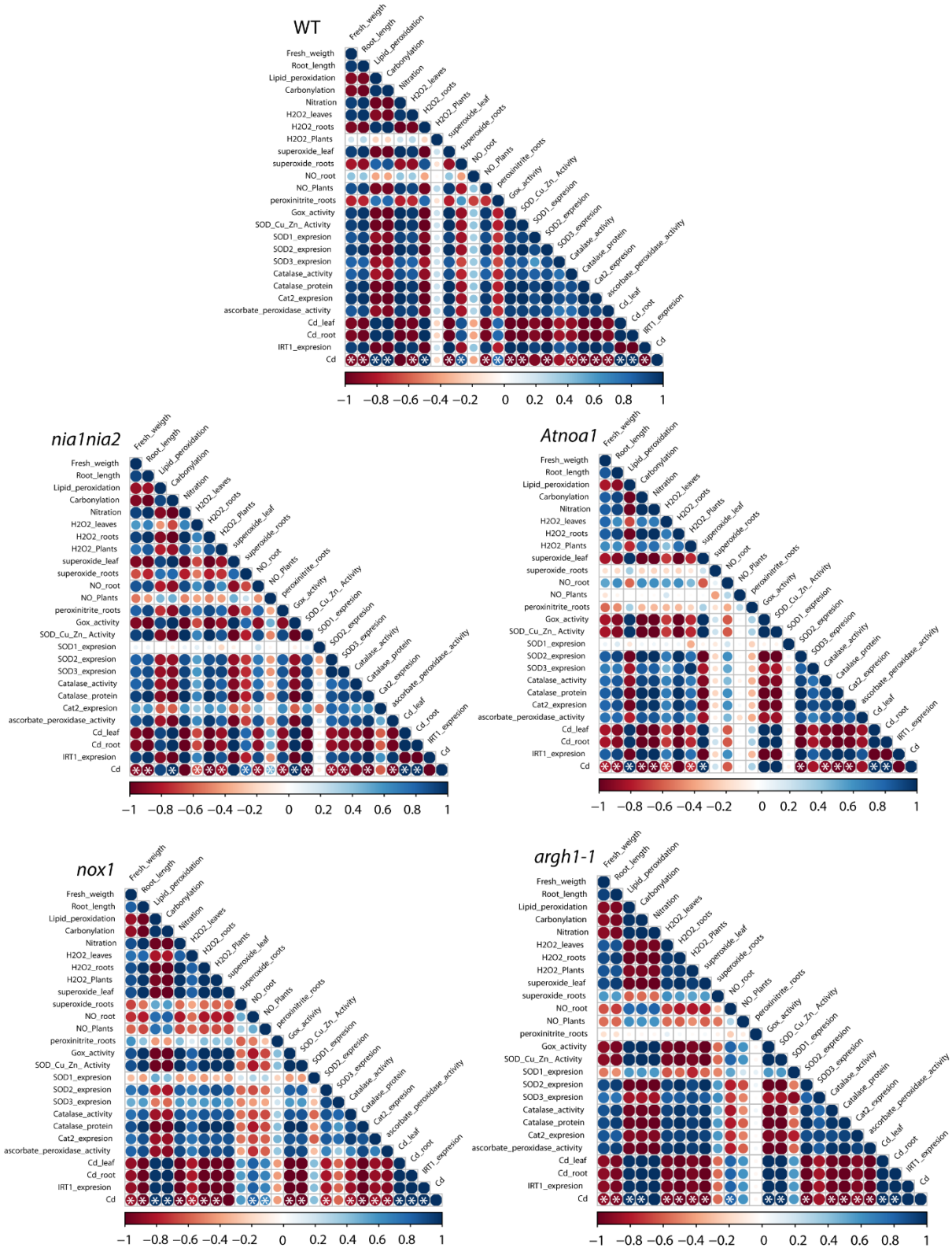


Fig. 4.2.9. Analysis of the data obtained by bioinformatics. Coefficient of correlation between variables in WT (A), *nia1 nia2* (B), *Atnoa1* (C), *nox1* (D) and *argh1-1* (E) was calculated as the Pearson's Product Moment Coefficient previous standard normalization of the variables using the corrplot R package.

4.2.4. Concluding remarks

We observed an increase in ROS (H_2O_2 and $O_2^{\cdot-}$) and RNS ($ONOO^-$) in Cd-treated WT seedling roots as compared to control conditions, which, in addition to inhibiting the antioxidant system, induced oxidative stress as demonstrated by an increase in carbonyl groups and lipid peroxidation (Fig. 4.2.10); both these markers correlated positively with Cd (Fig. 4.2.9A). This oxidative stress probably led to a reduction in the root length and fresh weight of Cd-treated seedlings, parameters that negatively correlated with the metal (Fig. 4.2.9A). Each NO-related mutant and possibly the source of NO affected respond differently to Cd, not only with respect to NO production but also in relation to ROS metabolism. The increase observed in ROS production in WT seedling roots grown under Cd conditions did not occur in *nox1* and *argh1-1* mutants. Nevertheless, the increased levels of NO in *argh1-1* and *nox1* mutants and the decline in the antioxidant system appear sufficient to induce oxidative damage, leading to a decrease in seedling growth. The decrease in the level of NO in response to Cd in *argh1-1* and *nox1* mutants, caused by the incubation with the NO production inhibitor AG, mitigates the increase in lipid peroxidation in these mutants, correlating NO with oxidative damage (Fig. 4.2.10). The *Atnoa1* mutants did not vary ROS and RNS production in seedling roots following Cd treatment. However, these smaller seedlings appeared to be subject to oxidative stress under control conditions, as total H_2O_2 content, lipid peroxidation, carbonyl group and nitration levels were higher than in WT seedlings, suggesting that *Atnoa1* mutants may not be appropriate option for future research in this field. Nevertheless, given that ascorbate peroxidase (APX) in *Atnoa1* mutant grown under Cd conditions was unaffected, NOA1 could provide APX protection leading to lower protein nitro-oxidative damage. Therefore, under Cd conditions, nitration declines and carbonyl groups remain at levels similar to those under control conditions in *Atnoa1* mutants (Fig. 4.2.9, Fig. 4.2.10). The mutant least affected by Cd was *nia1 nia2*. In this mutant, NO and $ONOO^-$ production fell in seedling roots with the treatment, while total H_2O_2 content and lipid peroxidation remained at levels similar to those under control conditions. Therefore, in *nia1 nia2* mutant a lower decrease in seedling fresh weight was observed. Catalase (CAT) in the *nia1 nia2* mutant was not down-regulated by Cd treatment. The protection of CAT together with the reduction in Cd accumulation in seedling roots may be responsible for the phenotype observed in *nia1 nia2*. An excess of endogenous NO appears to be detrimental to *nox1* and *argh1-1* mutants under Cd stress conditions, while the maintenance of the antioxidant system is crucial in order to prevent oxidative damage. A finely tuned balance between ROS and NO/RNS therefore appears to be necessary to regulate responses to Cd-treated seedlings.

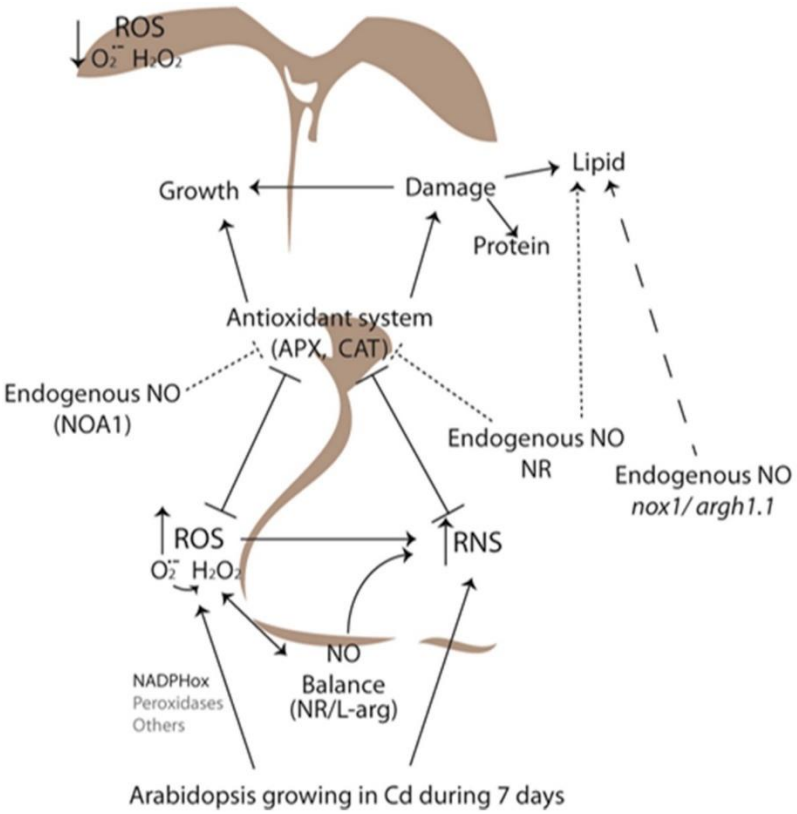


Fig 4.2.10. Scheme for NO functions in Arabidopsis seedlings expose to Cd. Arabidopsis seedlings grown in Cd showed an increase in ROS (H_2O_2 and $O_2^{\cdot-}$) and RNS production in WT seedling-roots while a decrease in ROS is observed in seedling-leaves. The increase in ROS seedling-roots is accompanied by an inhibition of the antioxidant system, such as catalase (CAT) and ascorbate peroxidase (APX). Nitrate reductase (NR) appears to be involved in CAT inhibition while NOA1 in the inhibition of APX. The excess of ROS in roots and the imbalance in the antioxidant system induces an oxidative damage in both, proteins and lipids being this one due in part to NR-NO dependent. On the other hand, an increase of L-arg-dependent endogenous NO also induces an increase in lipid peroxidation. Oxidative damage observed lead to a decrease in seedling fitness.

4.2.5. Acknowledgements

This study was co-funded by the ERDF and the Science, Innovation and University Ministry (BIO2015-67657-P and PGC2018-098372). L.C. T-C was supported by an FPU fellowship from the Spanish Ministry of Education, Culture and Sports. CLMS analyses were carried out at the EEZ-CSIC Technical Services facility. HK2-Green was kindly provided by Dr. Dan Yang. We also wish to thank Angela Tate and Michael O'Shea for proofreading the English manuscript.

4.2.6. References

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Chapter 4.3

4.3. Nitric oxide is essential for peroxule production and peroxisome proliferation in response to Cadmium stress

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Running title:

NO function in peroxisome dynamic and metabolism

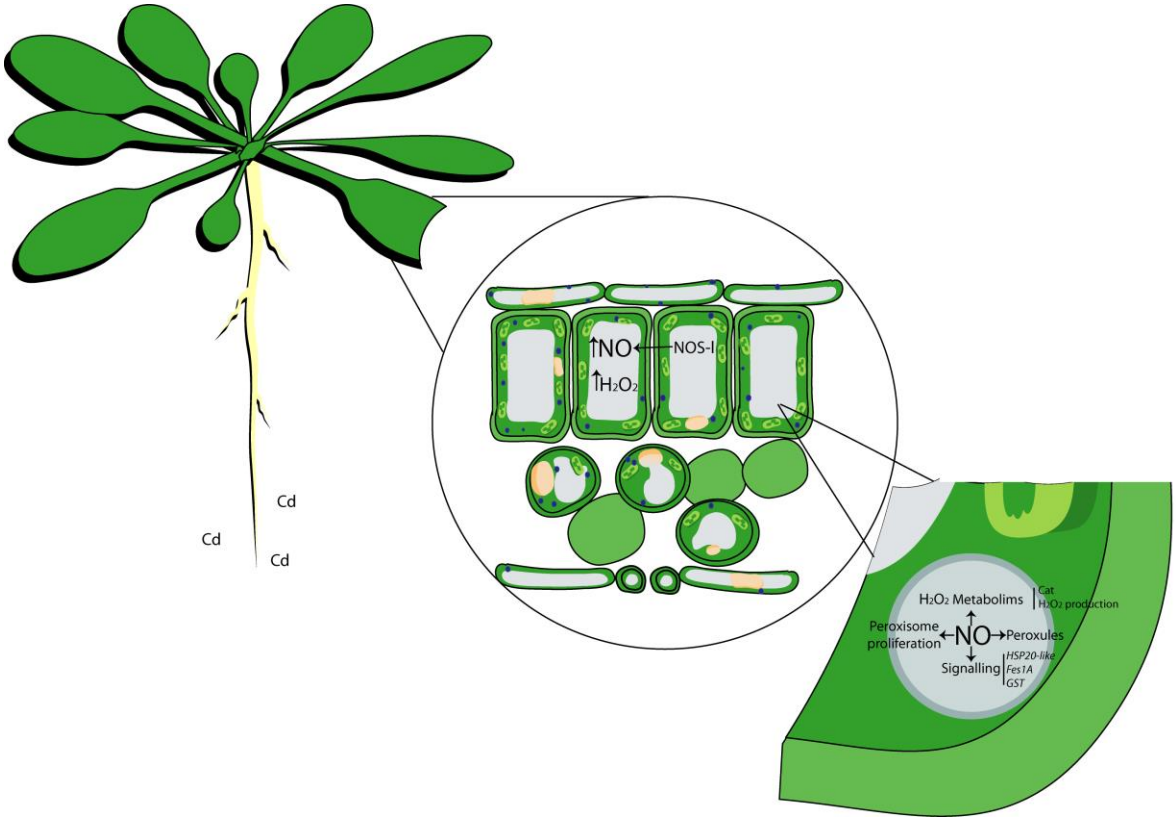
ABSTRACT

Nitric oxide (NO) has been shown to be produced in peroxisomes, as well as its derivatives although their impact on the organelles remains elusive. To determine whether NO regulates peroxisomal dynamics under Cd stress, we generated double and triple NO-related mutants expressing CFP-SKL (*nox1*x*px-ck* and nia1 *nia2*x*px-ck*), which enabled us to follow the organelles by confocal microscopy. We have shown that NO production induced at the early plant response to Cd stress is involved in peroxules production, which is highly compromised in *nia1 nia2* mutants, with lower NO levels. Peroxisomal proliferation after Cd treatment analysed by electron microscopy and by the accumulation of the peroxisomal marker PEX14, does not occur in *nia1 nia2* mutants, phenotype that is recovered by exogenous NO. Ultrastructure analysis of peroxisomes showed that the number of the organelles in cells and oxidative metabolism is altered in both NO-related mutants. Furthermore, the pattern of oxidation and *S*-nitrosylation of CAT, one of the main antioxidant enzymes in the organelle, is altered under control and Cd conditions in NO-related mutants. Finally, we have analysed peroxisomal-dependent signalling, which is also affected in NO-related mutants. Our results show that NO must be tightly regulated for an optimal peroxisomal function and signalling.

Key words: cadmium; carbonylation; catalase; nitric oxide; peroxisomes; peroxules; signalling; *S*-nitrosylation

Under review

GRAPHICAL ABSTRAC



4.3.1. Introduction

Peroxisomes are single-membrane bounded organelles initially considered as a sink of reactive oxygen species produced by normal metabolism in the cell (Hu *et al*., 2012). Peroxisomes are dynamic organelles that adapt their morphology, number and metabolism depending on the tissue, organ, and the nutritional state of the plant. In plants, peroxisomes play a crucial role in different biochemical pathways, including photorespiration, fatty acid β-oxidation, glyoxylate metabolism, amino-acid catabolism, polyamine oxidation and hormone biosynthesis (Reumann and Bartel, 2016). In fact, the variety of metabolic processes of peroxisomes in plants and their plasticity is remarkable, and recent studies carried out thanks to "-omic" techniques are allowing us to discover new functions of these organelles (Reumann and Bartel, 2016). In addition, peroxisomes are a key source of reactive oxygen and nitrogen species (ROS/RNS) and contain an extensive combination of antioxidant defences that regulate their accumulation (Sandalio and Romero-Puertas, 2015). Overproduction of ROS and RNS can cause a severe oxidative and nitrosative state of the cell, although these molecules can also act as signals regulating developmental processes and stress responses (Marinho *et al*., 2014; Mittler, 2017; Castillo *et al*., 2018). Peroxisomes can also play an important role in cellular redox homeostasis, which is, in turn, a key element in the regulation of cell metabolic pathways (Foyer and Noctor, 2003; Yun *et al*., 2012). Moreover, it has been demonstrated that ROS can regulate fast changes in the metabolism and dynamics of peroxisomes after environmental cues (Rodríguez-Serrano *et al*., 2009; Sinclair *et al*., 2009; Hu *et al*., 2012; Kao *et al*., 2018), suggesting that peroxisomes may function as an important decision-making platform in the cell (Sandalio and Romero-Puertas, 2015).

In particular, we have previously shown that cadmium (Cd) which is a toxic heavy metal causing important environmental and health concerns worldwide; produces time course-dependent changes in peroxisomal dynamics. Specifically, initial peroxisomal membrane extensions (the so-called peroxules) have been observed, followed by peroxisome proliferation, and finally the number of peroxisomes return to the number under control conditions whilst increasing the speed of movement (Rodríguez-Serrano *et al*., 2016). These changes were regulated mainly by NADPH oxidase (C and F)-related ROS production and the peroxin 11a (PEX11a) is essential for peroxule production (Rodríguez-Serrano *et al*., 2016). Results in mutants affected in PEX11a suggested that peroxules might be involved in regulating ROS accumulation and in ROS dependent gene expression in response to stress. Therefore, these results demonstrate that PEX11a and peroxule formation play a key role in regulating stress perception and fast cell responses to environmental cues (Rodríguez-Serrano *et al*., 2016; Fransen *et al*., 2017).

Nitric oxide (NO) is a free radical that functions as a ubiquitous inter- and intra-cellular signalling molecule involved in the regulation of plant defence and response to stress and plant development (Yu *et al*., 2014; Sanz *et al*., 2015; León *et al*., 2016). In recent years, the ability of NO to regulate different processes including hormonal metabolism and signalling through direct protein post-translational modifications has been shown (PTMs), such as *S*-nitrosylation (so called nitrosation) and nitration that may affect protein function, stability and localization (Martínez-Ruiz *et al*., 2011; Kovacs and Lindermayr, 2013; Romero-Puertas and Sandalio, 2016). Additionally, the function of NO is closely linked to reactive oxygen species (ROS), because it not only reacts rapidly with them but is also able to regulate ROS production and removal through PTMs of the enzymatic sources and antioxidant systems, respectively (Lindermayr and Durner, 2015; Romero-Puertas and Sandalio, 2016). In particular, NO is able to regulate peroxisomal proteins, antioxidants and ROS-producing, under normal and stress conditions such as Cd treatment (Gupta and Sandalio, 2012; Ortega-Galisteo *et al*., 2012; Sandalio *et al*., 2019).

Recently, NO has been found to be involved in the plant response to Cd and although a general protection against stress has been shown after exogenous NO application, the role of NO produced by the plant is not so clear (Romero-Puertas *et al*., 2019). Although an overall increase in endogenous NO production was observed in plant responses after short-term treatments, this tendency changes after long-term treatments (Terrón-Camero *et al*., 2019). In recent years, it has been suggested that NO may be related to some functions in plant response to Cd. Specifically, transcriptomic analysis modulating NO levels during plant response to Cd stress indicated that NO may be involved in the regulation of root growth, nitrogen assimilation, iron homeostasis and metabolic proteolysis (Besson-Bard *et al*., 2009; Romero-Puertas *et al*., 2019). However, the role of nitric oxide in metabolism and peroxisomal dynamics in plant response to Cd stress is unknown.

We have analysed peroxisome metabolism and dynamics in plant response to Cd stress by biochemical (using NO donors and scavengers) and genetic approaches. Therefore, two previously characterized mutants were used in this study: one affected in NO production as it is impaired in nitrate reductases (NR1/NIA1and NR2/NIA2; nia1 nia2), with a previously suggested role in NO biosynthesis (Yamasaki and Sakihama, 2000; Rockel *et al*., 2002; Desikan *et al*., 2002; Guo *et al*., 2003; Modolo *et al*., 2005; Moreau *et al*., 2008), and one indirect NO overproducer, impaired in a chloroplast phosphoenolpyruvate/phosphate translocator (*nox*1/*cue1*; Streatfield *et al*., 1999; He *et al*., 2004). The results obtained suggest that NO regulates peroxule production and peroxisome proliferation in cell response to Cd stress. The number of peroxisomes and peroxisomal ROS-related metabolism are also affected by different levels of NO in the plant.

4.3.2. Materials and methods

4.3.2.1. Plant material and growth conditions

Arabidopsis thaliana (Col-0) was the genetic background used in this study. Arabidopsis seeds (WT, *px-ck*, *nia1 nia2*, *nia1 nia2*x*px-ck*, *nox*1 and *nox1*x*px-ck*) were surface sterilized and stratified for 48 hours at 4ºC and then sown on Hoagland solid medium (0,5x) pH 5.6 (Hoagland and Arnon, 1950). The seeds were grown at 22ºC, 100 µE of irradiance, 60-65% relative humidity and 16/8 light/dark light conditions for 14 d. The seedlings were then transferred to Petri dishes with 9 mL of liquid Hoagland (0.5x) medium leaving roots of 15 seedlings submerged for 24 h, like in hydroponic conditions. Subsequently, initial Hoagland (0,5x) medium was substituted by 9 mL of liquid Hoagland medium containing 100 µM $CdCl_2$ and seedlings were harvested at different time-points (30 min, 1 h and 3 h), weighed and processed or frozen when necessary. *S*-nitrosoglutathione (GSNO; 1 mM and 0,25 mM) as a NO donor and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO; 0.5 mM), as a NO scavenger, were freshly prepared and added to the medium when indicated.

4.3.2.2. Generation of double and triple mutants with altered NO level and peroxisomal-targeted CFP

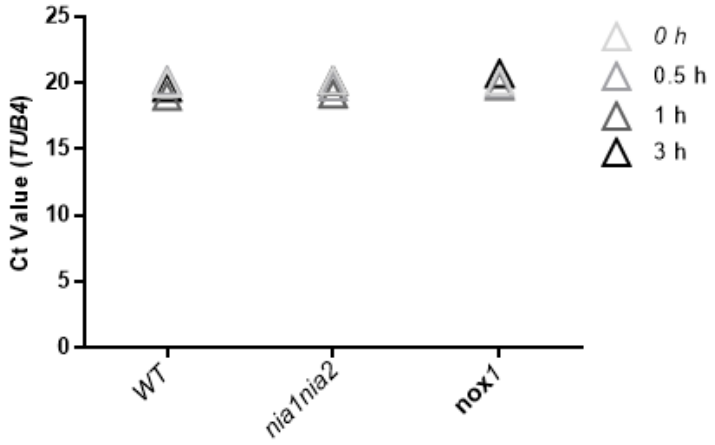
Emasculated flowers from *nia1 nia2* (Wilkinson and Crawford, 1993) and *nox1* (He *et al.*, 2004) were crossed with pollen from *px-ck*, containing a peroxisomal-targeted cyan fluorescent protein (CFP; Nelson *et al*., 2007). Crosses with emasculated flowers from *px-ck* and pollen from *nia1 nia2* or *nox1*, were also done with similar results. The F1 plants were self-pollinated, and double (*nox1*x*px-ck*) and triple mutants (*nia1 nia2*x*px-ck*) were selected from F2 and F3 using fluorescence microscopy and genotyped by PCR. Primers for *nia1 nia2* selection have been described before (Lozano-Juste and Leon, 2010). The *CUE1* gene is deleted in *nox1* mutant and homozygous plants were selected as described elsewhere (He *et al.*, 2004).

4.3.2.3. Confocal microscopy analyses

All the leaves analyzed in the experiments belonged to plants grown under the same conditions and were the same age. Arabidopsis leaves were sliced and mounted in PBS/glycerol 30:70% (v/v) as described elsewhere (Rodríguez-Serrano *et al.*, 2016). The abaxial sections were examined using a confocal laser scanning microscope model TCS SP5 (Leica Microsystems, Wetzlar, Germany). A minimum of seven confocal images were collected from one leaf of each plant, with at least five plants being used per experiment. Five independent experiments were carried out. At least 125 images were analyzed per treatment, meaning that thousands of peroxisomes were examined. The videos were generated by sequential images, taking 15 frames in the x, y, and t dimensions and the number of peroxisomes and peroxules were analyzed using Leica Lite software (Leica Microsystems; Rodríguez-Serrano *et al.*, 2016).

4.3.2.4. Gene expression by RT-qPCR

Total RNA was isolated by Trizol reagent (Invitrogen) and DNase was used according to the manufacturer's protocol (Ambion DNA free). 1 µg RNA was reverse transcribed with 5x primer scrip RT master mix (Takara) as described elsewhere (Rodríguez-Serrano *et al.*, 2016). Quantitative real-time PCR was performed on an iCycler iQ5 (Bio-Rad, Hercules, CA), using TB Green Premix Ex Taq (Takara). Amplification efficiency was calculated using the formula E = [10 (1/a) -1] × 100, where "a" is the slope of the standard curve. The relative expression of each gene was normalized to that of *TUB4*, and the results were analyzed using the method described by (Pfaffl, 2001). The *TUB4* gene was selected for normalization by the GrayNorm algorithm (Remans *et al.*, 2014) from five candidate reference genes as described before (Terrón-Camero *et al*., 2019). The stability of *TUB4* was checked under our conditions in all backgrounds (Suppl. Fig. S1). The primers used in this study are described in Suppl. Tables S1 and S2.



Suppl. Fig. S4.3.1. RT-qPCR Ct values of *TUB4* gene in our condition and all backgrounds analysed. Mean values.

4.3.2.5. Enzymatic and Western blot analyses

Whole seedlings were homogenized as described elsewhere (Rodríguez-Serrano *et al.*, 2016). Proteins were quantified with Bradford Protein Assay (Bio-Rad) and BSA (bovine serum albumin) was used in the standard curve. Extracts were used to measure catalase (CAT; EC 1.11.1.6) activity as described elsewhere (Aebi, 1984). For the detection of carbonylated catalase, immunoprecipitation of derivatized proteins with dinitrophenyl hydrazine (DNPH) using an anti-DNPH (1:40,000 dilution) and subsequent immune-detection with anti-catalase (1:5,000; Agrisera; Sigma;) was performed (Romero-Puertas *et al*., 2002). The Biotin-switch method was used to detect *S*-nitrosylated catalase (Romero-Puertas *et al.*, 2007). The anti-biotin antibody (1:10,000 Sigma-Aldrich) was used to immunoprecipitate *S*-nitrosylated proteins followed by an identification of CAT with anti-catalase (1:5,000; Agrisera). Anti-PEX14 (1:10,000 Agrisera) was used as a marker of peroxisomal accumulation (Calero-Muñoz *et al.*, 2019).

4.3.2.6. H_2O_2 and NO detection

To detect nitric oxide, the seedling-roots were incubated with 10 μM 4,5-diaminofluorescein diacetate (DAF-2 DA) as described before (Terrón-Camero *et al*., 2018). Specificity of the reaction was checked by pre-incubating samples with cPTIO (500 μM), an NO scavenger. Aminoguanidine (AG; 0.5 mM) and tungstate (TUNG; 0.5 mM) were used as inhibitors of NOS-like and nitrate reductase activities, respectively, to identify the possible NO source. GSNO (1 mM) was used as a NO donor when described. Hydrogen peroxide (H_2O_2) was measured by fluorimetry using homovanillic acid (excitation of 315 nm; emission of 425 nm) and horseradish peroxidase in 50 mM HEPES pH 7.5 (Romero-Puertas *et al.*, 2004). Standard curve of commercial H_2O_2 was used to quantify the samples. For NO detection, the seedling extracts were incubated with 20 μM DAF-2 for 2h at 37ºC (Excitation: 495, Emission: 515; Nakatsubo *et al.* 1998).

4.3.2.7. Cytochemical identification of peroxisomes

Cytochemical localization of peroxisomes was carried out as described before (Calero-Muñoz *et al.*, 2019). Leaves from the different genotypes were of the same age (15 days) and *nox1* x *px-ck* leaves were slightly smaller than *px-ck* at this stage. Briefly, Arabidopsis leaves (from *px-ck; nia1 nia2* x *px-ck* and *nox1* x *px-ck* seedlings) were cut into pieces of approximately 1 mm^2 and initially fixed with 0.5% glutaraldehyde (v/v), prepared in 50 mM potassium phosphate buffer, pH 6.8, for 2.5 hours at RT. Pieces were then washed with the same buffer. The samples were incubated in DAB solution (2 mg/ml) prepared in 50 mM Tris-HCl, pH 9.0 for 1.5 hours. After that, samples were incubated in a freshly prepared solution of DAB and 0.02% H_2O_2 at 37 °C for 3 h. Samples were then washed with 50% potassium phosphate buffer, pH 6.8, and stained with 1% (w/v) OsO_4. The samples were then dehydrated in a stepped ethanol series from 30 to 100%. Once the samples had been embedded in Spurr resin, semithin and ultrathin sections were obtained and contrasted for the analysis of the structure and ultrastructure by a light or a Transmission Electron Microscope (Zeiss ME 10C). The images were analyzed by Image-J (Fiji).

4.3.2.8. Statistical analyses

Mean values in the quantitative experiments described above were obtained from at least three independent experiments with no less than three independent samples each. Statistical analyses were performed using one or two-way ANOVA test when necessary; followed by a Student´s t-test (p-value < 0.05) or a Tukey's multiple comparison tests (p-value < 0.05), respectively. Analyses were carried out by using IBM SPPS Statistic 24 and GraphPad Prism 6.

Error bars representing standard error (SEM) or whiskers (maximum and minimum) are shown in the figures.

4.3.3. Results

4.3.3.1. Peroxule production in response to Cd in NO-related mutants

It has recently been shown that Cd induces peroxisomal membrane extensions, so-called peroxules, very soon after treatment followed by elongation of the peroxisomes, with constriction, beading and fragmentation into new peroxisomes; giving rise to peroxisome proliferation at 3 h (Rodríguez-Serrano *et al.,* 2016). To assess the possible involvement of nitric oxide (NO) in morphological changes observed in peroxisomes in response to Cd, we first analysed the production of NO at the time these changes occurred (0-3 h). We observed an increase in NO in WT seedlings of about 1.8 times after 30 min. and 1.5 times after 3h treatment respect to control samples (Fig. 1). We also observed a significant increase in NO production in response to Cd stress in NO-related mutants: *nia1 nia2*, affected in nitrate reductases (NR1/NIA1 and NR2/NIA2) and in the NO overproducer, *nox1/cue1* (*nox1* from now on; Fig. 4.3.1), although *nia1 nia2* showed the lower and *nox1* the higher NO levels compared to the WT plants (Fig. 4.3.1). These results suggest that nitrate reductase (NR) may not be the only source involved in the NO increase observed in the seedling response to Cd but NO level is highly compromised in *nia1 nia2* mutants. Furthermore, we used a NOS-l inhibitor (aminoguanidine), which decreases NO production in seedling roots in response to Cd and a NR inhibitor (tungstate), which does not decrease NO production but increases it in response to Cd (Suppl. Fig. S4.3.2).

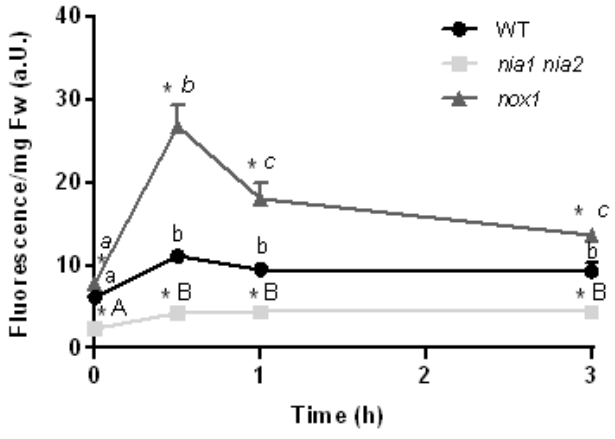
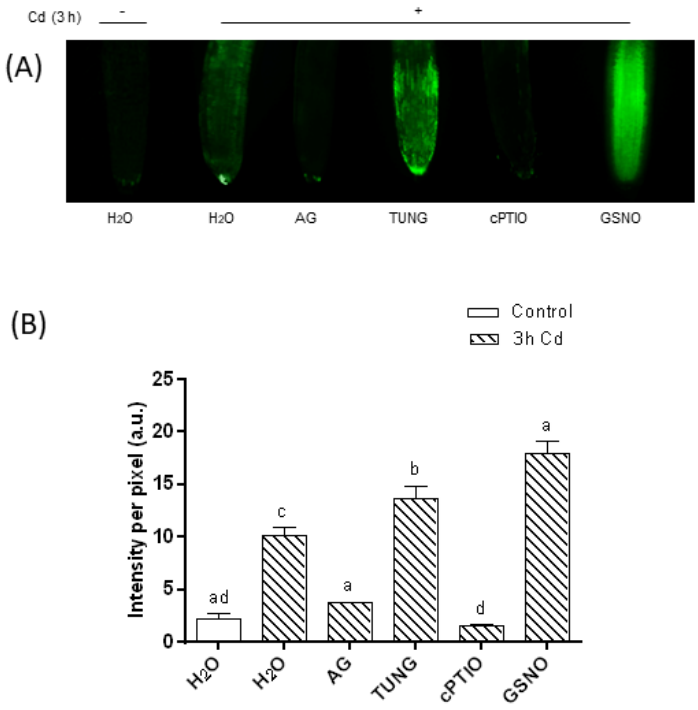


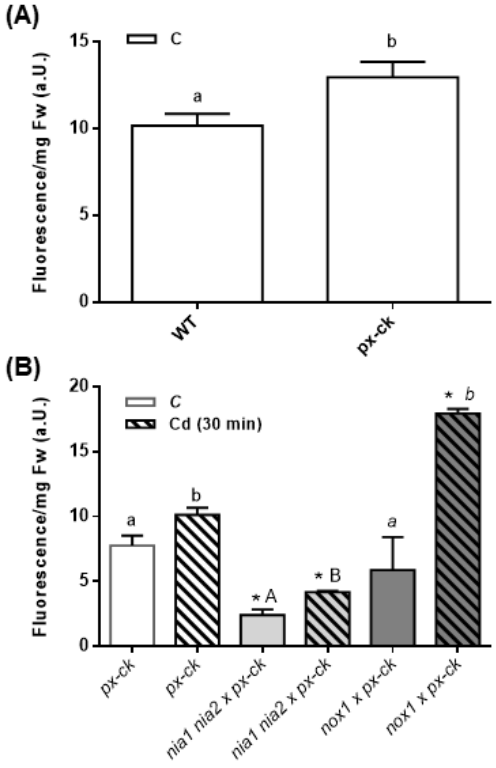
Figure 4.3.1. Effect of cadmium on NO accumulation in Arabidopsis seedlings. Total NO content determined by fluorimetry using DAF2-DA, under control (0 h) and Cd conditions in *nia1 nia2*, *nox1* and WT seedlings. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between time points of Cd treatment within the same genotype (WT: lower case; *nia1 nia2*: upper case and *nox1*: italics), obtained by the Tukey's multiple comparison tests (p-value <0.05). Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT within a time-point, according to Student´s t-test (p-value < 0.05).



Suppl. Fig. S4.3.2.: NO detection by CLMS in seedling roots in response to Cd. Specificity of DAF2-DA fluorescence for NO is verified by root incubation with the NO scavenger cPTIO (0.5 mM) and the NO donor , GSNO (0,5 mM). WT roots were pre-incubated with the NOS inhibitor, aminoguianidine (AG, 1 mM), the NR inhibitor, tungstate (TUNG, 1 mM) under Cd treatment. Data represent mean ± standard error (error bars) of three independent experiments. Letter denote significant differences determined by the T-test compare versus WT Cd amd ssteric denote significant differences determined by the T-test compare versus WT ($P<0.05$).

To follow peroxisomal dynamics we then crossed *nia1 nia2* and *nox1* with the peroxisomal marker line *px-ck*, giving rise to the triple *nia1 nia2*x*px-ck* and double *nox1*x*px-ck* mutants, respectively. *px-ck* lines showed a similar increase of NO production in response to Cd (30 min) as the non-*px-ck* lines (Suppl. Fig. S4.3.3). All mutants with CFP showed a slight increase in fluorescence, which may be due to CFP protein (Suppl. Fig. S4.3.3), although NO trend in plant response to Cd was not altered. We then observed peroxule production in all *px-ck* lines at 30 min (Fig. 4.3.2A), when Arabidopsis showed the higher percentage of peroxisomes producing peroxules after Cd stress (Rodríguez-Serrano *et al*., 2016), which match with the higher NO production observed in Fig. 4.3.1. To show whether NO is involved in peroxule formation, we treated Arabidopsis plants with the NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethy-limidaziline-1-oxyl-3-oxide (cPTIO), which has previously been shown to decrease NO accumulation in response to Cd (Besson-Bard *et al*., 2009). cPTIO caused a decrease of 75% in the number of peroxisomes which extended peroxules in response to Cd (Fig. 4.3.2B). Interestingly, the percentage of peroxule formation in response to Cd showed a strong decrease, about 75%, in *nia1 nia2*x*px-ck* mutants compared to *px-ck* (Fig. 4.3.2A and C; Suppl. Video S4.3.4 and S4.3.5), with only 5 % of the peroxisomes (in green) producing peroxules (Fig. 4.3.2A, 4.3.2C and Suppl. Video S4.3.5). No significant percentage of peroxule production was found in any of

the background analysed under control conditions (Suppl. Videos S4.3.1-S4.3.3; peroxisomes are showed in green). Furthermore, the percentage of peroxisomes producing peroxules increased to 18% when *nia1 nia2xpx-ck* plants were incubated with the NO donor, GSNO and Cd, simultaneously (Fig. 4.3.2C; Suppl. Video S4.3.6), similar to the percentage of peroxules in *px-ck* under Cd stress (Fig. 4.3.2C). Different NO donors, such as N-Acetyl-3-(nitrosothio)-DL-valine, S-Nitroso-N-acetylpenicillamine (SNAP) were also able to induce peroxule production (data non shown).



Suppl. Fig. S4.3.3. Effect of cadmium on NO accumulation in WT, *px-ck, nia1 nia2xpx-ck* and *nox1xpx-ck*. Total NO content determined by fluorimetry using DAF2-DA in: A) WT and *px-ck* under control conditions; and B) under C and Cd conditions in *nia1 nia2xpx-ck* , *nox1xpx-ck* and *px-ck* seedlings. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between: *px-ck* and WT in A); and time points of Cd treatment within the same genotype in B); *px-ck* : lower case; *nia1 nia2xpx-ck* : upper case and *nox1xpx-ck*: italics), according to Student´s t-test (p-value < 0.05). Asterisks denote significant differences between *nia1 nia2xpx-ck* or *nox1xpx-ck* and *px-ck* within a time-point, according to Student´s t-test (p-value < 0.05).

No increase in peroxule production was observed in WT incubated with GSNO and Cd simultaneously (Fig. 4.3.2C; Suppl. Video S4.3.5), suggesting that a minimum of NO is needed to reach the percentage of peroxule production in response to Cd and that an excess of NO does not increased it. In fact, the percentage of peroxule production in *nox1*x*px-ck* mutants, which showed higher NO levels, did not show significant differences with *px-ck* (Fig. 4.3.2A, 4.3.2C and Suppl. Video S4.3.8). We then checked peroxin 11a (*PEX11a*) expression as peroxule production has been shown to be regulated by this protein (Rodríguez-Serrano *et al.*, 2016). We found an

induction of *PEX11a* in WT seedlings in response to Cd treatment, which was similar in *px-ck* mutants suggesting that CFP does not alter PEX11a-dependent signalling (Suppl. Fig. S4.4.4). No significant changes were observed however, in *PEX11a* transcript in *nia1 nia2*, nor in *nox1* seedlings (Fig. 4.3.3), suggesting that an induction of the gene is not essential for the production of peroxules.

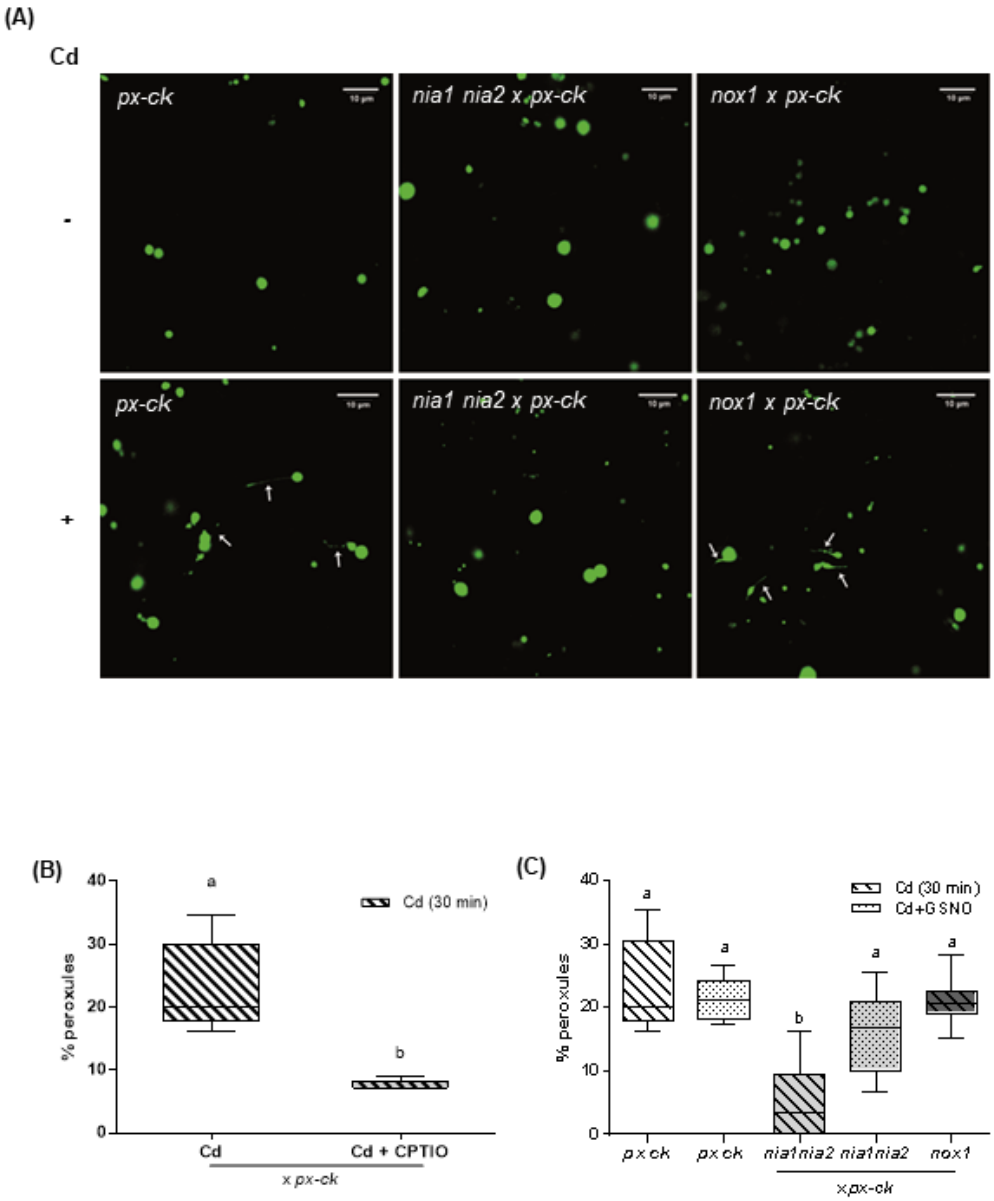


Figure 4.3.2. Effect of cadmium on peroxule formation in Arabidopsis seedlings. (A) Representative images of peroxisomes (in green) under control condition and after Cd treatment (30 min), producing peroxules (arrows). (B) Effect of cPTIO (0.5 mM), a NO scavenger, in peroxule formation induced in *px-ck* seedlings after Cd treatment (30 min.). (C) Peroxules formation in *px-ck*, *nia1 nia2*x*px-ck* and *nox1*x*px-ck* seedlings after Cd treatment and effect of NO donor, GSNO (0.5 mM) in peroxule formation in *px-ck and nia1 nia2*x*px-ck* after Cd treatment (30 min.). Data represent mean, distributions and whiskers (maximum and minimum) of at least three independent experiments. Different letters denote significant differences between different treatments according to Student´s t-test (p-value < 0.05) in (B); and between different treatments and genotypes according to the Tukey's multiple comparison tests (p-value <0.05) in (C).

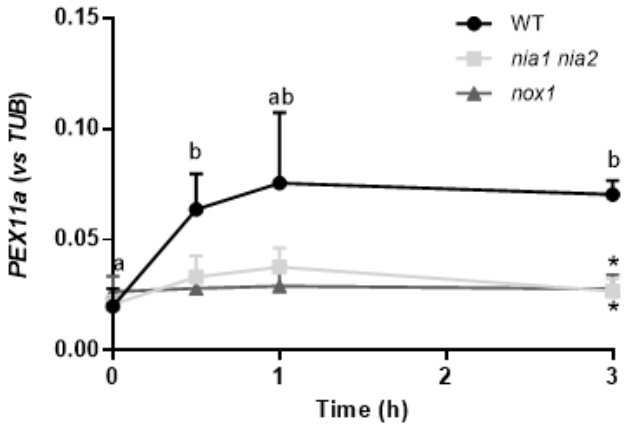
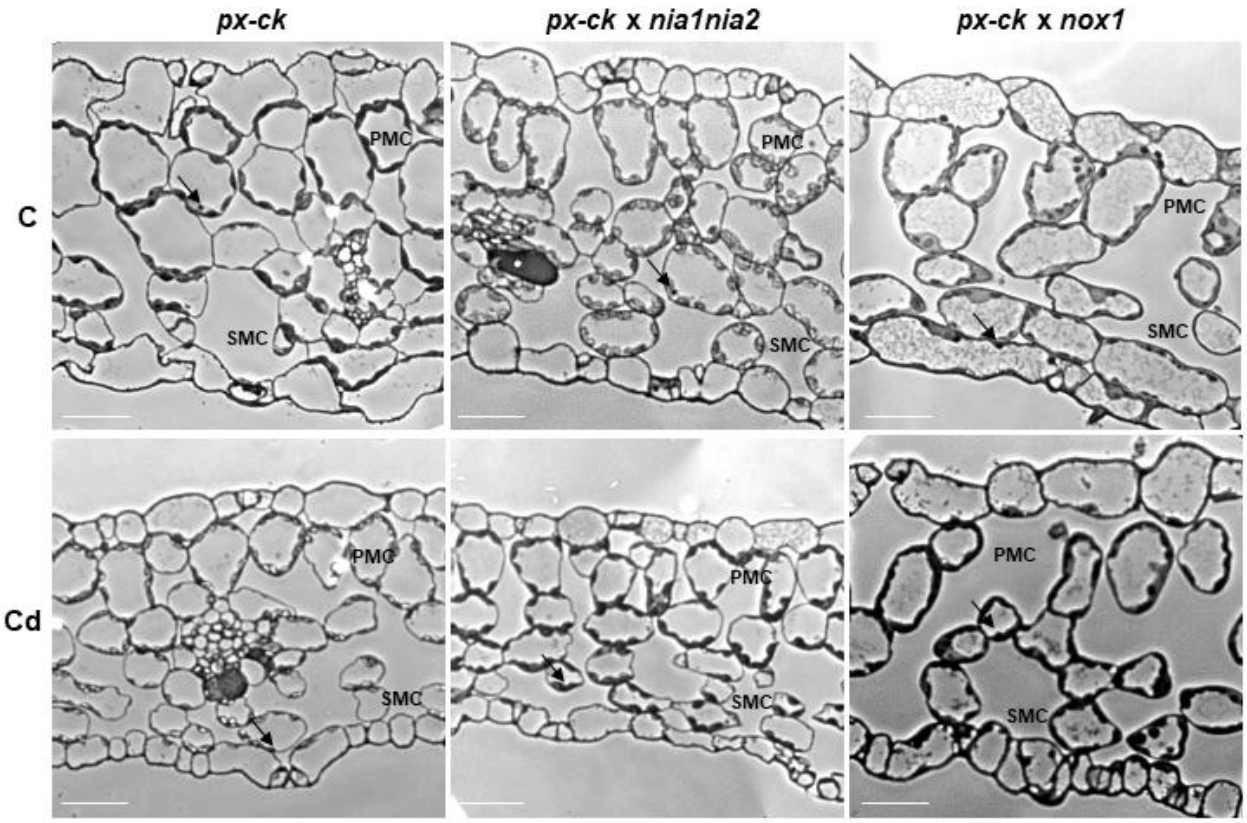


Figure 4.3.3. Effect of cadmium on *PEX11a* expression in Arabidopsis seedlings. Time course analysis of *PEX11a* expression by qRT-PCR under control (0 h) and Cd conditions in *nia1 nia2*, *nox1* and WT seedlings. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between time points of Cd treatment within the same genotype obtained by the Tukey's multiple comparison tests (p-value <0.05). NO letters mean no significant differences with Cd treatment within a genotype. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT within a time-point, according to Student´s t-test (p-value < 0.05).

4.3.3.2. Peroxisome proliferation in plant response to Cd in NO-related mutants

To assess if peroxisome proliferation in response to Cd is also affected by NO we used DAB histochemistry (Castillo, *et al*., 2008) to image and count the peroxisomes in cross sections of *nia1 nia2*x*px-ck*, *nox1*x*px-ck* and *px-ck* leaves by light microscopy (Suppl. Fig. S4.3.5). We observed an increase in the number of peroxisomes per cell slice in response to Cd, in *px-ck* and *nox1*x*px-ck* mutants but not in *nia1 nia2*x*px-ck* (Fig. 4.3.4A). We then supplied *nia1 nia2* mutants with GSNO, an NO donor, simultaneously to Cd treatment and analysed the protein accumulation of peroxin 14 (PEX14) used as a peroxisomal accumulation marker, which showed an increase after Cd treatment (3 h; Calero-Muñoz *et al.*, 2019). PEX14 protein accumulation was increased in *nia1 nia2* mutants after Cd and GSNO treatment (3 h; Fig. 4.3.4B, Suppl. Fig. S4.3.6), thus recovering the WT phenotype.



Suppl. Fig. S4.3.5. Optical microscopy analysis of catalase-mediated staining of peroxisomes with 3′3-diaminobenzidine (DAB) in thin leaf sections. Representative images of thin leaf-sections from seedlings (WT, *nia1 nia2* and *nox*1) treated or not with Cd (3h). Representatives peroxisomes are indicated with arrows. DAB-stained and OsO4-fixed peroxisomes are marked with arrows in the control image. Peroxisomes were detected in the palisade mesophyll cells (PMC) and spongy mesophyll cells (SMC). Bar=100 μM

Interestingly, the number of peroxisomes per cell slice is lower in *nox1*x*px-ck* mutants under normal conditions compared to *px-ck* (Fig. 4.3.4A). The distribution of peroxisomes per cell slice is also altered in *nox1*x*px-ck* mutants, as the percentage of cells with one peroxisome is 1.7-times higher than the percentage in *px-ck* whilst the percentage of cells with three peroxisomes is 0.5-times higher (Fig. 4.3.4C). It was also hard to find a cell with more than three peroxisomes in *nox1*x*px-ck* mutants (Fig. 4.3.4C). The number of peroxisomes per cell is also altered in *nia1 nia2*x*px-ck*, with the percentage of cells with one peroxisome being higher whilst the percentage of cells with two peroxisomes was lower than in *px-ck* (Fig. 4.3.4C).

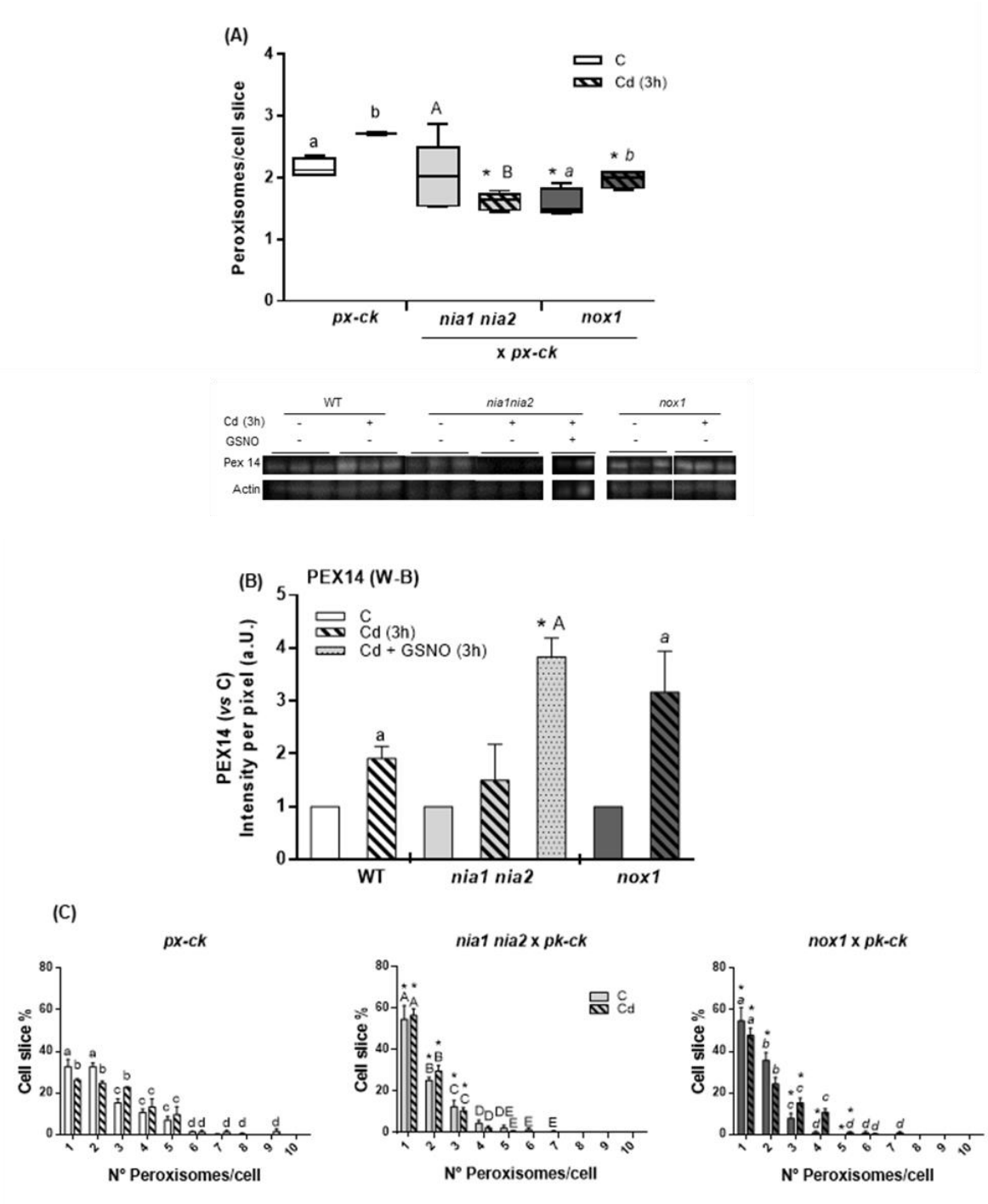


Figure 4.3.4. Effect of cadmium on peroxisome proliferation. Number of peroxisomes per cell slice under control and after Cd treatment (3 h) in *nia1 nia2*x*px-ck*, *nox1*x*px-ck* and *px-ck* seedlings, analysed by cytochemistry and optical microscopy (from thin leaf sections represented in Suppl. Fig. S5). (B) Representative Western-blot and quantification of peroxisome accumulation analysed by the marker protein peroxin 14 (PEX14) in WT, *nox1*, and *nia1 nia2*, after Cd treatment (3 h) and *nia1 nia2* supplemented with the NO donor, GSNO (0.5 mM). (C) Distribution of peroxisomes by the number of these organelles contained in each cell slice analysed by cytochemistry and optical microscopy. The percentage of cells containing from 1 to 10 peroxisomes is represented. Data represent distribution and whiskers (maximum and minimum) in A), and mean and standard error (error bars) in B) and C), of at least three independent experiments. In A) and C) different letters denote significant differences between Cd treatment and control within the same genotype according to Student´s t-test (p-value < 0.05). The asterisks denote significant differences between *nia1 nia2*x*px-ck* or *nox1*x*px-ck* and *px-ck* within a time-point according to Student´s t-test (p-value < 0.05). In B) the asterisks denote significant differences between Cd or Cd+GSNO treatments and control within the same genotype according to Student´s t-test (p-value < 0.05); and the letter denote significant differences between Cd+GSNO and Cd treatments in *nia1 nia2* mutants according to Student´s t-test (p-value < 0.05).

4.3.3.3. Peroxisome morphology in mutants with altered NO-metabolism

Taking into account that the number of peroxisomes per cell was different in mutants with altered NO-metabolism, we decided to check the morphology of peroxisomes by electron microscopy (Fig. 4.3.5A-F). Although *nox1*x*px-ck* leaves are slightly smaller than *px-ck*, we observed no significant differences in the size of the peroxisomes in any of the NO-dependent mutants compared to *px-ck* (Fig. 4.3.5A, B, C and G). Area of peroxisomes in *px-ck* mutants is around 0.4-0.5 μm^2, similar to WT plants showing that the presence of CFP do not affect size of peroxisomes (Castillo *et al*., 2008). Circularity in *nia1 nia2*x*px-ck* mutants was lower than in *px-ck* and in *nox1*x*px-ck* mutants it is slightly higher but not significant (Fig. 4.3.5A, B, C and H). We also observed that the number of peroxisomes showing precipitates, associated as described before, with oxidized CAT (Shibata *et al.*, 2013) and/or H_2O_2 (Romero-Puertas *et al.*, 2004), increased 7-times in *px-ck* peroxisomes with Cd treatment (Fig. 4.3.5A, D and I). Interestingly, peroxisomes from both mutants have these precipitates under control conditions: 25% of peroxisomes in *nia1 nia2*x*px-ck* mutants and 60% of peroxisomes in *nox1*x*px-ck* (Fig. 4.3.5A, B, C and I), suggesting that oxidative metabolism may be affected in both mutants. An increase in the percentage of peroxisomes with precipitates was also observed in both mutants with Cd treatment, being similar to *px-ck* under the same conditions in *nia1 nia2*x*px-ck* and double in *nox1*x*px-ck* (Fig. 4.3.5D, E and I).

4.3.3.4. Oxidative metabolism in NO-related mutants under Cd stress

While no significant changes in H_2O_2 content was observed in WT seedlings, a significant decrease in *nox1* at 1 h treatment was found, and then control levels were recovered at 3 h (Fig. 4.3.6A). Similarly, in *nia1 nia2* mutants a slight, but significant decrease was observed after 1 h of Cd treatment, which was sustained up to 3 h (Fig. 4.3.6A). We then analysed GST as a gene marker for H_2O_2-dependent signalling and observed an induction after Cd treatment in WT seedlings (3 h) but not in *nox1* mutants (Fig. 4.3.6B). Although an induction of GST was also observed in *nia1 nia2*, this occurred from the very beginning of the treatment with GST expression being significantly down regulated in these mutants compared to WT seedlings under control conditions (Fig. 4.3.6B). We then checked catalase (CAT), which is a peroxisomal enzyme that maintains H_2O_2 homeostasis. A decrease in CAT activity was observed in WT in response to Cd stress (Fig. 4.3.6C) although expression of *CAT2*, the gene mainly involved in this activity did not change with the treatment (Fig. 4.3.6D). In the *nox1* mutants, a decrease of CAT activity was also observed after Cd treatment and was only significantly different after 30 minutes' treatment recovering the activity after that time (Fig. 4.3.6C). This recovery was probably due to the initial induction of *CAT2* gene in *nox1* mutants (Fig. 4.3.6D). Under control conditions however, CAT activity in *nox1* mutants was half of the activity in WT seedlings. Interestingly, in *nia1 nia2* mutants CAT activity and *CAT2* expression was double than that in the WT under control conditions (Fig. 4.3.6C-D). A decrease in CAT activity was also observed in *nia1 nia2* mutants

with the metal treatment, although the activity in this mutant during the treatment was similar to that of the WT seedlings under control conditions (Fig. 4.3.6C). We also analysed *CAT2* expression in *px-ck* lines in plant response to Cd, which showed no differences respect to WT plants suggesting that differences observed in NO-related mutants are due to their own genotype and not to the presence of CFP (Suppl. Fig. S4.3.4). Similar result was observed with glycolate oxidase activity, one of the main peroxisomal enzymes involved in H_2O_2 production (Suppl. Fig. S4.3.4).

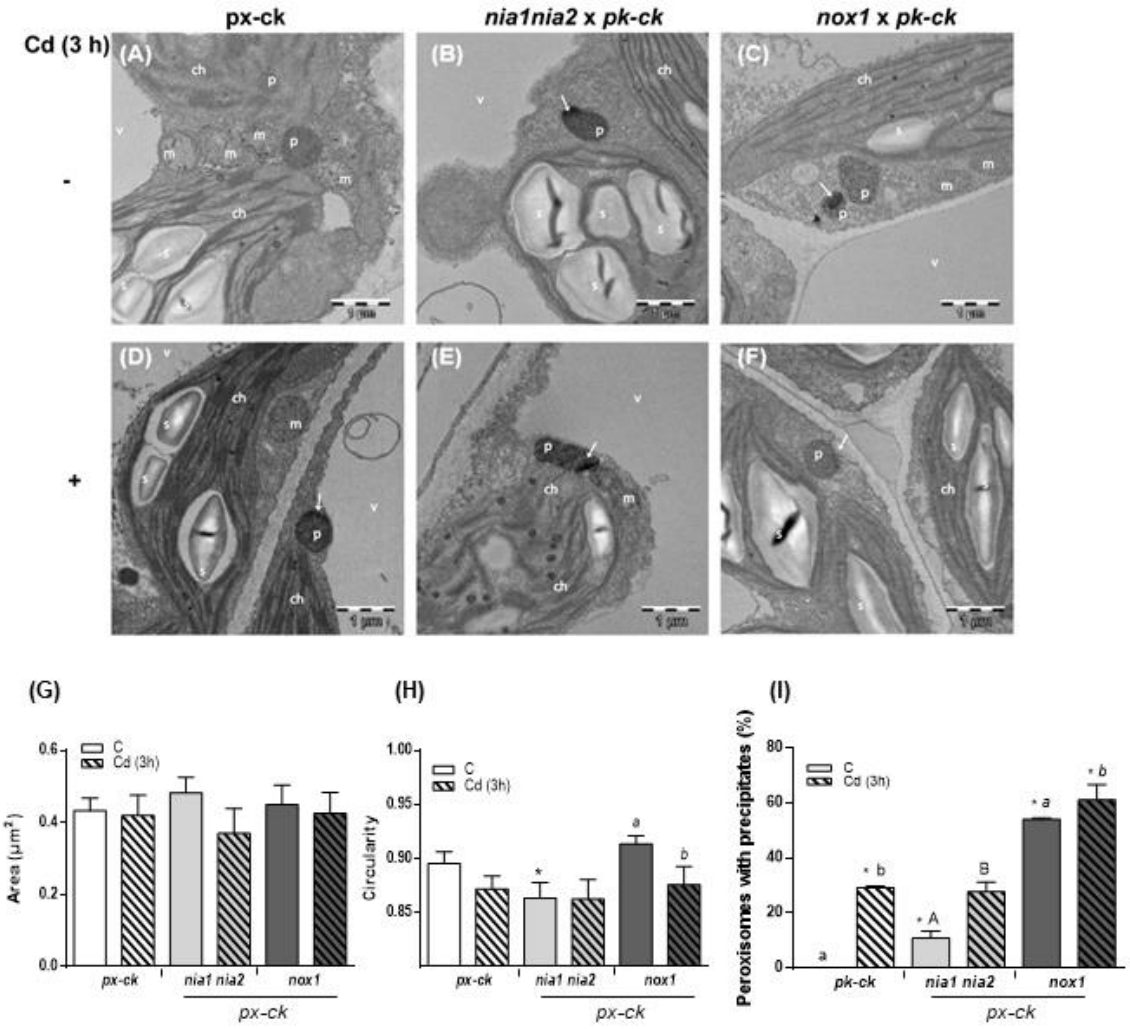


Figure 4.3.5. Peroxisome ultrastructure in Arabidopsis seedlings under control and Cd treatment. Representative micrographs of leaf sections from *px-ck*, *nia1 nia2*x*px-ck* and *nox1*x*px-ck* seedlings treated (D, E, F) or not (A, B, C) with Cd (3 h), analyzed by electron microscopy (scale bar 1 µm). (G) Area, (H) circularity and (I) percentage of peroxisomes with precipitates. At least 600 cells were analyzed per treatment, meaning that thousands of peroxisomes were examined. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between Cd treatment and control within the same genotype (*px-ck*: lower case; *nia1 nia2*x*px-ck*: upper case and *nox1*x*px-ck*: italics), obtained by the Student´s t-test (p-value <0.05). Asterisks denote significant differences between *nia1 nia2*x*px-ck* or *nox1*x*px-ck* and *px-ck*, within a time-point, according to Student´s t-test (p-value < 0.05). Abbreviations: ch, chloroplasts; m, mitochondria; p, peroxisome; v, vacuole; s, starch. The arrows indicate precipitates inside the peroxisomes.

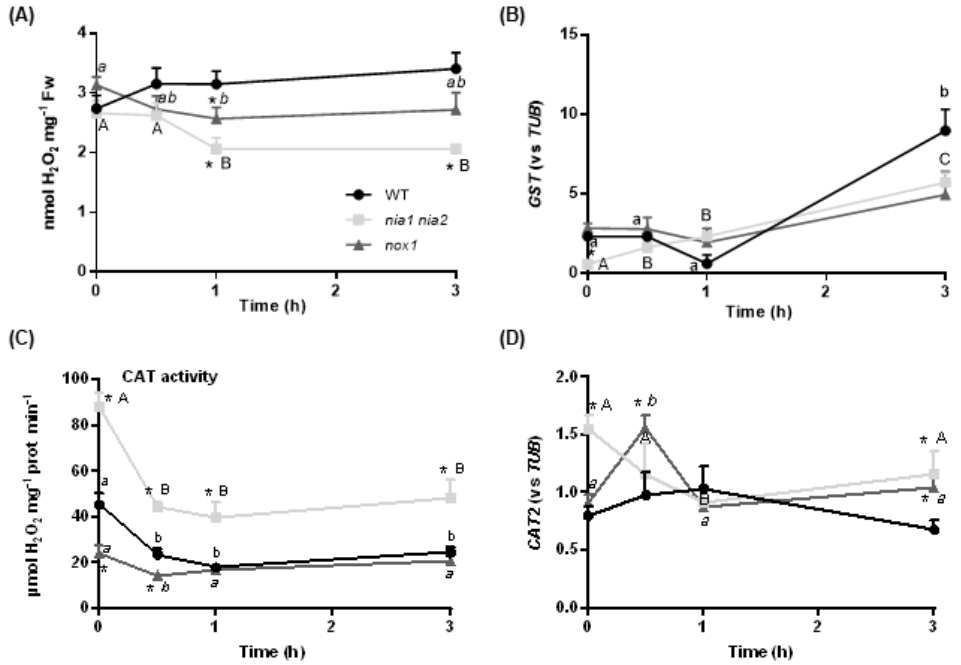
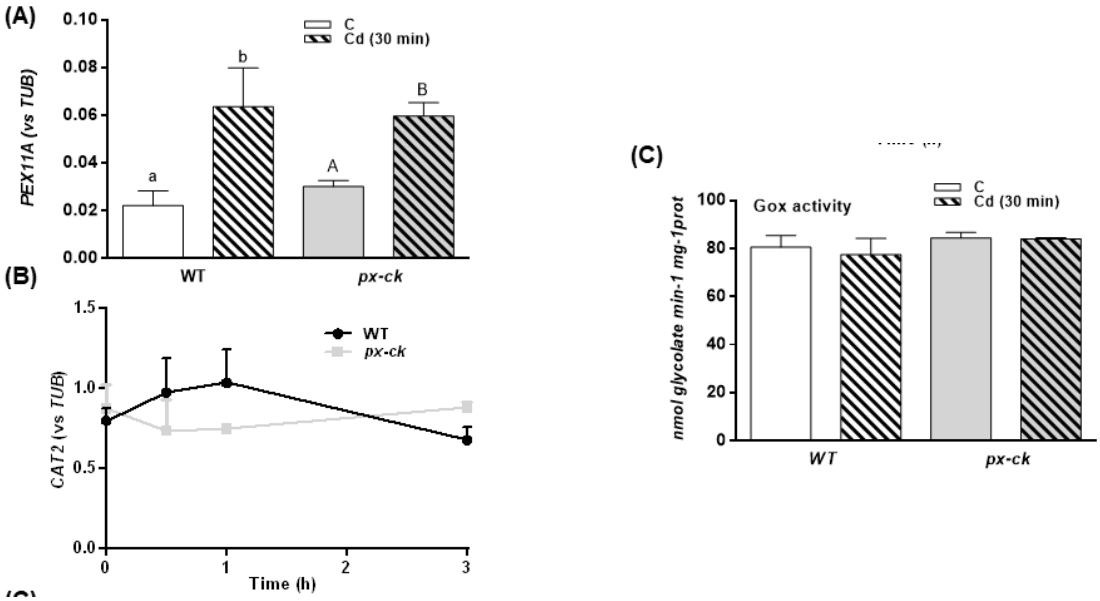


Figure 4.3.6. H_2O_2 metabolism in Arabidopsis seedlings under Cd stress. (A) H_2O_2 content determined by fluorimetry, (B) expression of *GST* by qRT-PCR (C) catalase activity and (E) expression of *CAT2* by qR-TPCR in WT, *nia1nia* and *nox1* seedlings under Cd treatment (0-3 h). Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between time points of Cd treatment within the same genotype (WT: lower-case; *nia1 nia2*: upper case and *nox1*: italics), obtained by the Tukey's multiple comparison tests (p-value <0.05). NO letters mean no significant differences with Cd treatment within a genotype. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT within a time-point, according to Student´s t-test (p-value < 0.05).



Suppl. Fig. S4.3.4: *PEX11a* and *CAT2* expression and Glycolate oxidase activity in WT and *px-ck*. *PEX11a* (A) and *CAT2* (B) expression by qRT-PCR and glycolate oxidase activity (GOX; C) under control and Cd conditions in WT and *px-ck* seedlings. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters (WT: lower-case; *px-ck*: upper case) denote significant differences between Cd treatment and control within the same line according to Student´s t-test (p-value < 0.05). No letters mean no significant differences with Cd treatment within a genotype according to Tukey's multiple comparison tests (p-value <0.05) in B). No significant differences have been found when comparing *px-ck* with WT according to Student´s t-test (p-value < 0.05).

4.3.3.5. Post-translational modifications of catalase in mutants with altered NO metabolism

CAT activity showed a decrease, especially after 30 minutes' Cd treatment, but this does not appear to be due to differences in *CAT2* expression in WT. As this protein has been shown to be highly regulated by post-translational modifications (PTMs), we then analysed oxidized and *S*-nitrosylated pattern of CAT under control and Cd treatment. We found that the carbonylation of CAT, used as a marker for the oxidation state of protein, was higher and the *S*-nitrosylated pattern did not change significantly under Cd treatment in WT seedlings compared with non-treated seedlings (Fig. 4.3.7A and B). Interestingly, CAT is highly oxidized and *S*-nitrosylated in *nia1 nia2* under control conditions and a decrease of both PTMs was observed in response to Cd treatment (Fig. 4.3.7A and B). In *nox1* mutants however, basal pattern of *S*-nitrosylation was maintained with Cd treatment and was higher than in WT seedlings (Fig. 4.3.7B), and oxidation state was decreased with the treatment (Fig. 4.3.7A), contrary to the behaviour of WT. CAT content did not change after 30 minutes' Cd treatment in any of the Arabidopsis backgrounds, although the CAT content in *nia1 nia2* mutants was higher than in the WT and *nox1* mutants (Suppl. Fig. S4.3.7).

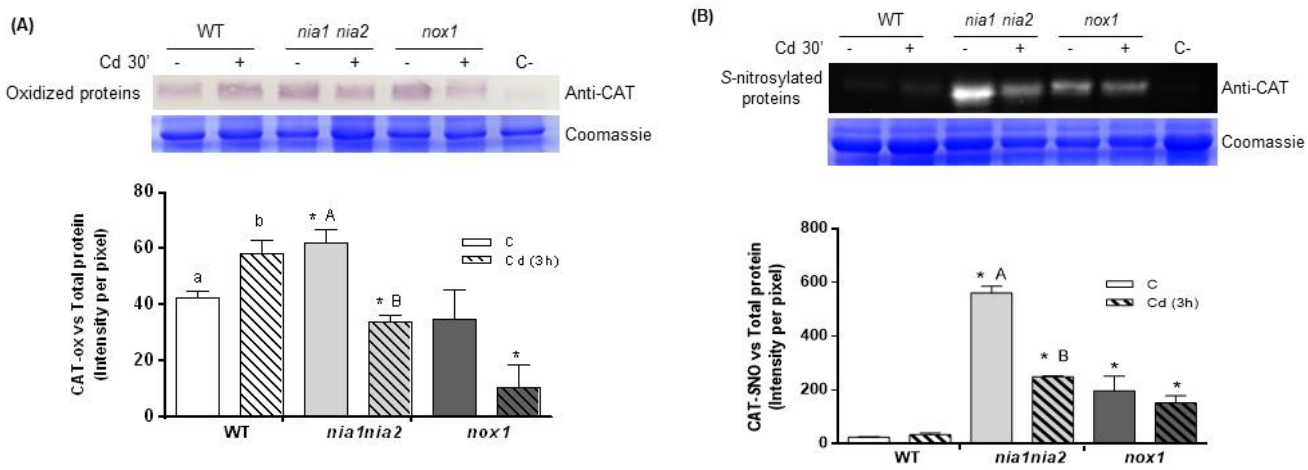
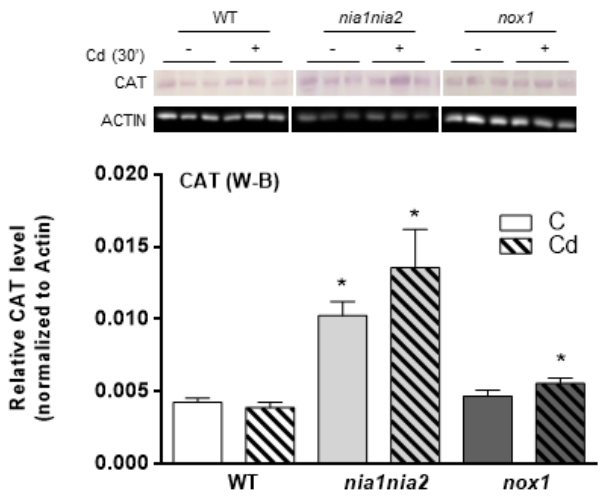


Figure 7. Effect of cadmium on catalase post-transcriptional modifications in Arabidopsis seedlings. (A) Representative Western-blot with oxidation pattern of CAT following Cd treatment (30 min.) in WT, *nia1 nia2* and *nox1* seedlings and quantification. Protein extracts from seedlings were subjected to derivatization with DNPH, immuno-precipitated with anti-DNPH (oxidized proteins) and CAT was then identified by a specific antibody. Non-derivatized samples from *nia1 nia2* were used as a negative control. (B) Representative Western-blot with *S*-nitrosylation pattern of CAT following Cd treatment (30 min.) in WT, *nia1 nia2* and *nox1* seedlings and quantification. Protein extracts from seedlings were subjected to the biotin-switch method. *S*-nitrosylated proteins were then purified by an anti-biotin antibody and subjected to Western blot analysis with anti-CAT antibody. Non biotinylated samples from *nia1 nia2* were used as a negative control. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters (WT: lower-case; *nia1 nia2*: upper case and *nox1*: italics) denote significant differences between Cd treatment and control within the same genotype according to Student´s t-test (p-value < 0.05). No letters mean no significant differences with Cd treatment within a genotype. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT within a time-point, according to Student´s t-test (p-value < 0.05).



Suppl. Fig. S4.3.7. Variation in CAT protein accumulation during Cd treatment. Representative Western-blot and quantification of CAT content in WT, *nia1 nia2* and *nox1* extracts used in Fig. 7. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT within a time-point according to Student´s t-test (p-value < 0.05).

4.3.3.6. Peroxisomal dependent signalling in response to cadmium

Mutants with altered peroxisomal dynamics show a disturbed peroxisomal ROS-dependent signalling (Rodríguez-Serrano *et al.*, 2016), so we analysed if peroxisomal dependent signalling was altered in NO-related mutants. We checked two genes specifically up-regulated after H_2O_2 production in peroxisomes (Sewelam *et al.*, 2014) and related with protein folding and repair: the heat shock protein 20-like (*HSP20-like*) and the Arabidopsis orthologs of the human Hsp70-binding protein (*FES1A*). We found that the very early response is affected in both mutants compared to WT, suggesting that these genes might not be related with peroxule production but rather with altered ROS metabolism within the peroxisomes (Fig. 4.3.8). Interestingly, although in *nia1 nia2* mutants the induction of the genes after 3 hours' treatment was similar to that in WT, in *nox1* mutants it was significantly lower (Fig. 4.3.8).

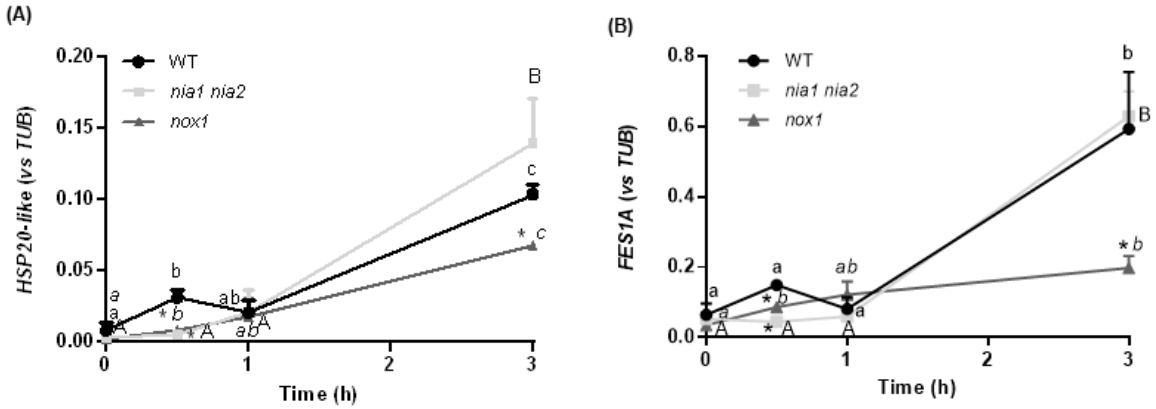


Figure 4.3.8. Role of NO in peroxisomal-dependent signalling in response to Cd. Expression of *HSP20-like* (A) and *FES1A* (B) by qRT-PCR in WT, *nia1 nia2* and *nox1*seedlings under Cd treatment (0-3 h). Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between time points of Cd treatment within the same genotype (WT: lower-case; *nia1 nia2*: upper case and *nox1*: italics), obtained by the Tukey's multiple comparison tests (p-value <0.05). Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT within a time-point, according to Student´s t-test (p-value < 0.05).

4.3.4. Discussion

Peroxisomes are essential organelles widely found from yeast to mammals and in particular in plants, which have been involved in response to different stresses (Baker and Paudyal, 2014; Fransen *et al.* 2017; Mathur, Shaikh and Mathur 2018). Although the signalling molecule NO has been involved in plant response to Cd (Besson-Bard *et al.,* 2009; Rodríguez-Serrano *et al.,* 2009) and peroxisomes are intimately related with NO metabolism (Sandalio and Romero-Puertas, 2015), our knowledge about NO function in peroxisome metabolism and dynamics under control and stress conditions is scarce.

4.3.4.1. NO regulates peroxule production in plant response to Cd stress

We observed an increase in NO production in the early response (30 min to 3 h) to Cd in Arabidopsis seedlings (Fig. 4.3.1). This result is in agreement with most of the studies analysing NO production in response to short-term Cd treatments in a wide-range of plant species, whilst the tendency observed after long-term treatments is a decrease (Bartha *et al*., 2005; Groppa *et al*., Benavides, 2008; Besson-Bard *et al.,* 2009; Romero-Puertas *et al.,* 2019; Terrón-Camero *et al*., 2020). We also observed an increase in NO in response to Cd in *nia1 nia2* mutants although in these mutants NO production is highly compromised compared to WT seedlings (Fig. 4.3.1) involving NR in part of the NO induction observed in WT. Other sources may also be involved in NO induction after Cd treatment as incubation with the arginine competitor, aminoguanidine (AG) diminished NO production after Cd treatment (Suppl. Fig. S4.3.2). Results with different species point to a crosstalk between different NO sources in plant response to Cd (Besson-Bard *et al.,* 2009; Rodríguez-Serrano *et al.,* 2009; Pérez-Chaca *et al.,* 2014; Chen *et al.,* 2018; Terrón-Camero *et al*., 2020; Terrón-Camero *et al.,* 2019). On the contrary to *nia1 nia2*, *nox1* mutants showed a higher level of NO during the treatment (Fig. 4.3.1). *nox1* mutants have been found to increase the arginine substrate for NOS-l activity (He *et al.,* 2004), suggesting that the arginine-dependent pathway may also be involved in increasing NO production under Cd stress in this mutant (Fig. 4.3.1 and Suppl. Fig. S4.3.2).

Interestingly, *px-ck* seedlings incubated with the NO scavenger (cPTIO), and *nia1 nia2*x*px-ck* seedlings, which produce less NO, lead to a significant decrease in peroxule production in response to Cd (Fig. 4.3.2B and 4.3.2C). The phenotype observed in *nia1 nia2*x*px-ck*, was recovered when supplying NO (Fig. 4.3.2C). Taken together, these data suggest that NO is a positive regulator of peroxule production in plant response to Cd stress including this signalling molecule in the complex network regulating early response to Cd. The cost of energy for producing peroxules or the existence of different peroxisomal populations (with specific role) may explain that an excess of NO does not increase percentage of peroxisomes producing peroxules although

this point needs further analysis. As far as we know, peroxule production has been associated to ROS production by exogenous application of H_2O_2 (Sinclair *et al.*, 2009; Barton *et al*., 2013) and by endogenous RBOHs-dependent ROS production in response to Cd stress (Rodríguez-Serrano *et al.,* 2016). Molecular events involved in peroxule extensions are not well understood, with the peroxine PEX11a being recently involved in this process (Rodríguez-Serrano *et al.,* 2016). We found an induction of PEX11a in WT seedlings in response to Cd as previously described (Rodríguez-Serrano *et al.,* 2016), but no changes in the expression of this peroxine were observed in the NO-related mutants suggesting that an induction of the gene might not be necessary for the production of peroxules and that redox-dependent PTMs may be involved. In fact, yeast peroxine Pex11p activation depends on redox changes of its cysteins (Knoblach and Rachubinski, 2010; Schrader *et al*., 2012). Other PTMs can not be ruled out however, as PEX11a has been identified as putative target of phosphorylation in Arabidopsis (Kataya *et al*., 2019; Sandalio *et al.,* 2019). Further proteomic analysis is required to clarify PEX11a PTMs dependence on the rapid regulation of peroxule formation and the possible involvement of NO.

4.3.4.2. NO regulates peroxisome proliferation

Peroxisome proliferation has been associated with ROS or stress involving ROS accumulation (Lopez-Huertas *et al*., 2000; Palma *et al*., 1991; Oksanen *et al*., 2003; Nila *et al*., 2006; Castillo *et al.,* 2008; Mitsuya *et al.,* 2010); and in particular, in plant response to Cd (Romero-Puertas *et al.,* 1999; Rodríguez-Serrano *et al.,* 2016; Calero-Muñoz *et al.,* 2019). Here we have shown that peroxisome proliferation in response to Cd is regulated by NO (Fig. 4.3.4A and B) and similar to that ocurring with peroxule production, it appears that NO is a positive regulator. Signalling upstream peroxisome proliferation is scarce and only phytochrome A (Phy A), the bZIP transcription factor HY5 HOMOLOG (HYH), and the peroxin PEX11b has been involved in this process during seedling photo-morphogenesis (Desai and Hu, 2008). On the contrary, jasmonic acid (JA) appears to be a negative regulator of peroxisome proliferation in the mesophyll cells of Arabidopsis (Castillo *et al.,* 2008). Antagonist effect between NO and JA in peroxisome proliferation is interesting and further work is needed to get a deeper insight into the mechanism involved.

Interestingly, although proliferation appears not to be affected in *nox1*x*px-ck* mutants, the number of peroxisomes per cell is always lower than in *px-ck* (Fig. 4.3.4C). This lower number of peroxisomes in *nox1* mutant does not appear to be associated with *PEX11a* expression however. An increase in both, size and number of peroxisomes was found in potato treated with high concentrations of JA (Ulloa *et al*., 2002), whilst a lower total number of enlarged peroxisomes were found in Arabidopsis leaves treated with JA (Castillo *et al.,* 2008). The mechanisms involved

in the regulation of peroxisome proliferation and elongation under JA treatment remains unclear although it also appears that they are neither dependent on *PEX11* or *DRP3A* (Castillo *et al.*, 2008).

4.3.4.3. NO regulates ROS metabolism in peroxisomes

We observed and increase in the number of peroxisomes containing precipitates in *px-ck* after 3hs' Cd treatment (Fig. 4.3.5D and I), which could be associated with H_2O_2 and/or CAT activity (Romero-Puertas *et al.*, 2004; Shibata *et al.*, 2013). An increase in H_2O_2 has been shown previously in response to Cd stress in pea leaf peroxisomes (Romero-Puertas *et al.*, 1999) and in Arabidopsis seedlings expressing the H_2O_2 biosensor HyPer in peroxisomes (Calero-Muñoz *et al.*, 2019). Interestingly, in *nia1 nia2*x*px-ck* and especially in *nox1*x*px-ck* mutants these precipitates were observed under control conditions, suggesting that H_2O_2 metabolism in the organelles might be altered in these mutants (Fig. 4.3.5B, C and I). Other organelles such as mitochondria and chloroplast have also been shown to be affected in *nox1* mutants (Voll *et al.*, 2003; Hourton-Cabassa *et al.*, 1998; Bona *et al*., 2007; Voll *et al.*, 2003; Hu *et al.*, 2014).

Interestingly, a significant decrease in H_2O_2 content was found in both NO-related mutants, which was maintained in *nia1 nia2* mutants (Fig. 4.3.6A) although no changes were observed in WT. An increase in GST transcripts after 3 hours' treatment was found at 3 h Cd treatment, as described before (Calero-Muñoz *et al*., 2019) and a similar result was described in mulberry plants (Xu *et al*., 2019). *nia1 nia2* mutants behave similarly to WT but no changes in GST expression has been observed in *nox1* seedlings (Fig. 4.3.6 B). These results suggest that NO is modifying H_2O_2 content and its dependent signalling. It is well known that exogenous addition of NO donors increases antioxidant activities such as GST after paraquat, As or Cd treatments (Nahar *et al.*, 2016; Hasanuzzaman *et al.*, 2018; Souri *et al*., 2020) while an excess of endogenous nitric oxide may inhibit antioxidant activities under Cd stress (Terrón-Camero *et al*., 2020).

Results obtained about CAT activity, expression (*CAT2*) and protein PTMs in NO-related mutants respect WT showed that oxidative metabolism in peroxisomes is regulated by NO. The decrease in CAT activity observed in WT under Cd stress may be explained by the increase in the oxidation state of the protein. CAT has been shown previously to be carbonylated and *S*-nitrosylated in pea plants in response to Cd, leading to the inhibition of its activity (Romero-Puertas *et al.*, 2002; Ortega-Galisteo *et al.*, 2012); and in the presence of H_2O_2, CAT is subjected to oxidation in multiple sites (Nguyen and Donaldson, 2005; Anand *et al*., 2009). CAT activity was also affected by the increase in NO and $O_2^{\cdot-}$ production in peroxisomes from Arabidopsis plants treated with Pb (Corpas and Barroso, 2017). Interestingly, CAT is more oxidized under control conditions in *nia1 nia2* mutants compared to WT and a decrease in the oxidation state of CAT, contrary to that occurring in WT, was observed in both NO-related mutants in response to

Cd, probably due in part to the decrease in the H_2O_2 content observed. *S*-nitrosylated CAT is also higher under control conditions in NO-related mutants compared to WT seedlings, suggesting a protection of CAT from oxidation by *S*-nitrosylation. Similarly, in sorghum leaves, salt stress promotes opposite patterns of carbonylation and *S*-nitrosylation of C4 phosphoenolpyruvate carboxylase (PEPCase; Baena *et al*., 2017); and specific patterns of carbonylation and *S*-nitrosylation under salt stress are essential in citrus plant vigour (Tanou *et al.,* 2014). It has also been suggested that *S*-nitrosylation of antioxidant enzymes prevents irreversible protein carbonylation during recalcitrant seed desiccation tolerance in *Antiaris toxicaria* (Bai *et al.*, 2011). Although in *nia1 nia2* mutants, CAT is more oxidized and *S*-nitrosylated in control conditions, which should affect its activity, a higher induction of the gene (*CAT2*) and protein content is observed, explaining the increase in CAT activity in these mutants.

4.3.4.4. Peroxisomal dependent signalling in response to Cd is altered in NO-related mutants

ROS act as secondary messengers, which activates a signal transduction pathway culminating in the regulation of gene expression, which is critical for plant subsistence (Foyer and Noctor, 2003; Suzuki *et al*., 2012; Mittler, 2017; Baxter *et al.*, 2014). It has been shown that peroxisomal H_2O_2 induces transcripts such as, *FES1A* and the chaperone *HSP20-like*, involved in protein repair responses leading to stress tolerance and plant survival under stress (Sewelam *et al.*, 2014; Zhang *et al.,* 2010). We observed that both NO-related mutants have affected the very early response to Cd, with the expressions of *FES1A* and *HSP20-like* being significantly lower than in WT (Fig. 4.3.8). Both mutants showed a decrease in H_2O_2 in response to Cd compared to WT and this might explain the lower expression of the repairing genes. After three hours of Cd treatment *nia1 nia2* mutants recover the induction of the genes in a similar way to WT, while *nox1* mutants were still affected, suggesting that an excess of NO is disturbing part of the peroxisomal-dependent signalling.

To sum up, peroxisomal distribution, metabolism and dynamics are affected by NO, which have an effect on organelle functionality and dependent signalling in plant response to Cd stress (Fig. 4.3.9). We have observed that *nia1 nia2* mutants showed lower level of NO respect to WT, which is essential for peroxule production and for peroxisome proliferation. Oxidative metabolism in the peroxisomes is disturbed in NO-related mutants, with CAT being one of the antioxidant altered at transcriptional and post-translational level. Peroxisomal dependent signalling in plant response to Cd stress is also affected in NO-related mutants (Fig. 4.3.9). Although further work is needed to describe the molecular mechanisms underlying the NO role in peroxisomes, we have shown that a tight NO-level regulation is needed for an optimal peroxisome function and signalling.

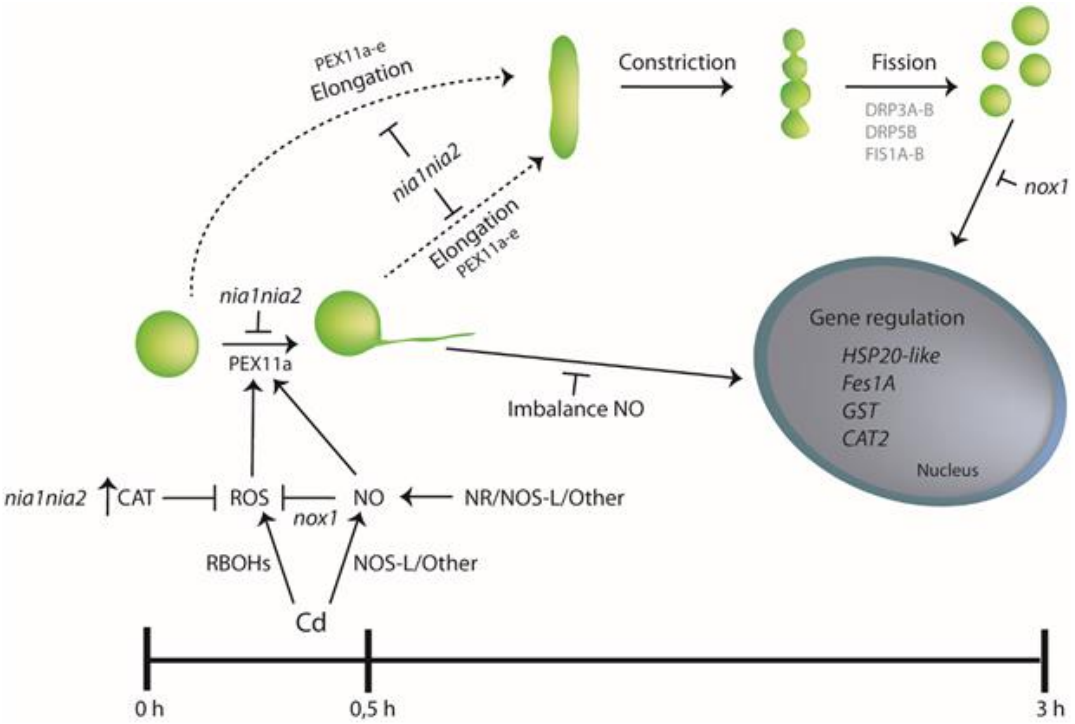


Figure 4.3.9. Model for NO function in peroxisomal dynamics in the early plant response to Cd treatment. Cd induces ROS and NO production, with *nia1 nia2* mutants showing a lower general level of NO. Therefore, *nia1 nia2* mutants are affected in peroxule production and peroxisome proliferation, and addition of NO recovers their phenotype. Altered levels of NO inhibit part of the peroxisomal dependent signalling after Cd treatment (30 min) and an excess of NO inhibits this signalling after 3h. Peroxisomal ROS metabolism is also altered in NO-related mutants being CAT levels and activity higher in *nia1 nia2* mutants than in WT plants.

4.3.5. Acknowledgements

This work was co-financed by the Ministry of Science, Innovation and Universities together with the European Regional Development Fund (MCIU/AEI/ERDF; PGC2018-098372-B-100 and BIO2015-67657-P). LC T-C was supported by a fellowship for Academic Staff (FPU) from the Spanish Ministry of Education, Culture and Sports. Confocal laser fluorescence microscopic analyses were carried out at the Technical Services of Granada University (CIC-UGR). We also wish to thank Angela Tate for proofreading the English manuscript. None of the authors have any conflict of interest to declare.

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Chapter 4.4

4.4. Nitric oxide function in *Fusarium oxysporum* infection to *Arabidopsis thaliana*

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Running title:

NO role in the Arabidopsis-Fusarium interaction

ABSTRACT

Plants continuously interact with fungi being some of them, such as *Fusarium oxysporum* lethal for the plant leading to lower crop yield. Recently, a regulatory role of nitric oxide (NO) in plant response to *F. oxysporum* has been evidenced, but the underlying mechanisms are poorly understood. Here, we show that the Arabidopsis mutant *nox1*, NO overproducer, showed a similar susceptibility to *F. oxysporum* than WT although a percentage of plants never die probably due to an earlier increase of NO-dependent defence mechanisms. In contrast, the double mutant *nia1 nia2*, affected in nitrate reductase activity, is highly sensitive to *F. oxysporum*. We have found that defence response is hardly affected in *nia1 nia2* while cell wall and cellulose content was highly diminished. Our results suggest that nitrate reductase-dependent decrease of cellulose synthesis and cell wall damage-dependent defences signalling facilitate *F. oxysporum* entrance in *nia1 nia2* leading to a higher and earlier mortality rates.

Key words: *Arabidopsis thaliana*, cellulose, *Fusarium oxysporum*, nitrate reductase, nitric oxide

4.4.1. Introduction

Plants interact with numerous microbes during their lives leading to negative or beneficial out-comes for plant fitness. In particular, fungi play a key role in both, natural and agricultural environments. Although mutualistic and neutral interactions are predominant, fungal plant pathogens cause large greenhouse and outdoors crops production losses (Masachis *et al*., 2016; Martínez-Medina *et al*., 2019). In fact, two Fusarium species (*F. graminearum* and *F. oxysporum*) have been placed as fourth and fifth in the Top 10 fungal plant pathogen list for Molecular Plant Pathology (Dean *et al*., 2012). In particular, *F. oxysporum* has a wide host range causing severe losses in around one hundred crops and also infect the model plant *Arabidopsis thaliana*. Infectious hyphae are able to sense signals from the plant, such as catalytic activity of secreted class III peroxidases, sugars and aminoacids, and enter the roots (Turrá *et al*., 2015). Then a crosstalk between the plant and the fungus is activated until the infection developed. Recently, NO production has been described in the interaction Arabidopsis-*F. oxysporum* (Gupta *et al*., 2014) and a regulatory role of nitric oxide (NO) in tomato response to *F. oxysporum* has been evidenced (Martínez-Medina *et al*., 2019a). Research from the last twenty years has shown NO relevance in plant metabolism acting as a signalling molecule (Brouquisse, 2019). Since the first reports, the function of NO has been closely linked to plant immunity (Delledonne *et al*., 1998; Durner *et al*., 1998; Bellin *et al*., 2013; Yu *et al*., 2014). In fact, an increment of NO has been largely observed during several processes such as microbe-associated molecular patterns (MAMP)-triggered immunity (PTI) and effector triggered immunity (ETI) responses where NO plays an important role in the hypersensitive response (HR) and systemic acquired resistance (SAR; Bellin *et al*., 2013; Wendehenne *et al*., 2014; Molina-Moya *et al*., 2019). Furthermore, damage-associated molecular patterns (DAMPs) have been shown to induce NO production, and show a feedback interaction with Ca^{2+} (Trapet *et al*., 2015). Not enough attention has been paid however to the specific roles of NO during the interaction of plants with pathogenic fungi, especially with root fungal pathogens probably due to difficulties of the below ground (Shelef *et al*., 2019). These interactions appear to be determined by the way of life of the pathogens: necrotrophic, biotrophic or hemi-biotrophic (Van Baarlen *et al*., 2004; Sarkar *et al*., 2014; Floryszak-Wieczorek and Arasimowicz-Jelonek, 2016; Martínez-Medina *et al*., 2019). Thus, in plant interactions with different fungal pathogens such as *Botrytis cinerea, Aspergillus nidulans, Macrophomina phaseolina*, *Verticillium dahliae* and *F. oxysporum* a differential concentration and spatio-temporal patterns of NO accumulation in the plant tissue have been described (Conrath *et al*., 2004; Wang and Higgins, 2005; Arasimowicz and Floryszak-Wieczorek, 2007; Turrion-Gomez and Benito, 2011; Sarkar *et al*., 2014; Martínez-Medina *et al*., 2019b). On the other hand, exogenous

NO application reduces the infection by *Rhizoctonia solani* in tomato plants (Noorbakhsh and Taheri, 2016).

Whereas NO production has been described during the plant interaction with *F. oxysporum*, the mechanisms underlying NO functions and sources in plant resistance to the fungus are scarce. We have analysed by a genetic approach, NO function in Arabidopsis resistance and defence response against *F. oxysporum*. Thus, two mutants with altered NO metabolism, such as the double mutant *nia1 nia2*, affected in nitrate reductases (NR1/NIA1 and NR2/NIA2), with a previously suggested role in NO biosynthesis (Yamasaki and Sakihama, 2000; Neill *et al*., 2002; Rockel *et al*., 2002; Guo *et al*., 2003), and one indirect NO overproducer, impaired in a chloroplast phosphoenolpyruvate/phosphate translocator *nox1*/*cue1*, *nox1* from now on (Streatfield *et al*., 1999; He *et al*., 2004), have been tested for defence response and resistance against *F. oxysporum*.

4.4.2. Materials and methods

4.4.2.1. Plant growth conditions and fungal strains

For infection assays, the pathogenic isolate *Fusarium oxysporum* f. sp. *conglutinans* PHW 699-3 (ATCC 58110) or a green fluorescent protein (GFP)-expressing derivative thereof (Hou *et al*., 2014) were used. Microconidia suspension was stored in glycerol 30% at -80ºC until use. For each experiment, microconidia were obtained from fungal cultures grown for 4 days in potato dextrose broth and glucose 20 g/L in Erlenmeyer flasks on a rotary shaker at 170 rpm. After that, spores were isolated and quantified with Neubauer chamber by optical microscopy (Di Pietro *et al*., 2004; Turrà *et al*., 2016).

Arabidopsis thaliana (Col-0) seeds: WT, *nia1 nia2* (Yamasaki and Sakihama, 2000; Rockel, 2002) and *nox1* (Streatfield *et al*., 1999; He *et al*., 2004), were surface-sterilized and stratified 48 hours at 4ºC and then sown on Hoagland solid medium (0.5x) pH 5.6 (Hoagland and Arnon, 1950). Seeds were grown at 22ºC, 100 µE of irradiance, 60-65% relative humidity and 16/8 h light/dark conditions for 14 days.

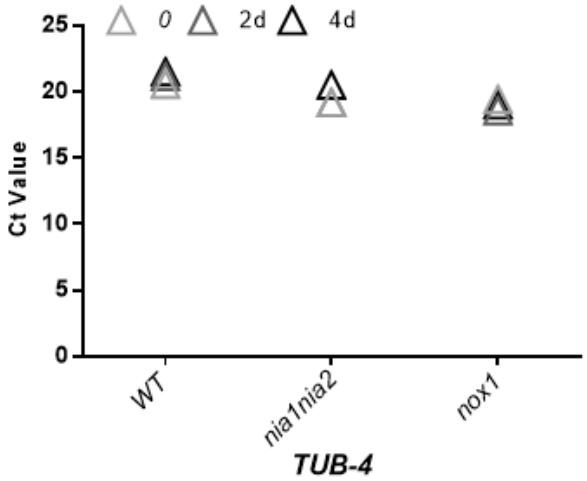
4.4.2.2. Plant infection assays

A. thaliana wild type (WT; Col-0) and mutants *nia1 nia2* and *nox1* root infection assays were performed as described previously (Masachis *et al*., 2016). Roots of two-week-old Arabidopsis seedlings were immersed for 30 min in a 5×10^6 microconidia ml^{-1} suspension of *F. oxysporum* f. sp. *conglutinans 699,* an isolate that has previously been shown to infect Arabidopsis (Hou *et al*., 2014) and planted in mini-pots with soil/vermiculite (1:1). At least, sixty plants per treatment and genotype were used in each experiment. Arabidopsis were then grown in a growth chamber at 24ºC, 120 µE of irradiance, 60-65% relative humidity and 16/8 h light/dark conditions

for 25 days. Survival was observed daily, calculated by the Kaplan-Meier method, and compared among groups using the log-rank test as described before (Masachis *et al*., 2016). GFP-expressing transformant of the fungal strain was used for microscopy experiment. For determination of the fungal burden, plants were harvested at 0, 2 and 7 days post infection (dpi) for DNA extraction.

4.4.2.3. Quantification of gene expression and fungal burden by real-time quantitative PCR (RT-qPCR)

To isolate total RNA, Trizol reagent (Invitrogen) was used according to the manufacturer's protocol and DNase treatment was then applied (Ambion DNA free). Reversed transcription with 5x primer script RT master mix (Takara) was carried out with 1.2 µg of RNA as a described previously (Rodríguez-Serrano *et al*., 2016). Gene-specific primers (Suppl. Table S1-S2) were used for RT-qPCR analysis of transcript levels by using iCycler iQ5 (Bio-Rad, Hercules, CA), and TB Green Premix Ex Taq (Takara). Amplification efficiency was calculated by the formula E = [10 (1/a) -1] × 100 where "a" is the slope of the standard curve. The relative expression of each gene was normalized to that of *TUB4*, whose stability is shown in Suppl. Fig. S4.4.1, and the results were analysed following the Pfaffl method (2001).



Suppl. Fig. S4.4.1. qRT-PCR Ct values of the final candidate reference genes in our condition (mean values).

Total genomic DNA was extracted from infected roots for quantification of fungal burden. RT-qPCR was carried out with *F. oxysporum*/Arabidopsis-specific primers *act2/TUB4*. Relative amounts of fungal DNA were calculated by comparative threshold cycle (ΔΔCt).

4.4.2.4. NO and H_2O_2 detection

To detect nitric oxide and H_2O_2, seedling were incubated with 10 µM 4,5-diaminofluorescein diacetate (DAF-2 DA) and 25 µM 2`7`-dichlorofluorescein diacetate (DCF-DA), respectively, as described elsewhere (Terrón-Camero *et al*., 2020). Aminoguanidine (AG)

and ascorbate were used for specificity of DAF-2DA and DCF-DA, respectively (Suppl. Fig. S4.4.2). Root fluorescence was examined under a confocal laser scanning microscope (Leica TCS).

4.4.2.5. Ferric-chelate reductase and peroxidase activities

Ferric-chelate reductase activity was determined in Arabidopsis seedlings at 1 h post infection (hpi). Seedlings were then transferred to 0.8% water noble-agar supplemented with 0.5 mM $CaSO_4$, 0.5 mM ferrozine and 0.5 mM EDTA Fe (III). After that, seedlings were placed on plates, incubated for 20 min at room temperature and imaged (Schmidt *et al*., 2000; Martínez-Medina *et al*., 2017). Peroxidase activity was determined in Arabidopsis seedlings infected or not with *Fusarium* (1 hpi). Seedlings were transferred on 0.8% water noble-agar supplemented with 0.91 mM ABTS and 2.5 mM H_2O_2. Plants were placed on plates, incubated for 45 min at 28ºC and imaged (Turrà *et al*., 2015).

4.4.2.6. Quantification of phenolic compounds from root exudates

The amount of phenolic compounds in the root exudates was quantified under UV light (365 nm; Berendsen *et al*., 2012; Stringlis *et al*., 2018). Briefly, 100 µL of water with roots exudates, produced by Arabidopsis inoculated or not with *F. oxyporum* for 1 h, were transferred into a 96 well microplate. Subsequently, fluorescence emitted (excitation at 360 nm; emission at 528 nm) was measured with a Varioskan LUX Multimode Microplate.

4.4.2.7. Quantification of cellulose content in roots

Roots were homogenized and centrifuged at 400 g and the supernatants were discarded. Pellets and acetic nitric reagent were mixed and boiled for 30 min. After that, samples were centrifuged at high speed for 5 min. Supernatants were discarded and pellets were washed with distilled water. Then, H_2SO_4 (67%) was added (v/v) and incubated for 1 h. Subsequently, samples were diluted 100 times, mixed with anthrone and boiled for 16 min. Finally, absorbance at 620 nm was measured by an spectrophotometer, at room temperature (Updegraff, 1969).

Cellulose staining was carried out in semithin sections of Arabidopsis roots. Samples were cut and placed in moulds with low melting point agarose at 5% in PBS for 3 h at 4ºC. Roots were then cut in 70 µm slides by a vibrating blade microtome VT1200S (Leica). Safranin 0.3% was prepared in ethanol 50% and slides were stained for 2 min and observed by light microscopy.

4.4.2.8. Statistical analyses

Mean values in the quantitative experiments described above were obtained from at least three independent experiments with at least three independent replicates each experiment.

Statistical analyses were performed using one or two-way ANOVA test when necessary. Mean values for the different treatments were compared using Tukey's multiple comparison tests ($P<0.05$) after the two-way ANOVA analysis or T-Student test after one-way ANOVA analysis, using IBM SPPS Statistic 24 and GraphPad Prism 6. Error bars representing standard error (SEM) are shown in the figures.

4.4.3. Results and discussion

4.4.3.1. *nia1 nia2* survival is compromised after *Fusarium oxysporum* inoculation

We set out to investigate the function of NO in *F. oxysporum* infection on Arabidopsis plants. Initially, we monitored NO production in Arabidopsis roots, WT and NO-related mutants *nox1* and *nia1 nia2*, over time post inoculation (0-3 days post inoculation; dpi). We observed an increase of NO of about 1.7-1.8 times in plants infected with *F. oxysporum* from early on up to 1 day in WT, followed by a significant decrease of NO at 3 dpi. This is consistent with previous reports showing a peak of NO at the onset of Arabidopsis infection with *F. oxysporum* (Gupta *et al*., 2014) and other plant-root fungal interactions, such as olive-*Verticilium dahliae* (Espinosa *et al*., 2014) and tomato-*Rizoctonia solani* (Noorbakhsh and Taheri, 2016). Different fungal elicitors also induced an increase in NO levels (Wang and Wu, 2004; Srivastava *et al*., 2009; Martínez-Medina *et al*., 2019b). We also observed a significant increase of NO production in response to *F. oxysporum* in *nia1 nia2*, affected in nitrate reductases (NR1/NIA1 and NR2/NIA2), following the same trend as WT over the time (Fig. 4.4.1), suggesting that NR is not the main enzyme involved in this NO peak. Additionally, we used aminoguanidine (NOS-l inhibitor), which decreases NO production in seedling roots in response to *F. oxysporum* (Suppl. Fig. S4.4.2A). These results are consistent with the decrease of NO production observed in *Taxus chinensis* culture cells treated with different *F. oxysporum* elicitors and the NOS inhibitor L-NNA (L-N^G-Nitroarginine; Wang and Wu, 2004). In other systems however, such as *Verticillium dahliae* toxins-Arabidopsis, NO production is mainly dependent on NR (Shi and Li, 2008). On the contrary, the NO overproducer *nox1*, which showed higher NO level under control conditions, as previously described (He *et al*., 2004; Hu *et al*., 2014; Zhan *et al*., 2018), showed a significant initial decrease in NO level at 6 hours post infection (hpi). After that, no changes in NO levels were observed until 3 dpi with a decrease, similar to the one observed in WT (Fig. 4.4.1).

To test whether a change in NO level and/or the absence of NR have an effect on fungal virulence, roots of Arabidopsis plants (WT, *nia1 nia2* and *nox1*) were inoculated with *F. oxysporum*.

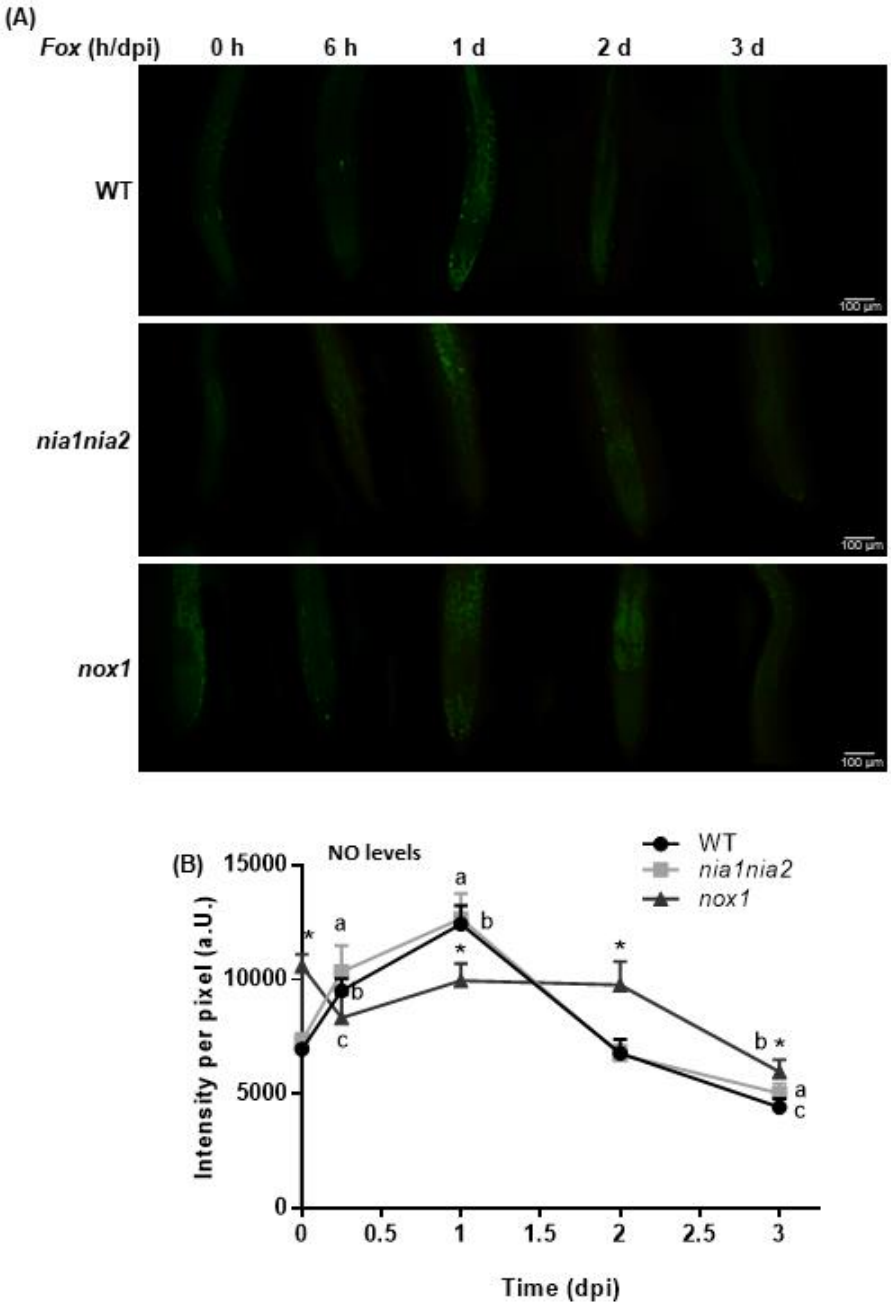
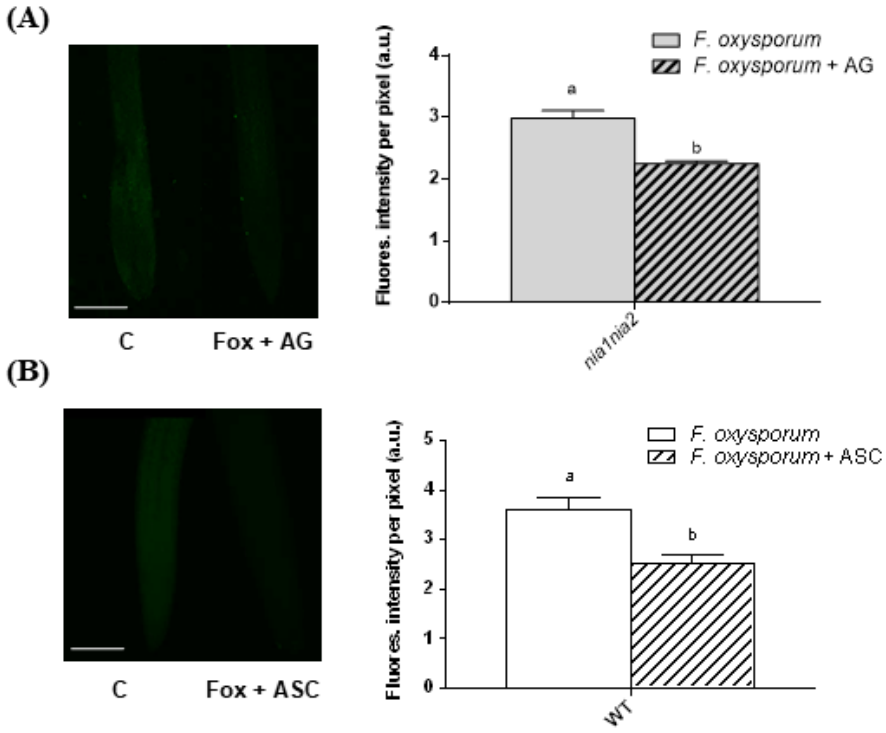


Figure 4.4.1. Effect of *F. oxysporum* on NO accumulation in Arabidopsis-roots. (A) Representative images of NO-dependent fluorescence in Arabidopsis seedling roots in WT, *nia1 nia2* and *nox1* and image quantification (B) of NO in roots determined by confocal microscopy using DAF2-DA before inoculation and 6 h and 1 to 3 d after *F. oxysporum* inoculation (h/dpi). Data represent mean and standard error (error bars) of at least three independent experiments with at least 10 roots on each sample analysed per experiment. Different letters denote significant differences between different time points of *F. oxyporum* treatment and 0 h within the same background. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT according to Tukey's multiple range test ($P<0.05$).



Suppl. Fig. S4.4.2. NO and H_2O_2 detection by CLMS controls. (A) Specificity of DAF2-DA fluorescence for NO is verified by root incubation with the NOS inhibitor aminoguianidine (AG; 1 mM) under *F. oxysporum* infection in *nia1 nia2* roots. **(B)** Specificity of DCF-DA fluorescence for H_2O_2 is verified by root incubation with ascorbate (1 mM) in WT roots. Data represent mean ± standard error (error bars) of three independent experiments. Different letters denote significant differences determined by the T-test ($P<0.05$).

Representative images of plants inoculated or not with *F. oxysporum* at 24 dpi, are shown in Suppl. Fig. S4.4.3 *nox1* plants followed a survival curve similar to WT (Fig. 4.4.2A) and interestingly, a percentage (5-10%) of *nox1* plants always survives to *F. oxysporum* infection. In contrast, *nia1 nia2* plants showed a higher and earlier mortality rate (Fig. 4.4.2A) and contained more fungal biomass (Fig. 4.4.2B) than WT and *nox1* plants (Fig. 4.4.2A-B). In fact, 40% of WT plants died at 15 dpi and 100% at 20 dpi while in *nia1 nia2* 40% of plants died at 7 dpi and 100% at 15 dpi. Thus, the infection by *F. oxysporum* is promoted in *nia1 nia2* plants. To corroborate this result, we used a GFP-marked line of *F. oxysporum* (Hou *et al*., 2014) and observed at the confocal microscope infection evolution in the different backgrounds (Suppl. Fig. S4.4.3B and Fig. 4.4.2C). We found that *F. oxysporum* started to enter Arabidopsis root tissue at 3 hpi (Suppl. Fig. S3B). Accordingly with our previous results, GFP-associated to *F. oxysporum* at 24 hpi is higher in *nia1 nia2* plants than in WT and *nox1* (Fig. 4.4.2C) suggesting a massive infection in *nia1 nia2*.

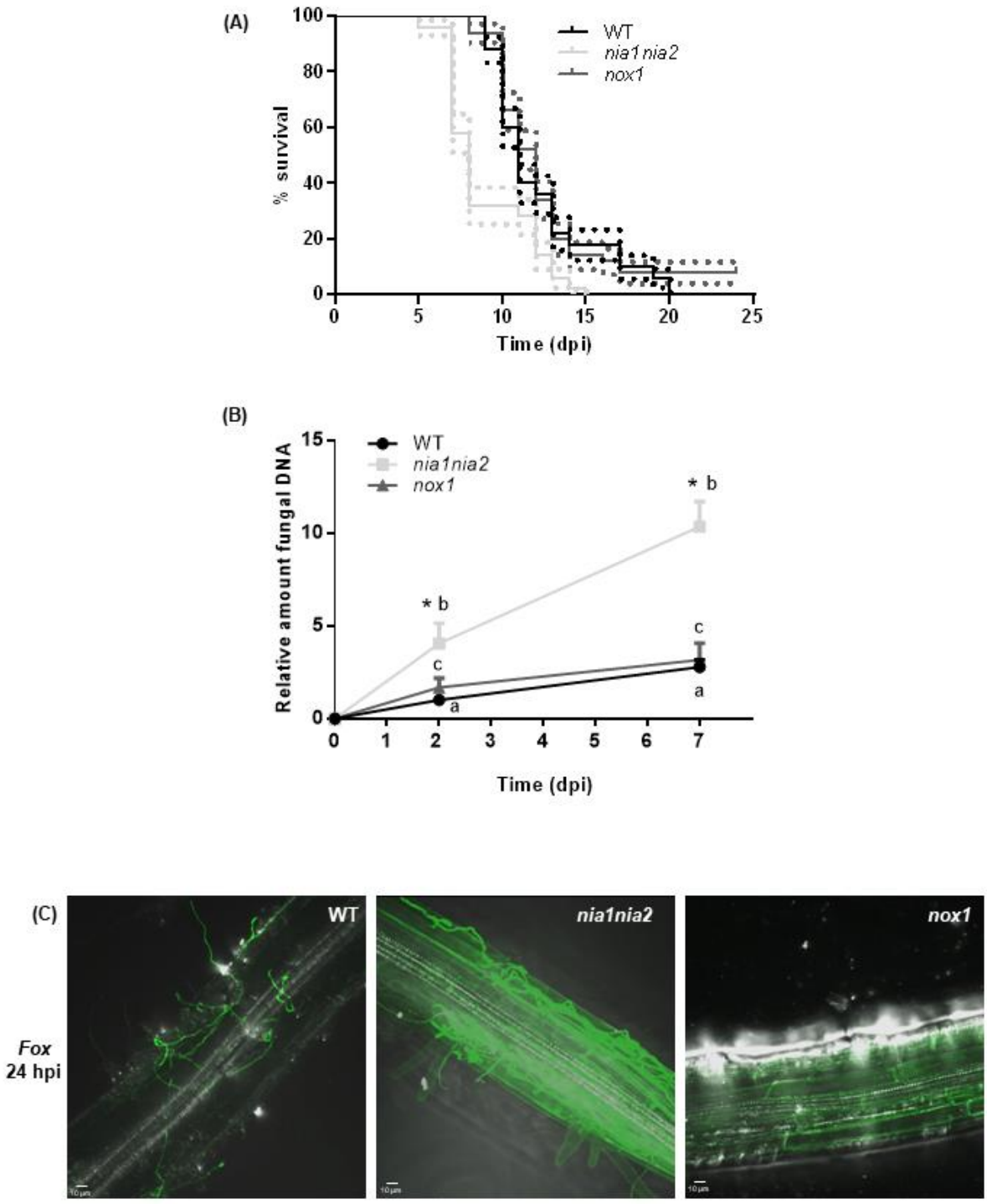
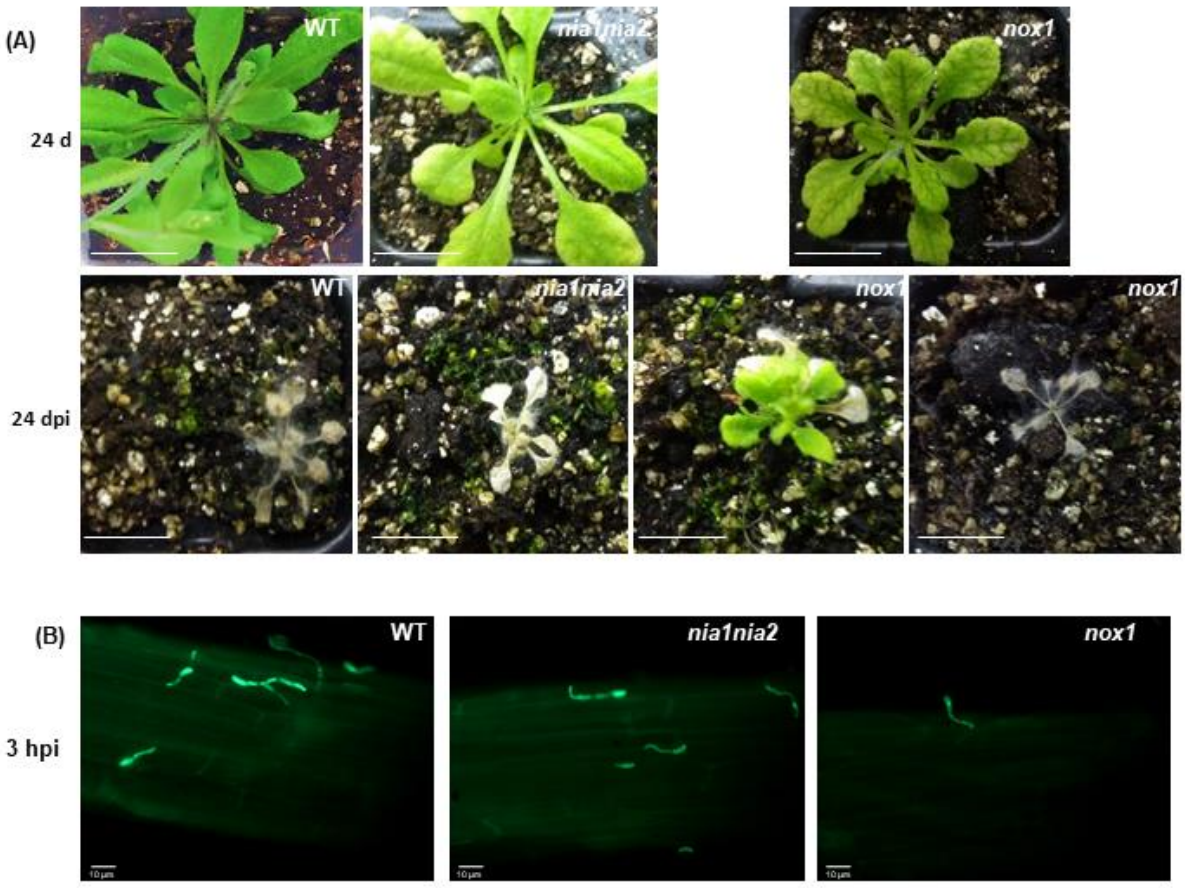


Figure 4.4.2. Infection development after *F. oxysporum* inoculation in Arabidopsis. (A) Kaplan–Meier plot of survival of Arabidopsis plants WT, *nia1 nia2* and *nox1* infected with *F. oxysporum*. Mortality was significantly higher in *nia1 nia2* ($P < 0.0001$) according to the log-rank test. Data correspond to one representative experiment from five independent experiments. (B) Quantification of *F. oxysporum* in Arabidopsis seedling. Relative amount of *F. oxysporum* DNA was determined 2 and 7 d after inoculation by quantitative RT-PCR analysis of the *F. oxysporum Actin* gene relative to the Arabidopsis *TUB2* gene. (C) Roots of Arabidopsis WT, *nia1 nia2* and *nox1* plants were observed by confocal microscopy 24h after inoculation with *F. oxysporum* expressing GFP. Scale bars, 10 µm. Data in (B) represent mean, and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between different time points of *F. oxysporum* treatment and 0 h within the same background. Asterisks denote significant differences between *nia1 nia2* or *nox1* and *WT* according to Tukey's multiple range test (C; $P<0.05$).



Suppl. Fig. S4.4.3. Infection development after *F. oxysporum* inoculation in Arabidopsis. (A) Representative plants from WT, *nia1 nia2* and *nox1* were imaged 24 days after inoculation with *F. oxysporum*. Scale bar, 1 cm. (B) Roots of Arabidopsis WT, *nia1 nia2* and *nox1* plants were observed by confocal microscopy 3 h after inoculation with *F. oxysporum* expressing GFP. Scale bar, 10 μm.

In the interaction tomato-*F. oxysporum*, fungal chemotropism to roots is mediated by root peroxidases (Turrá *et al*., 2015). To further explore if *nia1 nia2* showed higher chemo-attraction for *F. oxysporum* we analysed peroxidase activity exuded by Arabidopsis roots into the adjacent medium. We found an increase in peroxidase activity in WT roots 1 hpi and this activity in *nox1* mutants is similar to that of WT (Fig. 4.4.3A-B). Interestingly, *nia1 nia2* mutants showed significantly lower peroxidase activity in roots independently of inoculation or not with *F. oxysporum* (Fig. 4.4.3A-B), suggesting that peroxidase-dependent chemo-attraction to the fungus is not involved in the higher infection observed in *nia1 nia2*.

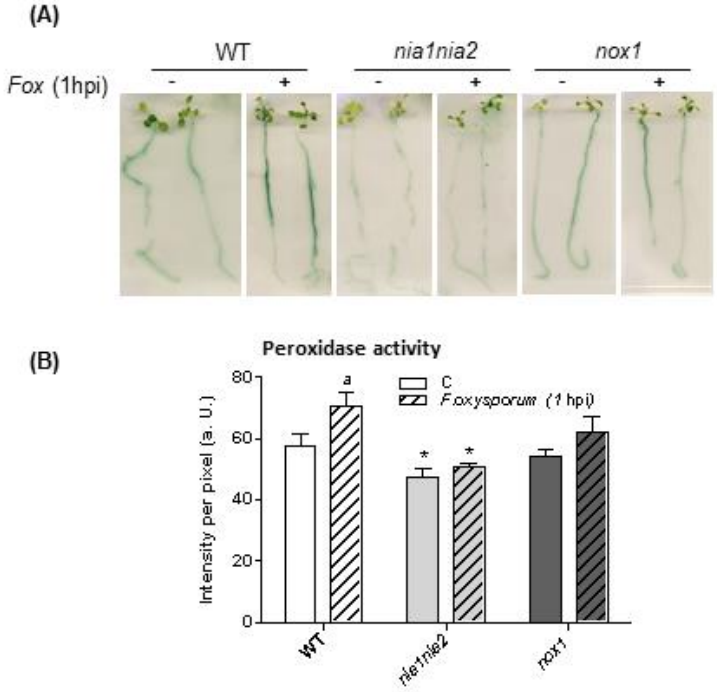


Fig. 4.4.3. Effect of *F. oxysporum* on peroxidase activity in Arabidopsis seedlings. (A) Representative images of peroxidase activity in Arabidopsis seedling roots in WT, *nia1 nia2* and *nox1* and image quantification of peroxidase activity (B) in roots before inoculation and 1 h after *F. oxysporum* inoculation. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between different time points of *F. oxysporum* treatment and 0 h within the same background. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT according to Tukey's multiple range test ($P<0.05$).

4.4.3.2. Arabidopsis defence response to *F. oxysporum*

In the defence response of *Arabidopsis thaliana* against *Pseudomonas syringae* has been shown that NR is required for the transcriptional modulation and bactericidal activity of nitric oxide (Vitor *et al*., 2013). We next asked how the fungus induces defence response during plant infection in our system. Phenols are compounds produced via phenylpropanoid pathway and have been shown to be involved in cell wall lignification inhibiting fungus penetration and may also act as antimicrobial molecules having immunity benefits for the host plant (Noorbakhsh and Taheri, 2016; Stringlis *et al*., 2018). *F. oxysporum* triggers phenols in WT roots exudates 1 hpi (Fig. 4.4.4A) and similar induction was observed in *nia1 nia2* mutants, while no changes were observed in *nox1* mutants (Fig. 4.4.4A). Interestingly, phenols level in both mutants is half-content of the level observed in WT suggesting that an optimal NO level is necessary for phenols production. Data about phenols synthesis and NO are scarce although it has been recently shown that exogenous NO increases phenols content in the tomato-*Rhizoctonia solani* interaction, independently of the susceptibility of the cultivar used (Noorbakhsh and Taheri, 2016).

Early plant defence responses to root pathogens also include H_2O_2 production, directly involved in the reinforcement and cross-linking of cell walls and defence (Heller and Tudzynski,

2011). We detected a transient burst of H_2O_2 in WT roots 4 hpi with *F. oxysporum* (Fig. 4.4.4B-C). After this first H_2O_2 peak, H_2O_2 production oscillated in time, showing another peak at 2 dpi. In any case, H_2O_2 levels were always higher in inoculated WT roots compared to non-inoculated roots (Fig. 4.4.4B-C). *nia1 nia2* mutants followed the same trend as WT after *F. oxysporum* inoculation although H_2O_2 levels in *nia1 nia2* roots were 30% lower than in WT (Fig. 4.4.4B-C). On the other hand, *nox1* showed a continuous increase of H_2O_2 levels after the first peak (Fig. 4.4.4B). In accordance, an early increase in H_2O_2 production after *F. oxysporum* inoculation or *F. oxysporum*-derived elicitor treatment has been described previously in Arabidopsis roots and *T. chinensis* culture cells, respectively (Wang and Wu, 2004; Gupta *et al*., 2014).

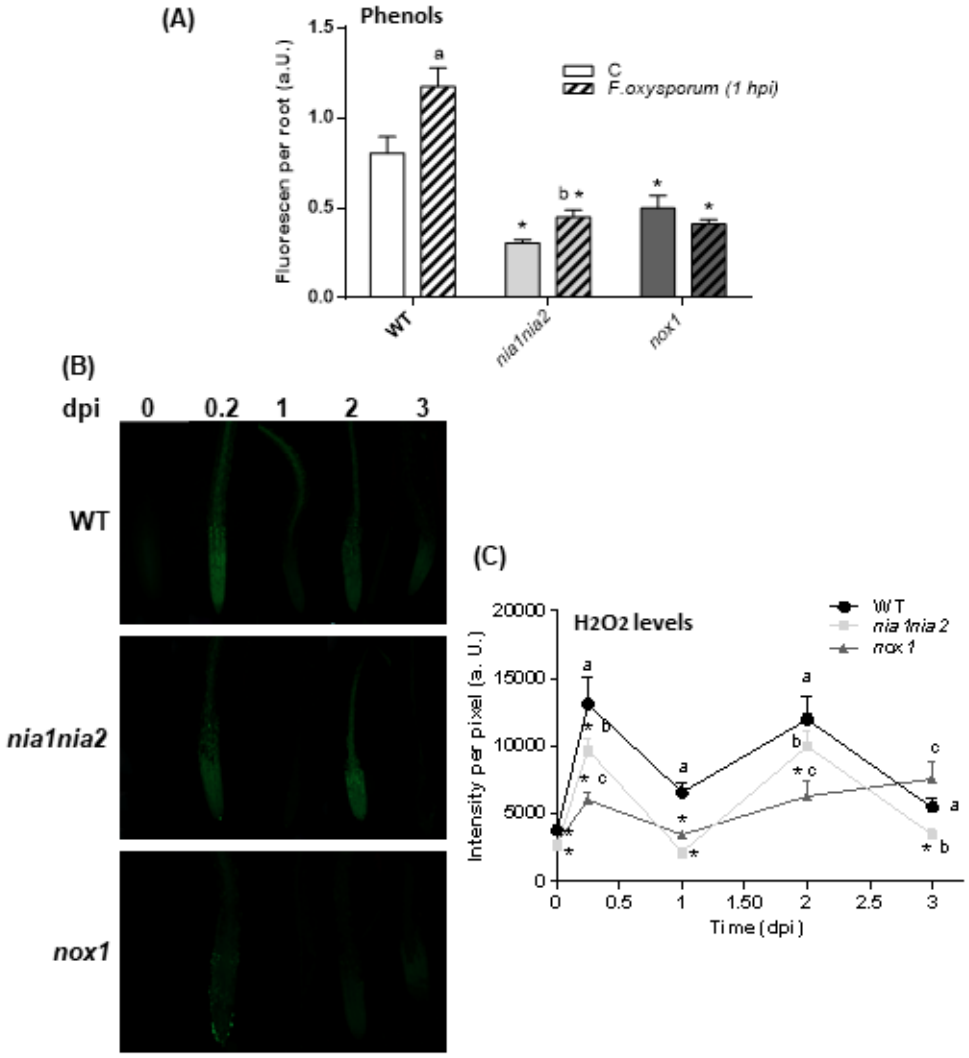


Fig. 4.4.4. Effect of *F. oxysporum* on phenols and H_2O_2 accumulation in Arabidopsis seedlings. (A) Quantification of total exudated phenolic compounds determined by fluorimetry under UV light (365 nm). (B) Representative images of H_2O_2-dependent fluorescence in Arabidopsis seedling roots in WT, *nia1 nia2* and *nox1* and image quantification (C) of H_2O_2 in roots determined by confocal microscopy using DCF-DA before inoculation and 6 h, and 1 to 3 d after *F. oxysporum* inoculation. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between different time points of *F. oxysporum* treatment and 0 h within the same background. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT according to Tukey's multiple range test ($P<0.05$).

Furthermore, in *T. chinensis* culture cells a decrease in the H_2O_2 peak was observed when an exogenous scavenger or inhibitor of NO was applied (Wang and Wu, 2004). In pea guard cells, chitosan-induced NO production occurs downstream of ROS (Srivastava *et al*., 2009). In our hands, oscillations in NO and H_2O_2 production appear to be inversed suggesting a regulation from one to each other. Interestingly, *nox1* mutants, with higher initial NO levels, showed the lowest levels of H_2O_2 throughout the infection process (Fig. 4.4.4B-C).

We further explore transcript levels of Arabidopsis immunity marker genes *PDF1.2*, *VSP2*, *PR-1* and *PR-5* (Chen *et al*., 2014; Thatcher *et al*., 2016). We observed an increase in the expression of the JA-related defence protein *PDF1.2* at 4 dpi, similar to results previously reported in Arabidopsis (Chen *et al*., 2014; Thatcher *et al*., 2016), while in our hands, no significant differences were observed in *VSP2* at this time (Fig. 4.4.5A-B).

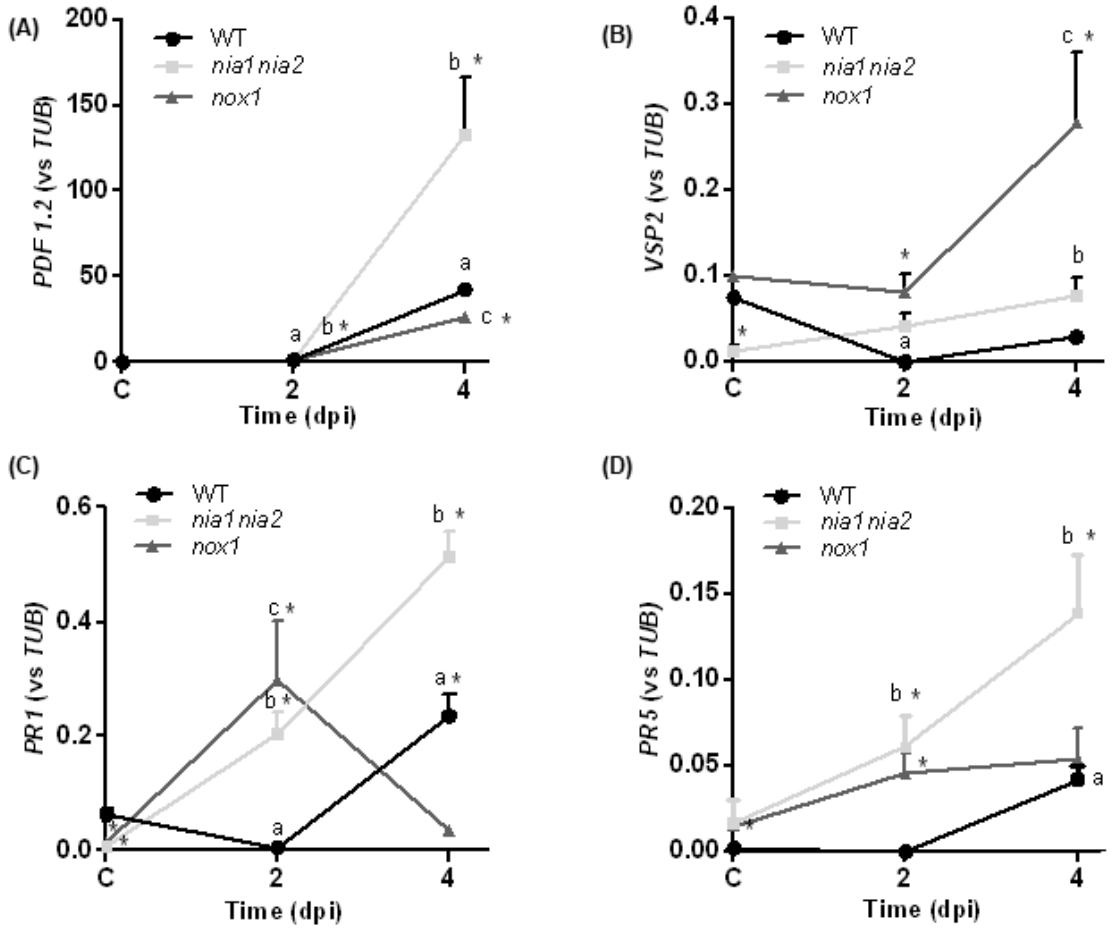


Figure 4.4.5. Effect of *F. oxysporum* on *defence genes* expression in Arabidopsis seedlings. Analysis of *PDF 1.2* (A)*; VSP2* (B)*; PR1*(C) and *PR5* (D) expression by qRT-PCR under control (0 h) and *F. oxysporum* inoculation after 2 d and 4 d in *nia1 nia2*, *nox1* and WT seedlings. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between different time points of Cd treatment and 0 h within the same background. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT according to Tukey's multiple range test ($P<0.05$).

A slightly lower induction of *PDF1.2* was observed in *nox1* mutants although in *nia1 nia2*, the induction was much higher than in WT. WT roots also showed an induction of NO/SA-dependent genes *PR1* and *PR5* at 4 dpi (Fig. 4.4.5C-D). Interestingly, induction of these genes was earlier (at 2 dpi) in mutants *nox1* and *nia1 nia2*, being also higher in *nia1 nia2* at 4 dpi (Fig. 4.4.5C-D). Earlier induction of NO-dependent defence genes is probably related with 5-10% increase of survival observed in *nox1* mutants. All results together showed that apparently defence response is not affected in *nia1 nia2* mutants respect to WT plants, and even gene induction is greater, suggesting that the higher fungal burden observed in *nia1 nia2* offset plant response.

4.4.3.3. Iron metabolism in Arabidopsis plants in response to *F. oxysporum*

Regulation of iron homeostasis is a main strategy in host-pathogen interactions. Plants use scavenging strategies to decrease pathogen accessibility and therefore virulence (Verbon *et al*., 2017). Arabidopsis exploits the root-specific Strategy I, which increase Fe uptake when necessary (Yi and Guerinot, 1996; Zhang *et al*., 2019). Initially, an acidification of the soil is produced by H^+-ATPases localized in the plasma membrane, to enhance solubility of Fe (Colangelo and Guerinot, 2004). The transcription factor FER-LIKE IRON DEFICIENCY (FIT) is a master regulator of this Strategy and regulates the expression of different Fe uptake genes such as the one coding for the enzyme FERRIC REDUCTION OXIDASE2 (FRO2) and the affinity IRON REGULATED TRANSPORTER1 (IRT1), which reduces soluble Fe^{3+} to Fe^{2+} and transport Fe^{2+} into the plant root, respectively (Colangelo and Guerinot, 2004). We observed and induction of these three genes in WT roots at 2 dpi, while no changes were observed in *nia1 nia2* and *nox1* suggesting that an optimal NO level is needed for this induction (Fig. 4.4.6A-C). An increase on the FRO activity was observed in WT roots 1 hpi and in *nox1* mutants while in *nia1 nia2* no activity was found (Fig. 4.4.6 D). Interestingly, basal activity was observed in *nox1* mutants under control conditions (Fig. 4.4.6 D). It has been shown that the NO scavenger cPTIO inhibits the induction of *FIT1*, *FRO2* and *IRT1* and that the presence of NO inhibits FIT1 degradation (Meiser *et al*., 2011). Furthermore, exogenous application of ET and NO induce *FRO2* and *IRT1* in Arabidopsis plants, enhancing iron uptake (García *et al*., 2010). In our hands however, higher endogenous NO avoid the induction of Fe-related genes suggesting that endogenous and exogenous NO may have different effect on the plant, and that a tight NO level is needed for an accurate induction of Strategy I. Other mechanisms involved in regulation of Fe-related genes cannot be ruled out, however. Apparently, a crosstalk between different hormones is involved in regulation of Fe-dependent genes as SA induces *FRO2* and *IRT1* (Shen *et al*., 2016), while JA inhibits their induction independently of *FIT1* (Cui *et al*., 2018). In any case, *nia1 nia2* behave in a similar way to *nox1* respect to Fe-dependent genes, suggesting that this behaviour is not related with the higher sensitivity to *F. oxysporum* observed in *nia1 nia2* mutants.

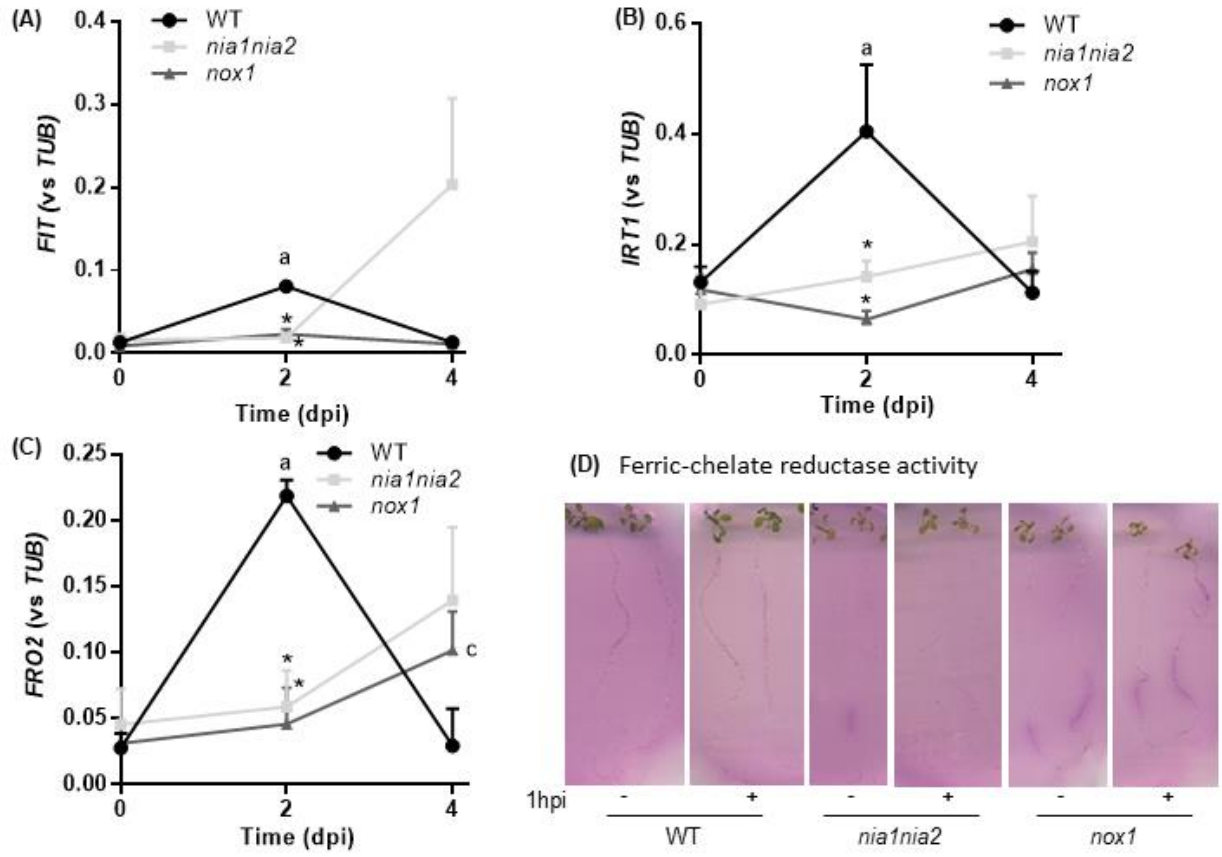


Figure 4.4.6. Effect of *F. oxysporum* on iron metabolism in Arabidopsis seedlings. Analysis of *FIT* (A), *IRT1*(B) *and FRO2* (C) gene expression by qRT-PCR under control (0 h) and *F. oxysporum* inoculation after 2 d and 4 d in *nia1 nia2*, *nox1* and WT seedlings. (D) Representative images of ferric-chelate reductase activity in Arabidopsis seedling roots in WT, *nia1 nia2* and *nox1* under control (0 h) and *F. oxysporum* inoculation after 1 h. Data represent mean and standard error (error bars; in A, B, and C) of at least three independent experiments. Different letters denote significant differences between different time points of Cd treatment and 0 h within the same background. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT according to Tukey's multiple range test (P<0.05).

4.4.3.4. Cellulose metabolism of Arabidopsis in response to *F. oxysporum*

Cellulose is composed of β-(1,4)–linked glucose chains, which are synthesized and exported to the apoplasts by cellulose synthase (CesA) complexes, localized in the plasma membrane (McFarlane *et al*, 2014). It is an essential component of plant cell wall, which is directly involved in the sense, transduction and response to different stimuli, being the first barrier for pathogens (Kesten *et al*., 2017). In fact, pathogenic fungi such as *Fusarium graminearum* have a number of genes encoding cellulases, which are induced during plant infection (Zhang *et al*., 2016), suggesting the need to decompose cellulose and weaken the cell wall to facilitate pathogen entry in the cells (Kesten *et al*., 2017). To determine if higher entry of *Fusarium oxysporum*, in *nia1 nia2* mutants, was due to a higher cell wall weakness, we visualized cellulose by safranin. We observed a depletion of cellulose content in *nia1 nia2* roots respect WT and *nox1* (Fig. 4.4.7A-D). We then quantify cellulose concentration by spectrophotometry and found a decrease of about 60% in *nia1 nia2* mutants respect WT (Fig. 4.4.7 E), supporting data obtained by microscopy. It

has been described previously that *nia1 nia2* showed a decrease in shoot and root growth under normal conditions (Kolbert *et al*., 2015), recovering root growth when the medium is supplemented with NO (Lozano-Juste *et al*., 2010). On the other hand, dwarf phenotypes have been described in mutants affected in cellulose synthase genes (CesAs; McFarlane *et al*., 2014; Chen *et al*., 2018; Gigli-Bisceglia *et al*., 2018).

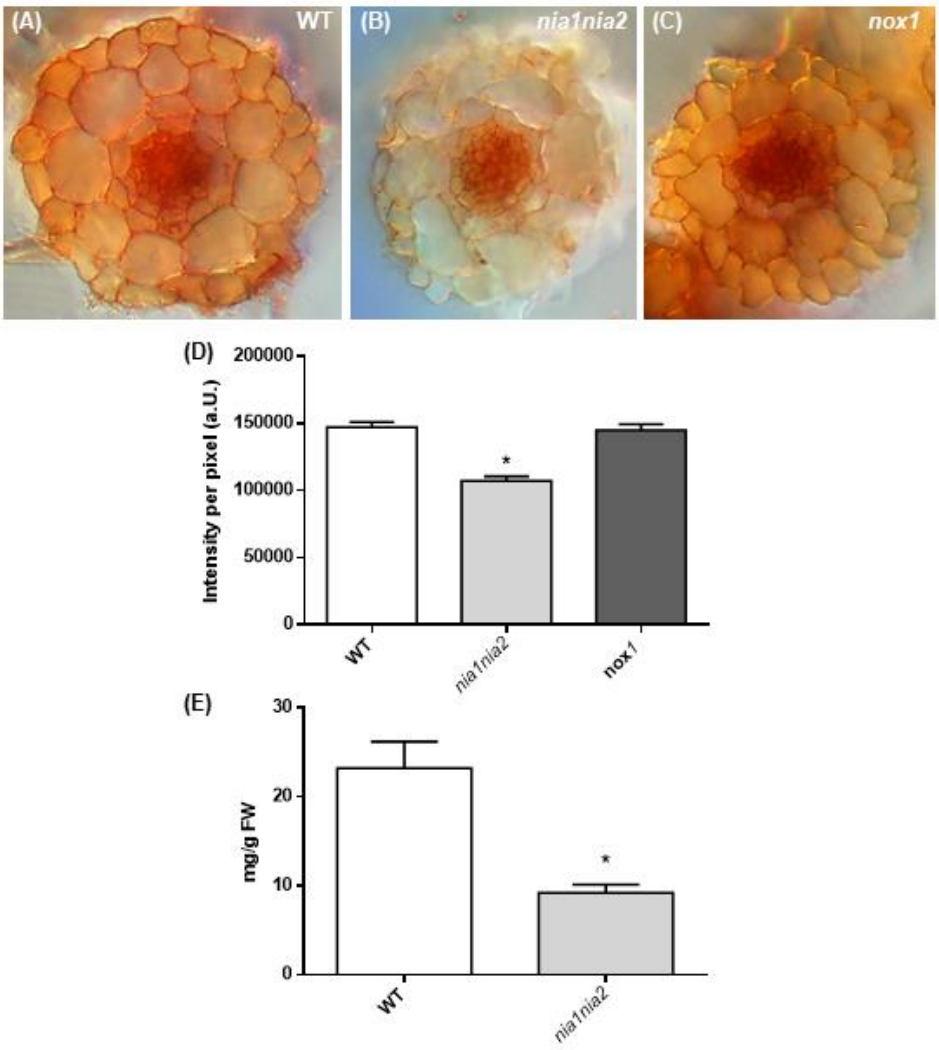
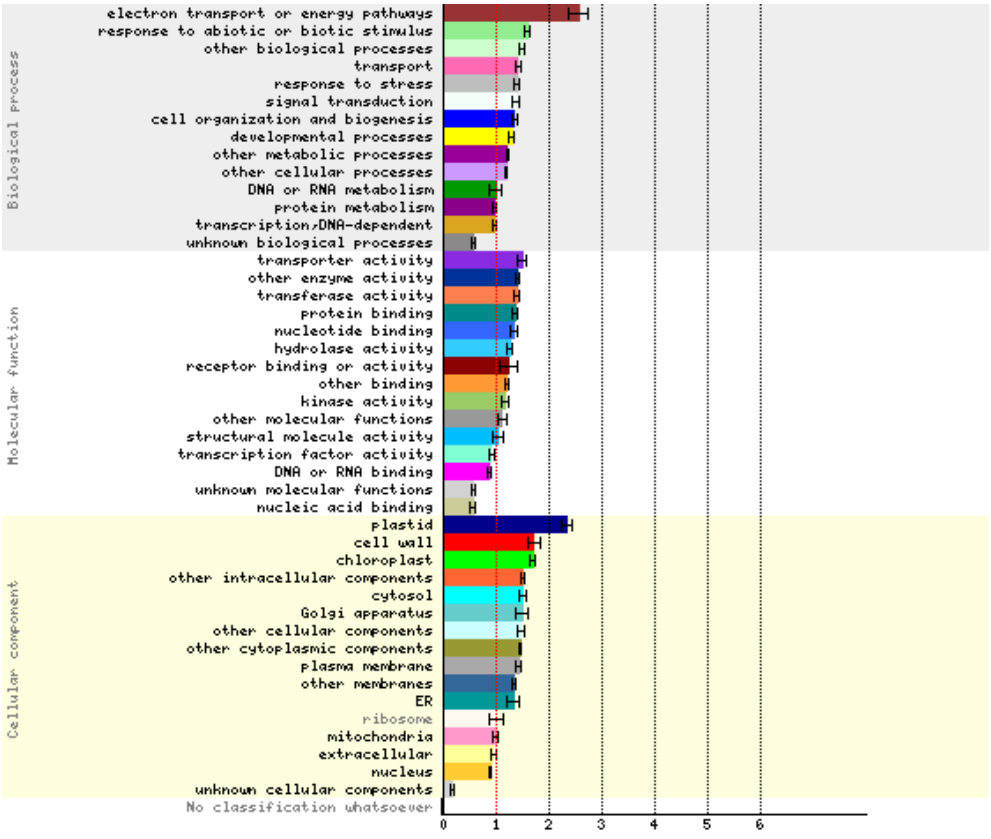


Figure 4.4.7. Cellulose content in Arabidopsis seedlings. (A-C) Representative images of cellulose content and quantification (D), in WT, *nia1 nia2* and *nox1*seedling roots. (E) Cellulose content measured in WT and *nia1 nia2* root extracts. Data represent mean and standard error (error bars; in D and E) of at least three independent experiments. Different letters denote significant differences between different time points of Cd treatment and 0 h within the same background. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT according to Tukey's multiple range test ($P<0.05$).

We then look for altered genes in *nia1 nia2* mutants respect to WT, from published data (Gibbs *et al*., 2014) and classified by GO (http://bar.utoronto.ca/ntools/cgi-bin/ntools/classification_superviewer.cgi; Suppl. Fig. S4.4.4). We found that 6.6 % of the altered genes (from a total of 262 genes) in *nia1 nia2* respect to WT are related to cell wall, being differences in this category statistically significant (Suppl. Fig. S4.4.4). We searched databases to look for genes in this group related with fungus, being only cellulose synthase A4 (*Ces4A*) classified in this category.



Suppl. Fig. S4.4.4. Gene ontology (GO) categories in *nia1 nia2* genes. Classification of genes which present altered expression in *nia1 nia2* respect to WT from published data (Gibbs *et al*., 2014) and classified by GO (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi). Bold titles indicate genes categories represented with significant statistical differences compared to whole Arabidopsis genes. Red bard show Cell Wall as one of the categories which shows significant differences.

We then analysed *CES4A* expression during the infection process and found an inhibition in WT plants from the beginning, being significant at 4 dpi (Fig.4.4.8A). Accordingly, expression angler database (http://bar.utoronto.ca/eplant/) showed an inhibition of the gene in plant response to *Phytophthora infestans* spores and *Botrytis cinerea* conidiospores. Furthermore, mutations in CESA subunits required for secondary cell wall formation (CESA4, CESA7 and CESA8), conferred to Arabidopsis enhanced resistance to the necrotrophic fungus *Plectosphaerella cucumerina* and the soil-borne bacterium *Ralstonia solanacearum* (Hernandez-Blanco *et al*., 2007) suggesting a role for this enzymes in activation of defence pathways. No significant differences where observed in *nox1* and remarkably, an opposite pattern, respect to WT, was found

in *nia1 nia2* where we observed a two-fold induction of *CES4A* at 4 dpi (Fig. 4.4.8A). It has been recently shown that *CES4A* is regulated by the transcription factor *MYB46* (Kim *et al*., 2014). In fact, MYB46 has been proposed as a transcriptional activator of secondary cell wall biosynthesis and lignin deposition (Zhong *et al*., 2007) and recently, has been shown to function as a disease susceptibility modulator to *B. cinerea* being able to integrate cell wall remodelling and downstream activation of secondary lines of defence (Ramírez *et al*., 2011). We then analysed MYB46 expression pattern during infection. Interestingly, while no changes in *MYB46* expression were found in WT and *nox1*, around a 10-fold induction was observed in *nia1 nia2*, similarly to that occurring with *CES4A* (Fig. 4.4.8 B). All results together suggest that in *nia1 nia2* mutants cellulose synthesis and regulation of *MYB46* and *CesA4* after *Fusarium* inoculation is altered leading to a higher susceptibility. Further work however, is needed to clarify mechanisms underlying cellulose synthesis and NR dependence, which affect plant resistance.

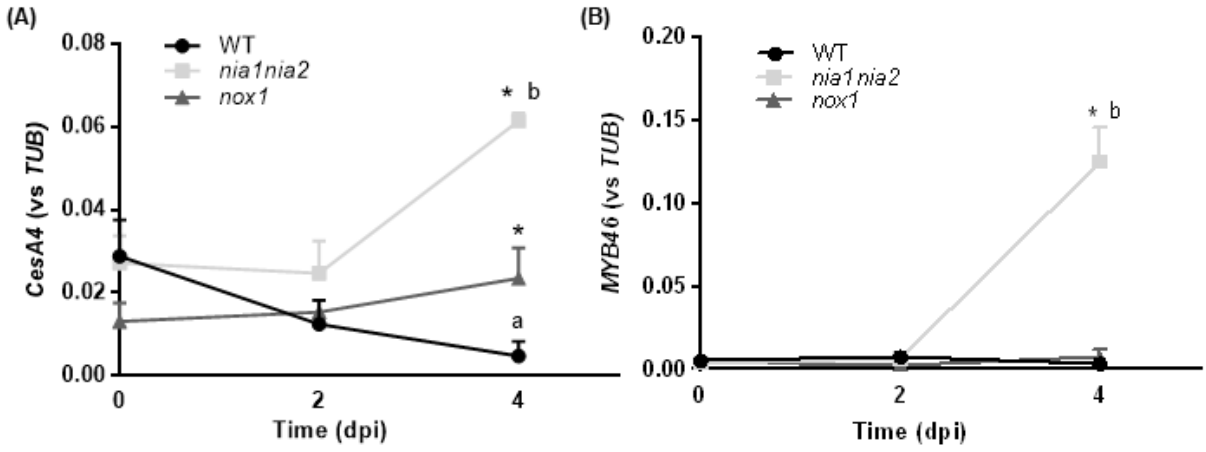


Figure 4.4.8. Effect of *F. oxysporum* on cellulose-related genes in Arabidopsis seedlings. Analysis of *CesA4* (A) *and MYB46* (B) gene expression by qRT-PCR under control (0 h) and *F. oxysporum* inoculation after 2 d and 4 d in *nia1 nia2*, *nox1* and WT seedlings. Data represent mean and standard error (error bars) of at least three independent experiments. Different letters denote significant differences between different time points of Cd treatment and 0 h within the same background. Asterisks denote significant differences between *nia1 nia2* or *nox1* and WT according to Tukey's multiple range test ($P<0.05$).

4.4.4. Conclusion

We demonstrated that fine-tuned NO accumulation is required for proper plant response to *Fusarium oxysporum* infection as both Arabidopsis mutants, *nox1* and *nia1 nia2* showed altered defence response (Fig. 4.4.9). Although plant response through defence genes is enhanced in both mutants, while in *nox1* this boost maybe leading to an increased survival respect to WT; in *nia1 nia2* is not sufficient to overcome plant death, probably due to weakness in the cell wall. How nitrate reductase and thus NO, is affecting cell wall synthesis under control and defence response is an important issue to decipher.

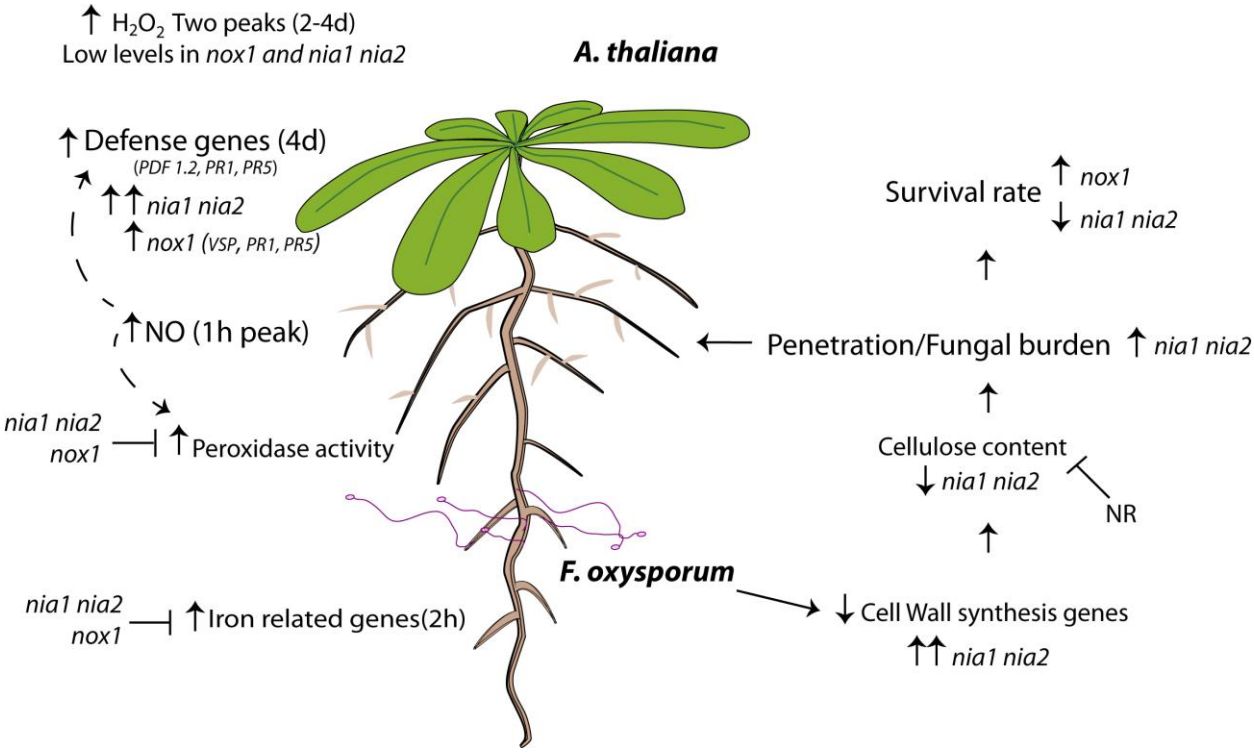


Figure 4.4.9. Scheme showing Arabidopsis, WT and NO-related mutants, in response to *Fusarium oxysporum*. *Fusarium oxysporum* infection induces plant defence response through increasing NO and H_2O_2 levels. Timing in NO is altered in *nox1* mutant while in *nia1 nia2* is very similar to that of the WT. H_2O_2 timing and level is mainly altered in *nox1*, showing lower levels of H_2O_2 during the infection, although a first peak is observed. An induction of peroxidase activity and iron related metabolism is also observed in plant response to Fusarium, but it is affected in both mutants suggesting a fine-tune regulation of NO for a proper response. Finally, an induction of defence genes is detected, enhanced in both mutants and probably leading to a higher survival in *nox1* mutants. Interestingly, in *nia1 nia2* a weakness in cell wall is observed, which probably boost fungal penetration and plant death despite the plant defence response display.

4.4.5. Acknowledgements

This research was supported by grants EX12-BIO296, from Junta de Andalucía and PGC2018-098372-B-100 from the Ministry of Science, Innovation and Universities together and the European Regional Development Fund co-funding (MCIU/AEI/ERDF). LC T-C and E M-M were supported by fellowships for Academic Staff from the Spanish Ministry of Education, Culture and Sports (FPU14/00622 and FPU17/04303, respectively), and MA P-V was supported by a fellowship from MCIU/AEI/ERDF (BES-2016-076518). *Fusarium oxysporum* isolates were kindly donated by Prof. Di Pietro (University of Cordoba, Spain). Confocal laser fluorescence microscopic analyses were carried out at the EEZ. None of the authors have any conflict of interest to declare.

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Chapter 4.5

4.5. Plant response to cadmium and Fusarium: crosstalk and NO role.

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Highlights

ABSTRACT

Plants in their habitats are continuously affected by wide range of biotic stresses and abiotic stress being some of them simultaneous. Therefore, plants present complex signalling pathways to survive against single stresses or stress combination. Some stress combinations may have positive effects on plants when compared with stresses applied individually. Here, we analyzed crosstalk between plant response to cadmium and *Fusarium oxyporum* and the role that the signal molecule nitric oxide (NO) may play in Arabidopsis response to stress. Our results suggest that Arabidopsis enhanced resistance to *F. oxysporum* pre-treated with Cd may be explained by a cluster of genes altered related by Cd stress that could be regulated by NO and *F. oxysporum*. The regulation of important signalling pathways such as MAPK, hormone signalling, secondary metabolism, and pathogen-related proteins could be responsible for plant resistance.

Key words: cadmium; *Fusarium oxysporum;* nitric oxide; reactive oxygen species; reactive nitrogen species; transcriptome.

4.5.1. Introduction

Plant biodiversity, crop yield and global food security are seriously influenced by abiotic and biotic stresses. Plants, as sessile organisms in their natural habitats, are threaten by a variety of biotic stresses such as the one caused by fungus, bacteria, phytoplasms, oomycetes, nematodes, and non-cellular pathogens; and abiotic stress factors such as the ones related with water, temperature, light, contamination, etc. (Mantri *et al*., 2014; Nejat and Mantri, 2017), being some of them simultaneous.

Complex signalling pathways are involved in plant defence to survive against single stresses or stress combination. Plants are able to coordinate wide transcriptional reprogramming and display an unique program of transcript response to the stress that may be not similar to either stresses individually, although there may be a significant overlap in response pathways to combined environmental stresses (Mantri *et al*., 2010; Atkinson 2013; Prasch and Sonnewald 2013; Rasmussen *et al*., 2013; Sham *et al*., 2015). It appears that a significant overlap pathways and several defence genes are commonly involved in response to multiple biotic and abiotic stresses as shared general stress-responsive genes (Mantri *et al*., 2010; Massa *et al*., 2013; Narsai *et al*., 2013; Shaik and Ramakrishna 2013; Sham *et al*., 2014, 2015; Zhang *et al*., 2016).

Several studies showed that an abiotic stress have a negative effect on biotic stress resistance and can lead to enhanced plant susceptibility (Wang *et al*., 2009; Atkinson, 2012; Prasch and Sonnewald 2013; Sewelam *et al*., 2014; Suzuki *et al*., 2014; Kissoudis *et al*., 2014). It might be possible that the activation of certain genes such as receptor like kinases (RLK) or some transcription factors (TFs) by abiotic stress may have an opposite effect against a biotic stress (Zhao *et al*., 2013; Jun *et al*., 2015; Bai *et al*., 2018). However some stress combinations, may have positive effects on plants, when compared with stresses applied individually (Suzuki *et al*., 2014). Mao and collaborators (2009) performed a meta-analysis of 1094 microarrays of Arabidopsis and revealed that genes involved in photosynthesis, protein synthesis and response to oxidative stress are also largely involved in response to environmental stresses. Others studies also shown the importance of ROS/RNS, MAP-kinase cascades, heat shock proteins (HSPs), ion transporters, autophagy components and hormone signalling pathways in the plant response to both, biotic and abiotic stress (Poschenrieder *et al*., 2006 Atkinson and Urwin 2012; Kissoudis *et al*., 2014; Perez and Brown, 2014; Schwessinger *et al*., 2015; Wang *et al*., 2015; Bai *et al*., 2018).

Moreover, several factors such as epigenetic mechanism, plant species and variety, tissues, developmental stage, stress intensity, timing, climate, soil properties and human activity also modulate different by depending on a single or a combination of stresses (Wu *et al*., 2009; Mantri *et al*., 2010; Skibbe *et al*., 2010; Cramer *et al*., 2011; Weston *et al*., 2011; Massa *et al*., 2013; Shaik

et al., 2013; Asai and Shirasu, 2015; Li *et al*., 2015; Pandey *et al*., 2015). All these data show the complex nature of interactions and exhibit the complex mechanisms comprising overlap and specific signalling pathways fine-tune plant responses.

Heavy metals are an interesting example for in-depth study as an abiotic stress. It has been suggested that several plant species may capture high concentration of metals from the substrate as a self-defence mechanism against pathogens and herbivores (Poschenrieder *et al*., 2006; Llugany *et al*., 2018). These authors propose at least five different modes of action of metal defence against biotic stress whenever plants showed some metal resistance: 1. Phytosanitary effects; 2. Metal defence as antifeedant or a plant systemic pesticide (hyperaccumulator plants), 3; Trade-off of organic defences; 4. Metal therapy by substitution of a defective signalling system, and 5. Fortification of defences (Michaud and Angela, 2003; Coleman *et al*., 2005; Matyssek *et al*., 2005;). It should be noted that heavy metal, among others stresses, induced antioxidant defence to diminish ROS/RNS-derived damage (Poschenrieder *et al*., 2006; Choudhury *et al*., 2017; Terrón-Camero *et al*., 2019).

Understanding-signalling crosstalk between mechanisms of the plant defence pathways under combined stress conditions and the potential beneficial effects that would mild aggressive future stress in plants, is still limited, being even more limited in response to heavy metals and pathogens root fungi (Martinez-Medina *et al*., 2019; Poschenrieder *et al*., 2006). In this work, we get a deeper insight into an in-house transcriptomic analysis in plant response to Cd stress and search for transcripts differentially expressed in response to the heavy metal and related with biotic stress. We then showed that previous treatment with Cd can improve plant resistance to *Fusarium oxysporum*. After that, we complete a second layer of analysis searching for Cd-responsive genes related with biotic stress that might be regulated by NO, wich has been addressed widely as a good molecule for improving plant response to stress and specially to biotic stress (Choudhur *et al*., 2017; Begum *et al*., 2019; Martinez-Medina *et al*., 2019; Terrón-Camero *et al*., 2019). Finally, we narrowed gene selection by searching for genes specifically regulated in plant response to Cd and Fusarium.

4.5.2. Material and methods

4.5.2.1. Plant Cd treatment

Arabidopsis thaliana seedlings grown in solid Hoagland medium (0.5x) for 10 days were incubated 24 h in liquid Hoagland medium (0.5x). After that time, seedlings were treated with liquid Hoagland medium (0.5x) + 100 µM of Cd or not (as a control) for 24 h. Subsequently, Arabidopsis seedlings were transferred to Hoagland medium (0.5x) for 48 h before the infection.

4.5.2.2. Plant infection assay

A. thaliana infection was performed as described previously (Masachis *et al*., 2016). Root of two-weeks Arabidopsis seedlings previously treated or not with Cd as described before, were immersed for 30 min in a 5×10^6 microconidia ml^{-1} suspension of *F. oxysporum* f. sp. *conglutinans 699*, an isolate that has previously been shown to infect Arabidopsis (Hou *et al*., 2014), and planted in mini-pots with soil/vermiculite (1:1). At least, 60 plants per treatment and genotype were used in each experiment. Arabidopsis seedlings were then grown in a growth chamber at 24ºC, 120 µE of irradiance, 60-65% relative humidity and 16/8 h light/dark conditions for 25 days. Survival was observed daily, calculated by the Kaplan–Meier method, and compared among groups using the log-rank test as described before (Masachis *et al*., 2016).

4.5.2.3. Transcriptomic- and meta-analyses.

A transcriptomic analysis was previously carried out in our laboratory, in *Arabidopsis thaliana* (WT; Col 0) seedlings (14 days) in response to Cd 50 µM during 0 h, 30 min and 24 h. Three biological replicates and their corresponding controls were hybridized to ATH1 microarrays (Affymetrix). The files were corrected according to the background and normalized using the Robust Multiarray Averaging (RMA) procedure (Irizarry *et al*., 2003), with a custom chip definition file (CDF), using the Bioconductor "affy" package in the programming language R. This CDF remaps the individual probes on the Affymetrix chip to their corresponding genes, using recent sequencing information from the Arabidopsis Information Resource (TAIR). For each sample pair (control/ 30 min or control/24 h of Cd treatment), and for each gene a two-way t test was performed, with mean expression (log_2). Those genes with a p value less than 0.05 and an expression greater than 2 times respect its control were considered differentially expressed.

We then sourced in Geo expression omnibus (https://www.ncbi.nlm.nih.gov/geo/) and ISI web of Science (https://www.fecyt.es/es/recurso/web-science) to search for transcriptome analyses related with changes in endogenous or exogenous NO levels in *Arabidopsis thaliana* (Table 4.5.1; Table 4.5.2) or *Fusarium oxysporum* infection. Genes differentially expressed respect their control provided by de authors were organized in a database according to treatments and

mutants. Different gene groups were compared by Venny 2.0 application (http://bioinfogp.cnb.csic.es/tools/venny/index.html) and Bioinformatics and evolutionary genomics app (http://bioinformatics.psb.ugent.be/webtools/Venn/) to obtain differences and similarities between treatments selected.

Genes of interest were analysed and classified by "Classification Super Viewer" in different Gen Ontology (GO) categories (http://bar.utoronto.ca/ntools/cgi-bin/). Other programs such as StringDB (https://string-db.org/), GeneMania (https://genemania.org/), and KEGG (https://www.genome.jp/kegg/) were used to obtain a deeper insight into pathways and processes where the genes of interest were involved.

4.5.3. Results and discussion

4.5.3.1. Transcriptome analysis of *Arabidopsis thaliana* in response to Cd

Arabidopsis thaliana seedlings (WT; Col 0) treated with Cd 50 µM for 30 min or 24 h showed 495 genes or 923 genes differentially expressed respect to their control, respectively. The intersection of both dataset showed that 65 genes changed their expression at both times analysed (Fig. 4.5.1).

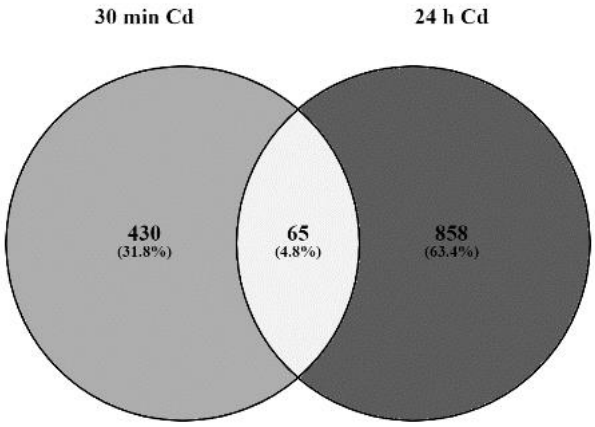

Fig. 4.5.1. Common genes regulated in plant response to Cd stress at different timepoints. Venn diagram with selected genes regulated after 30 min and 24 h of Cd treatment compared to control in WT (Col-0) seedlings, obtained by a transcriptome analysis.

Genes regulated at 30 min Cd treatment were classified into three functionall categories by Gen Ontology (GO): 1. Biological processes, 2. Molecular function and 3. Cell component (http://bar.utoronto.ca/ntools/cgi-bin/). Inside biological process, 13 categories were significantly regulated, i.e.: response to stress (11%), response to abiotic or biotic stimulus (9%) and signal transduction (6%; Fig. 4.5.2 A and Fig. 4.5.3). 12 significant categories were classified in molecular function group such as, transcription factor (9%), protein binding (11%) or transport activity (2%; Fig. 4.5.2 B and Fig. 4.5.3). Main categories significantly represented from a total of

14, in the group of group of cellular component, are nucleus (24%), other membranes (17%), and other cytoplasmic components (13%; Fig. 4.5.2 C and Fig. 4.5.3).

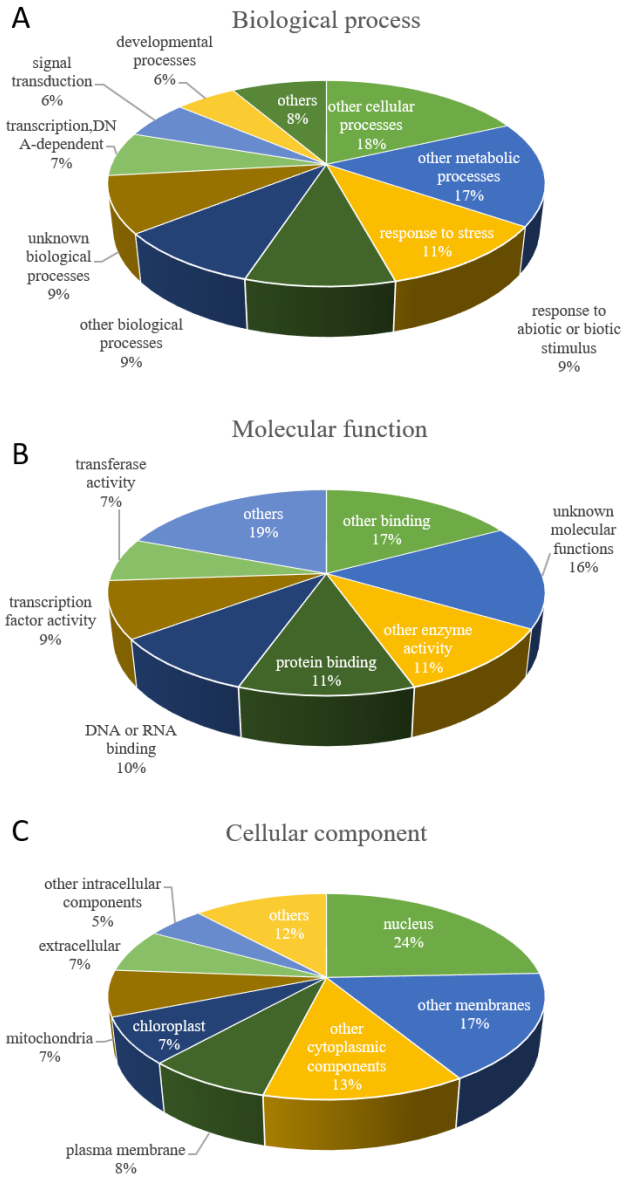

Fig. 4.5.2. Gene ontology clasification of genes regulated by Cd 30 min. (A) Pie-shart percentages werecalculated using the absolute number of genes categorized in one or more Geneontology established groups into Biological function, (B) Molecular functions (C) and Cellular component. Others in Biological Process: protein metabolism, cell organization and biogenesis, transport, electron transport or energy and DNA or RNA metabolism; in molecular function: nucleotide binding, hydrolase activity, receoptor binding or activity and structural molecule activity; in cellular component: unknown cellular componets, cell wall cytosol, ER, golgi apparatus, plastid and ribosome. Significant categories with absolute values in Arabidopsis were obtained from Gene Ontology (http://bar.utoronto.ca/ntools/cgi-bin/).

Although, several transcriptome analyses have been made in plant response to Cd stress, not many are related with short-time treatments. In *oryza sativa*, however, a transcriptome analysis was performed in roots after 1 h treatment showing 1772 genes with altered expression levels

involved in hormone signalling, transcriptional regulation and transport. Dataset obtained was compared with other transcriptomic analysis made by the same authors and plant response to different stresses obtaining that only 104 genes were specifically regulated under Cd exposure (Tan *et al*., 2017). In general, short-term Cd treatment is mainly associated with signalling pathways related with hormone metabolism and transcription factors regulation (Annexe 8.1; Table 8.1.1).

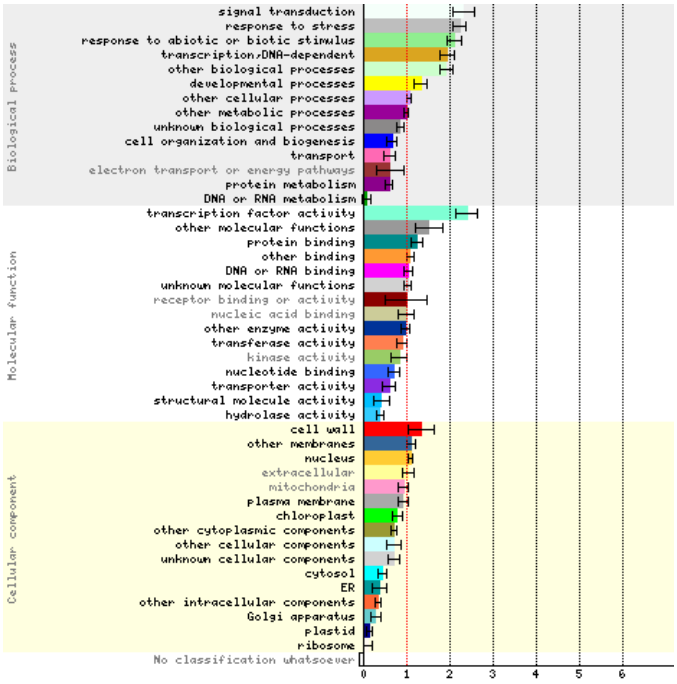

Fig. 4.5.3. Gene ontology clasification of genes regulated by Cd for 30 min treatment. Significant categories normed to frequency of class over all ID numbers in Arabidopsis set obtained from Gene Ontology (http://bar.utoronto.ca/ntools/cgi-bin/;± bootstrap Standard Deviation, p-value) are marked in bold.

Differentially regulated genes after 24 h of Cd treatment showed common significant categories within biological process as in 30 min treatment such as, response to stress (8%), response to abiotic or biotic stimulus (7%) and transport (4%; Fig. 4.5.4 A and Fig. 4.5.5). Interestingly, the electron transport or energy pathway, which was not included earlier, is the most represented within the significant categories after 24 h Cd treatment (Fig. 4.5.4 A and Fig. 4.5.5). Within molecular function category, one of the most represented is related with transporters activity, which increased respect to 30 min treatment; while transcription factor activity decreased from 30 min to 24 h Cd treatment (Fig. 4.5.4 B and Fig. 4.5.5). In term of cellular components, 15 categories were significantly represented. The most abundant was other membranes (16%), followed by other citoplasmatic components (13%) and nucleus (13%; Fig. 4.5.4C and Fig. 4.5.5). Interestingly, extracellular components appear as a new significant category at 24 h, respect to the 30 min treatment (Fig. 4.5.4 C and Fig. 4.5.5). Several transcriptome analyses have been made in different species after long-term Cd treatment. It is difficult however, to obtain a comprehensive

understanding of global plant responses to Cd due to the variety of: 1) methodologies used, involving hydroponics, plates, soil, etc.; 2) concentration and timing of the treatment; and 3) species and tissue analysed. In general, genes up regulated in response to Cd stress have been categorized as genes involved in: a) response to biotic stress, b) detoxification and oxidative stress, c) protein metabolism and d) transport. Down regulated genes have been categorized as genes involved in: a) photosynthesis and b) carbon metabolism (Ma *et al*., 2018; Corso *et al*., 2018; Rui *et al*., 2018). For more details, see Annexe 8.1 and Table 8.1.

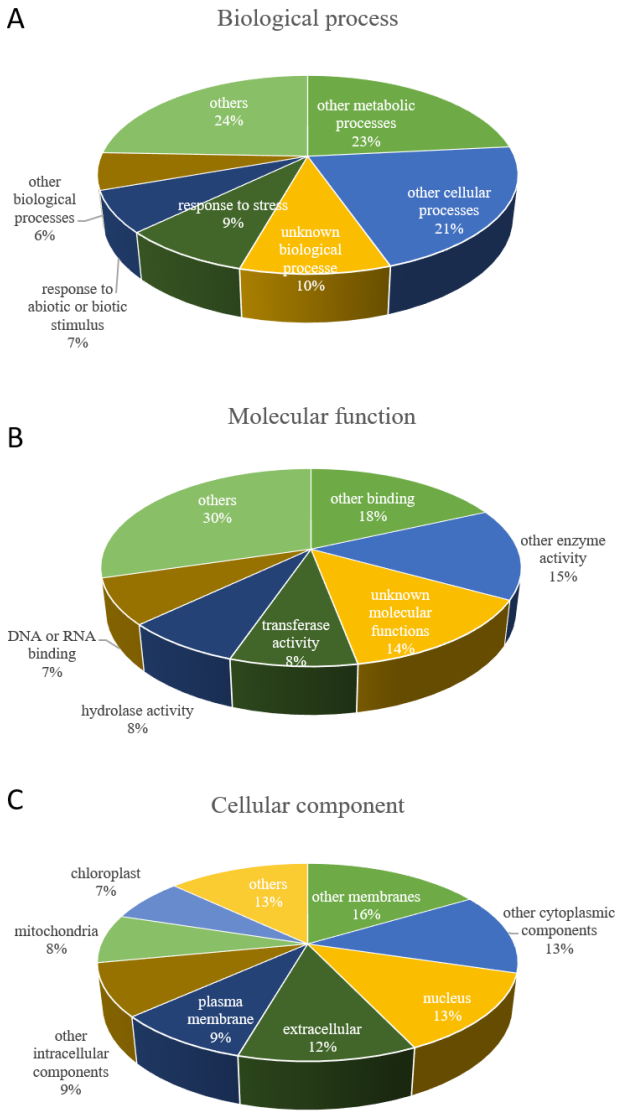

Fig. 4.5.4. Gene ontology clasification of genes regulated by Cd 24 h. (A) Pie-shart percentages werecalculated using the absolute number of genes categorized in one or more Geneontology established groups into Biological function, (B) Molecular functions (C) and Cellular component. Others in Biological process: protein metabolism, developmental processes, transcriptional, DNA dependent, transport, signal transduction, cell organization and biogenesis and electron transport or energy pathways;in molecular function: protein binding, transport activity, nucleic acid binding, kinase activity, structural molecular activity and receptor binding or activity; in cellular component: unknown cellular component, cytosol, cell wall, ER, plastid, golgi apparatus and ribosome. Significant categories with absolute values in Arabidopsis were obtained from Gene Ontology (http://bar.utoronto.ca/ntools/cgi-bin/).

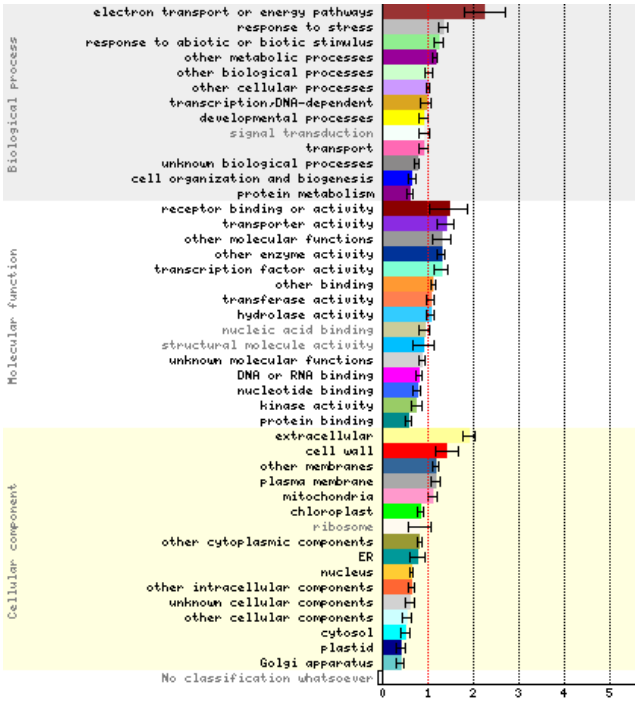

Fig. 4.5.5. Gene ontology clasification of genes regulated by Cd 24 h. Significant categories normed to frequency of class over all ID numbers in Arabidopsis set obtained from Gene Ontology (http://bar.utoronto.ca/ntools/cgi-bin/;± bootstrap Standard Deviation, p-value) are marked in bold.

To delve further into genes differentially regulated in response to Cd, we analysed our dataset with String DB and genemania software. Interestingly, at least 36 and 60 genes from the total of 495 and 923 genes differentially expressed after 30 min and 24 h Cd treatment, respectively (http://bar.utoronto.ca/ntools/cgi-bin/) were linked to biotic stress. In particular, 13 and 16 genes, respectively were linked to plant-fungal interaction (Table 4.5.1.).

Previously, different genes have been shown to be involved in plant response to both, biotic and abiotic stress, such as members of the WRKY transcription factors family (Imran *et al*., 2018) and we have found two members of this family, one at 30 min and another at 24 h Cd treatment (Table 4.5.1 and 4.5.2.). In *Salix matsudana* was also observed a significant number of genes downregulated in response to Cd and related to biotic stress (Yang *et al*., 2015). Furthermore, there are some transcriptome analyses made in plant response to both, biotic and abiotic stress, applied at the same time and independently. Thus, chickpea transcriptome was analysed in response to drought, cold, high salinity and to the necrotrophic fungal pathogen *Ascochyta rabiei*. 51 transcripts were differentially expressed in the shoots in response to *A. rabiei*, 21 transcripts of which were shared in plant response to the fungal pathogen and one or more of the abiotic stresses, although no transcript was regulated in the four stresses evaluated in common. Plant response to *A. rabiei* and high salinity shared the largest number of genes differentially regulated; while *A. rabiei* and cold, and *A. rabiei* and drought shared the least number (Mantri *et al*., 2010).

Table 4.5.1. Genes related with plant response to fungus and 30 min of Cd treatment." List of genes described by Gene Onlo which are regulated in response to 30 min of Cd treatment.

ID	GO	Description
At1g15010	[GO:0050832] defence response to fungus	
At1g51090	[GO:0050832] defence response to fungus	AtHMAD1__Heavy metal transport/detoxification supe
At1g56650	[GO:0050832] defence response to fungus	ATMYB75_AtPAP1_MYB75_PAP1_SIAA1__product
At3g45390	[GO:0050832] defence response to fungus	LecRK-I.2__Concanavalin A-like lectin protein kinase f
At4g31800	[GO:0050832] defence response to fungus	ATWRKY18_WRKY18__WRKY DNA-binding protei
At4g34410	[GO:0050832] defence response to fungus	ERF109_RRTF1__redox responsive transcription factor
At5g52020	[GO:0050832] defence response to fungus	Integrase-type DNA-binding superfamily protein
At2g46240	[GO:0009817] defence response to fungus, incompatible interaction	ATBAG6_BAG6__BCL-2-associated athanogene 6
At3g25780	[GO:0009620] response to fungus	AOC3__allene oxide cyclase 3
At4g02330	[GO:0009620] response to fungus	AtPME41_ATPMEPCRB_PME41__Plant invertase/pec superfamily
At2g34930	[GO:0050832] defence response to fungus	disease resistance family protein / LRR family protein
At5g57220	[GO:0050832] defence response to fungus	CYP81F2__cytochrome P450, family 81, subfamily F, p
At1g56510	[GO:0009817] defence response to fungus, incompatible interaction	ADR2_WRR4__Disease resistance protein (TIR-NBS-I

Table 4.5.2. Genes related with plant response to fungus and 24 h of Cd treatment." List of genes described by Gen Onlology are regulated in response to 24 h of Cd treatment.

ID	GO	Description
At4g11393	[GO:0050832] defence response to fungus	DEFL202__Defensin-like (DEFL) family protein
At4g22212	[GO:0050832] defence response to fungus	Arabidopsis defensin-like protein
At4g22230	[GO:0050832] defence response to fungus	Arabidopsis defensin-like protein
At5g33355	[GO:0050832] defence response to fungus	Defensin-like (DEFL) family protein
At5g43580	[GO:0050832] defence response to fungus	UPI__Serine protease inhibitor, potato inhibitor I
At5g61890	[GO:0050832] defence response to fungus	Integrase-type DNA-binding superfamily protein
At5g64810	[GO:0050832] defence response to fungus	ATWRKY51_WRKY51__WRKY DNA-binding
At5g65530	[GO:0050832] defence response to fungus	AtRLCK VI_A3__Protein kinase superfamily pro
At3g05360	[GO:0002238] response to molecule of fungal origin	AtRLP30_RLP30__receptor like protein 30
At2g23620	[GO:0009817] defence response to fungus, incompatible interaction	ATMES1_MES1__methyl esterase 1
At2g43580	[GO:0009620] response to fungus	Chitinase family protein
At3g22231	[GO:0009620] response to fungus	PCC1__pathogen and circadian controlled 1
At2g35000	[GO:0050832] defence response to fungus	ATL2G_ATL9__RING/U-box superfamily prote
At3g13403	[GO:0050832] defence response to fungus	Defensin-like (DEFL) family protein
At3g25820	[GO:0050832] defence response to fungus	ATTPS-CIN_TPS-CIN_TPS23__terpene synthas
At3g25830	[GO:0050832] defence response to fungus	ATTPS-CIN_TPS-CIN_TPS-CIN_TPS27__terpe cineole
At3g26830	[GO:0050832] defence response to fungus	CYP71B15_PAD3__Cytochrome P450 superfam

A similar study in *A. thaliana* showed that mRNA expression profile in response to *Botrytis cinerea* shares more genes differentially expressed with the response to osmotic stress, than with the response to heat or salt stress (Sham *et al*., 2015). Furthermore, 13 induced and 29 repressed genes were responsive to all four stresses (Sham *et al*. 2015). A RNA-Seq in *Solanum tuberosum* infected with *Phytophthora infestans*, showed several genes differentially regulated and involved with abiotic stress (salinity, drought and heat) and plant hormone treatment (abscisic acid, 6-benzylaminopurine, gibberellic acid and indole-3-acetic acid), suggesting again an overlap in the defence response to pathogen infection and abiotic stresses (Massa *et al*., 2013). Additionally, 50% of the differentially expressed genes were not specific and were regulated in response to two or more stress conditions, while the 50% remaining were stress specific only regulated in response to a single environmental factor (Massa *et al*., 2013). *Erysiphe graminis* infection and heat stress also share the regulation of 9 miRNAs (Xin *et al*., 2010), suggesting a central role of these genes in wheat response to both, biotic and abiotic stress. However, the function and regulatory mechanism of miRNA in response to combined stresses are practically unknown and deserve more research.

4.5.3.2. Cadmium pre-treatment protects Arabidopsis against *F. oxysporum* infection

Taking into account that Cd treatment induced a group of genes related with biotic stress, we analysed plant survival rate to *Fusarium oxysporum* after Cd pre-treatment. A significant increase in the survival rate was observed in plants pre-treated 24 h with Cd vs water, used as control (mock; Fig. 4.5.6). We found that plant mortality occurs later and 50% of the plants pre-treated with Cd survive to the infection compared to mock treatment (Fig. 4.5.6).

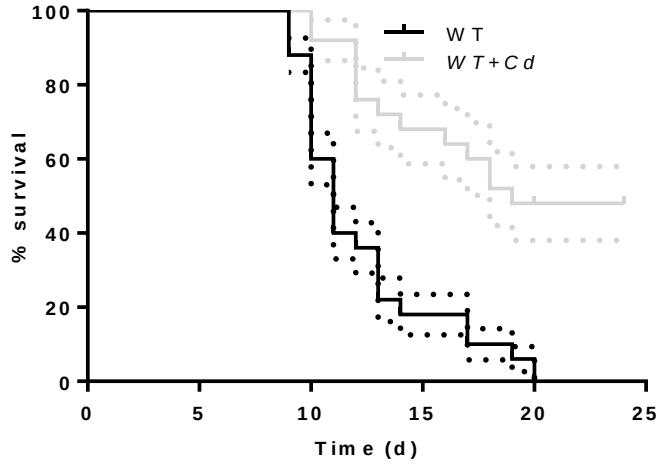

Fig. 4.5.6. Survival of plant pretreated with Cd, and them infeted with *F. oxysporum*. Kaplan–Meier plot of survival of Arabidopsis plants WTand then pre-treated with Cd or not infected with *F. oxysporum*. Differences in mortality were significant ($P < 0.0001$), according to the log-rank test. Data show a representative experiment from a total from 3.

Narusaka and collaborators (2004) described that treatment of *A. thaliana* with copper (Cu), or the infection with a necrotrophic pathogens *Alternaria alternate* and *Alternaria brassicicola* cause a significant overlap in regulated genes, mainly related to P450 genes, suggesting that ROS are the common signals triggering similarity in the response and supporting that heavy metals may enhance plant resistance to fungi. In our case, we were interested in NO role in increasing plant protection against *Fusarium oxysporum* by Cd.

4.5.3.3. A database of putative NO-regulated genes

The importance of NO in response to biotic and abiotic stress has been widely studied. NO has been described as a signalling molecule able to modulate transcriptional response mainly by post-translational modification of key transcription factors that control plant response (Gibbs *et al*., 2014; Begara-Morales *et al*., 2018; Cui *et al*., 2018). In addition, NO has been described as a protective molecule via induction of antioxidant system with ROS scavenging activity in response to several stresses (Sami *et al*., 2018; Nabi *et al*., 2019; Terrón-Camero *et al*., 2019).

To obtain a dataset of putative genes regulated by NO, we sourced in different databases for transcriptomic studies in *A. thaliana* related with NO metabolism and/or with plant supply of NO donors and/or scavengers. We found 8 analyses containing 39 different treatments (Table 4.5.3). One of this analyses used mutants altered in NO metabolism such as, *nia1 nia2*, *Atnoa1* and the triple mutant *nia1 nia2 Atnoa1* (Table 4.5.4), while the others were carried out by using NO donors or scavengers (Table 4.5.5). A total of 13688 genes that may be regulated by NO were found, being 8552 not repeated.

Table 4.5.3. Summary of literature analyzed to search genes regulated by NO.

Culture conditions	Treatment	Methodology	Tissue	Gen UP	Gen Down	Gen analized	Reference
***Arabidopsis thaliana* Col. 0, 18 days, Long day, MS Medium.**	SNP(250uM) 3h	Microarray	Root	9	30	39	**Badri, 2009**
***Arabidopsis thaliana* Col. 0, 5 weeks, shot day in last 3 weeks, 2 weeks in MS medium plantes; Sand 3 weeks, Hidroponic cultive 4 days.**	L-Name 4 mM + Cd 30mM 24 h / L-Name 4mM 24 h	CATMA array	Root	26/25	17/18	43	**Besson-Bard, 2009**
***Arabidopsis thaliana* Col. 0, 28 days, Long day, PM05 medium.**	SNP 0,1mM SNP 1mM 3 h	ATH microarray	Leaf	424	83	507	**Parani *et al*., 2004**
***Arabidopsis thaliana* Col. 0, 4/5 weeks, 10 h in dark conditions, Cell cultive in PS.**	NO gas 1250 ppm 10 min/ 0,5 mM NOR3	Microarray agilent ½	Cell cultive and plant	172	26	198	**Palmieri *et al*., 2008**
***Arabidopsis thaliana* Col. 0, 10,12 true leaves, short day.**	SNP 1 mM 10 min, 1 h y 3 h	AFLP	Leaf	68	2	70	**Polverari *et al*., 2003**
***Medicago truncatula*, long day, in soil. Diferent stage in leaves.**	SNP y GSNO (Buchanan-Wollaston *et al*., 2005)	AFLP comparado con Microarray	Leaf	10	3	13	**De Michele *et al*., 2009**
***Arabidopsis thaliana* Col. 0, 14 days in sand/vermiculite, 16 days hidroponic cultive Short day.**	GSNO 1mM 3h	Libreria mRNA	Root and Leaf	781	485	1380	**Begara-Morales *et al*., 2014**
***Arabidopsis thaliana Col.* 0, *NIA1 NIA2, NOA* and triple mutants, 5 days , long days**		ATH1 microarray	Plant	5328	6060	11388	**Gibbs *et al*., 2014**
					Total	13638	

Table 4.5.4. Summary of comparison between NO studies using muntants and 30 min and 24 h Cd treatment. Gibbs and co
nia2, *Atnoa1* and *nia1 nia2 Atnoa1* (Table 4.5.3). Number of genes resultity from comparison between Gibbs *et al*., 2014 and our tra
in Cd transcriptome. Down: down-regulated in Cd transcriptome. Up: Up-regulated in Cd transcriptome. Down: down-regulated in

NO dependent genes		Gibbs *et al*., 2014 *Nia1 nia2*	*Atnoa1*	*Nia1 nia2/ Atnoa1*
Cd 30´	Up	44	17	45
	Down	12	16	15
Cd 24 h	Up	62	12	49
	Down	28	40	50

Table 4.5.5. Summary of comparison between NO studies related with donors/scavengers and 30 min and 24 h Cd treatme
Arabidopsis with exogenous supply of NO or scavengers treatment (Table 4.5.3). Number of genes resultity from comparison of t
presented. Up: Up-regulated in Cd transcriptome. Down: down-regulated in Cd transcriptome.

NO dependent genes		Badri, 2009	Besson-Bard, 2009		Parani *et al*., 2004			Palmieri *et al*., 2008			Polverari *et al*., 2003	De Michele *et al*., 2009	
			L-name + Cd 30	L-Name	0,1 mM SNP	1 mM SNP	Comons	Cellular cultivate	Plant	Commos	SNP 3 h		R
Cd 30´	Up	0	1	2	25	41	11	4	20	2	0	0	1
	Down	0	1	0	0	0	0	1	0	0	2	0	
Cd 24 h	Up	1	4	3	6	24	2	7	4	4	0	1	2
	Down	2	3	4	0	0	0	1	0	0	1	0	

4.5.3.4. NO-regulated genes in Arabidopsis thaliana in response to Cd: crosstalk with Fungi

We compared the dataset of NO-regulated genes obtained in paragraph 4.5.3.3 with genes regulated after 30 min and 24 h Cd treatment described in paragraph 4.5.3.1 Results showed 17 common genes in these 3 groups, 184 and 202 genes in common in NO-regulated genes and plant response to Cd 30 min and 24 h, respectively (Fig. 4.5.7).

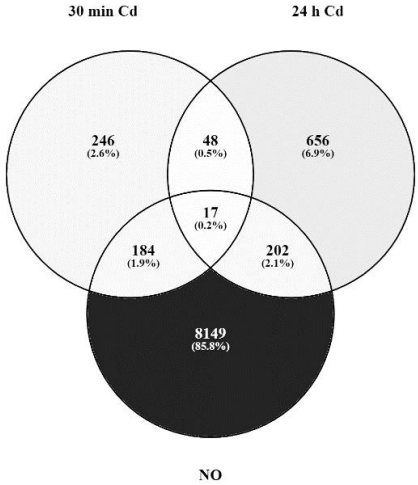

Fig. 4.5.7. Common genes in plant response to Cd and probably regulated by NO. Venn diagram with selected genes regulated after 30 min and 24 h of Cd treatment compared to control in WT (Col-0) seedlings, obtained by an in-house transcriptome analysis compared with the dataset of NO-regulated genes obtained in paragraph 4.5.3.1 from database.

We further analysed if the genes identified in response to Cd, and related to fungi, by BAR Toronto webpage (Table 4.5.1, Table 4.5.2), may be regulated by NO. Results showed that 9 and 6 genes regulated in plant response to Cd 30 min and 24h treatment respectivenly and related with fungi, might be regulated by NO (Fig.4.5.8, Table 4.5.5).

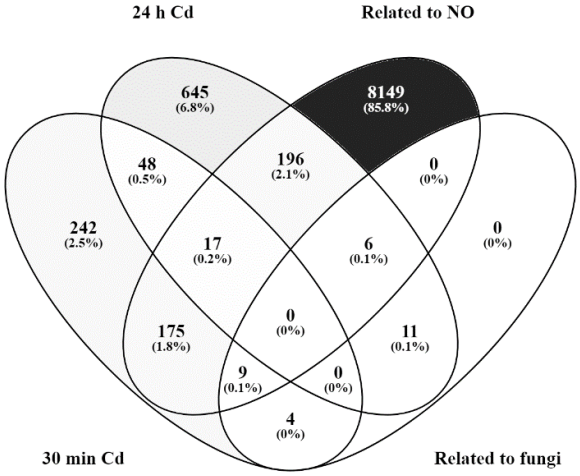

Fig. 4.5.8. Common genes regulared by Cd, related with fungi and NO. Venn diagram with genes regulated after 30 min and 24 h of Cd treatment (Col-0) seedlings, obtained by an in-house transcriptome analysis compared with the dataset of NO-regulated genes obtained in paragraph 4.5.3.3 and related to fungus infections.

Table 4.5.5. Common genes regulated by Cd at 30 min and 24 h , fungi and NO.

ID	GO	Description
At1g56510	[GO:0009817] defence response to fungus, incompatible interaction	ADR2_WRR4__Disease resistance protein (T
At3g25780	[GO:0009620] response to fungus	AOC3__allene oxide cyclase 3
At4g02330	[GO:0009620] response to fungus	AtPME41_ATPMEPCRB_PME41__Plant in inhibitor superfamily
At1g51090	[GO:0050832] defence response to fungus	AtHMAD1__Heavy metal transport/detoxific
At1g56650	[GO:0050832] defence response to fungus	ATMYB75_AtPAP1_MYB75_PAP1_SIAA1 pigment 1
At2g34930	[GO:0050832] defence response to fungus	disease resistance family protein / LRR family
At4g31800	[GO:0050832] defence response to fungus	ATWRKY18_WRKY18__WRKY DNA-bind
At4g34410	[GO:0050832] defence response to fungus	ERF109_RRTF1__redox responsive transcrip
At5g57220	[GO:0050832] defence response to fungus	CYP81F2__cytochrome P450, family 81, sub
At3g05360	[GO:0002238] response to molecule of fungal origin	AtRLP30_RLP30__receptor like protein 30
At3g22231	[GO:0009620] response to fungus	PCC1__pathogen and circadian controlled 1
At3g26830	[GO:0050832] defence response to fungus	CYP71B15_PAD3_Cytochrome P450 superfa
At4g22212	[GO:0050832] defence response to fungus	Arabidopsis defensin-like protein
At5g43580	[GO:0050832] defence response to fungus	UPI__Serine protease inhibitor, potato inhibit
At5g65530	[GO:0050832] defence response to fungus	AtRLCK VI_A3_Protein kinase superfamily

Enrichment analyses, made by String database (https://string-db.org/), showed that common genes in 30 min Cd, NO and fungus are related with flavonoids and secondary metabolites and plant hormonal signal transduction (Fig 4.5.9.).

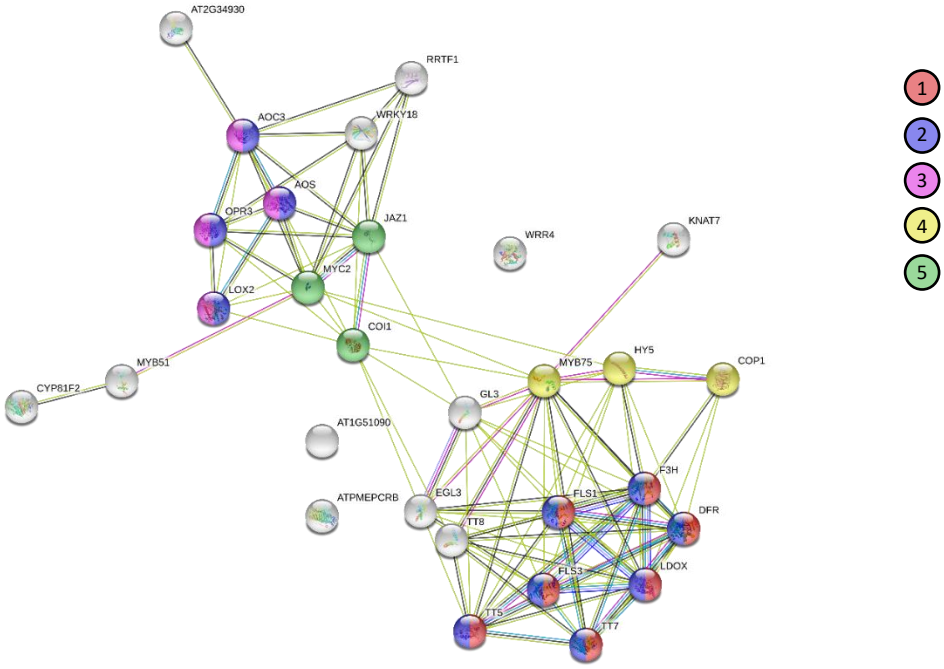

Fig. 4.5.9. Enrichment analysis of common genes in plant response to Cd 30 min, NO and fungi. 9 selected genes have been analyzed with StringDB (https://string-db.org)/, considering 5 genes and 5 genes in 1st and 2nd shells, respectively, as maximun number of interactors to show. Pathways represented significantly in this analysis are indicated in different colors according to KEGG Pathways: 1. Flavonoid biosynthesis; 2. Biosynthesis of secondary metabolites; 3. Alpha-Linolenic acid metabolism 4. Circadian rhythm and 5. Plant hormone signal transduction.

In fact, the role of different secondary metabolites in plant response to *F. oxysporum* infection including deposition of callose, that prevent pathogen attack as a physical and chemical barrier, has been widely shown (Aist *et al*., 1991; Yadela and Thomma, 2013). In vitro assays also showed that low flavonoid concentrations exhibit antimicrobial properties against *Fusarium oxysporum* f. sp. *lycopersici* affecting microconidial germination and fungal growth (Steinkellner and Mammerler, 2007). Moreover, in yellow lupine it was described that flavonoid biosynthesis enhanced plant resistance in the early phase of infection (Morkunas *et al*., 2011). In *Allium cepa*, detected changes in flavonoids levels after *F. oxysporum* infection were associated with defence response (Lee *et al*., 2012). Furthermore, the induction of phenylpropanoids by different elicitors, lead to an enhanced resistance to *F. oxysporum* in different species such as tomato and banana (Troncoso-Rojas *et al*., 2013; Fortunato *et al*., 2014). On the other hand, plant hormones have been widely described as key molecules in plant-*F. oxysporum* interaction (Kidd *et al*., 2011; Chen *et al*., 2014), although different role have been described for specific hormones. Accordingly, it has been shown that salicylic acid

increase plant resistance (Edgar *et al*., 2006; Berrocal-Lobo 2004) while jasmonic acid may promote the infection in some cases (Thatcher *et al*., 2009; Cole *et al*., 2014).

Enrichment analyses showed that common genes in 24 h Cd, NO and fungus are related with MAPK signalling pathway and plant pathogen interaction (Fig. 4.5.10). MAPK signalling is one of the earliest pathways activated in plant response to pathogens and in particular to fungi in a variety of species (Hamel *et al*., 2012; Molina-Moya *et al*., 2019). Accordingly, MAPK signalling pathway, calcium signalling and salicylic acid-mediated hypersensitive response were activated after *F. oxysporum* infection in *Brassica oleracea* roots (Xing *et al*., 2016). *F. oxysporum* also induces the expression of different MAPKs in wild soybean, while in cultivated soybean this induction is not observed (Chang *et al* 2019). In cotton, the MAPK GhMPK20, is negatively regulated by MKK4, MPK20 and WRKY40 cascade in response to *F. oxysporum* (Wang *et al*., 2018).

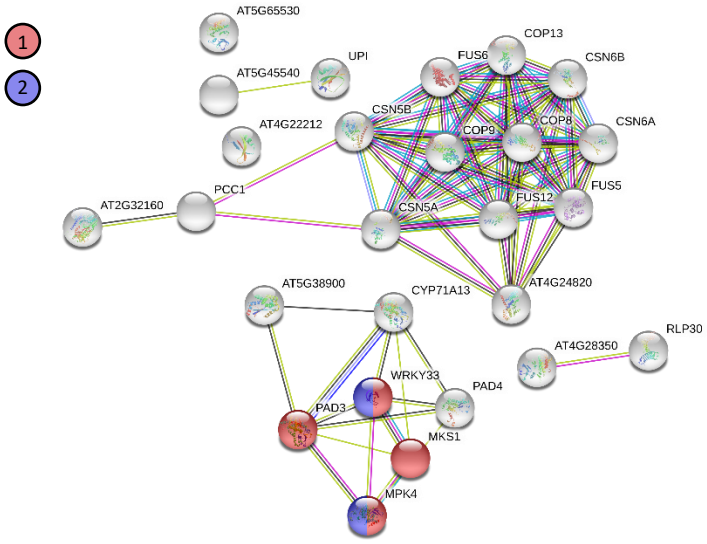

Fig. 4.5.10. **Enrichment analysis of common genes in plant response to Cd 24 h, NO and fungi**. 6 selected genes have been analyzed with stringDB (https://string-db.org/), considering 5 genes and 5 genes in 1st and 2nd shells ,respectively, as maximun number of interactors to show. Pathways represented significantly in this analysis are indicated in different colors according to KEGG Pathways: 1. MAPK signalling pathway plant; 2. Plant pathogen interaction.

4.5.3.5. NO-regulated genes in *Arabidopsis thaliana* treated with Cd or *F. oxysporum* infection.

For a more specific study, we analized available data by Zhu *et al.* (2013), who carried out a transcriptome analysis by RNA-seq in Arabidopsis plants after *Fusarium oxysporum* infection. They found 177 and 571 genes up-regulated; and 30 and 125 genes down-regulated at 1 and 6 days-post-inoculation, respectively. We compared our dataset

of genes regulated by Cd and NO with 123 genes provided by Zhu *et al*. (2013), which were in common at both time-points after *F. oxysporum* infection (Fig. 4.5.12). We found 3 common genes in all datasets, 3 other genes in common with *F. oxysporum* response, 30 min of Cd treatment and NO (Fig. 4.5.12 and Table 4.5.6); and 16 genes in common with *F. oxysporum* response, 24h of Cd treatment and NO (Fig. 4.5.12 and Table 4.5.6).

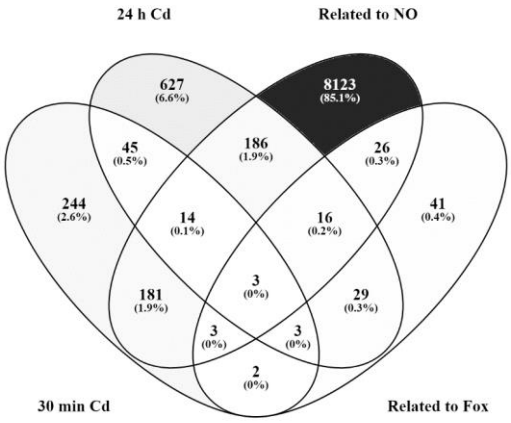

Fig. 4.5.12. Common genes regulated by Cd, NO and *F. oxysporum*. Venn diagram with genes regulated after 30 min and 24 h of Cd treatment, obtained by an in-house transcriptome analysis compared with the dataset of NO-regulated genes obtained in paragraph 4.5.3.3 and genes regulated in plants after *F. oxysporum* infected 1 and 6 (Zhu *et al*., 2013).

Enrichment analyses, made by String database (https://string-db.org/), showed that common genes regulated by Cd (30 min), NO and *F. oxysporum* are grouped in 5 KEGG pathways in a significant way: 1. starch and sucrose metabolism; 2. metabolic pathways; 3. biosynthesis and secondary metabolites; 4. sugar metabolism and 5. alpha-linolenic acid metabolism (Fig. 4.5.13). We found 7 KEGG pathways in common genes regulated by Cd (24 h), NO and *F. oxysporum*: 1. carbon metabolism; 2. glyoxilate and dicarboxylate melabolism; 3. metabolic pathways; 4. biosynthesis of secondary metabolism; 5. glycine, serine and threonine metabolism; 6. Peroxisome; and 7. pyruvate metabolism (Fig. 4.5.14).

Table 4.5.6. Common genes obtained after comparison data set in paragraphs: 4.5.3.1 an 4.5.3.3 and data from Zhu *et al* 20

ID	GO	Description
At4g23810	[GO:0010150] leaf senescence	ATWRKY53_WRKY53__WRKY family transcription fac
At3g29970	[GO:0005739] mitochondrion	B12D protein
At1g57630	[GO:0071456] cellular response to hypoxia	Toll-Interleukin-Resistance (TIR) domain family protein
At1g24140	[GO:0004222] metalloendopeptidase activity	AT3-MMP__Matrixin family protein
At3g43190	[GO:0005829] cytosol	ATSUS4_SUS4__sucrose synthase 4
At4g37390	[GO:0009733] response to auxin	AUR3_BRU6_GH3-2_GH3.2_YDK1__Auxin-responsive
At3g25250	[GO:0004672] protein kinase activity	AGC2_AGC2-1_AtOXI1_OXI1__AGC (cAMP-dependen protein kinase C) kinase family protein
At3g28580	[GO:0016787] hydrolase activity	P-loop containing nucleoside triphosphate hydrolases supe
At2g43570	[GO:0004568] chitinase activity	CHI__chitinase, putative
At2g43620	[GO:0004568] chitinase activity	Chitinase family protein
At1g66700	[GO:0010029] regulation of seed germination	PXMT1__S-adenosyl-L-methionine-dependent methyltran protein
At1g02220	[GO:0007275] multicellular organism development	ANAC003_NAC003__NAC domain containing protein 3
At2g29460	[GO:0005829] cytosol	ATGSTU4_GST22_GSTU4__glutathione S-transferase ta
At2g15490	[GO:0005829] cytosol	UGT73B4__UDP-glycosyltransferase 73B4
At1g14540	[GO:0046872] metal ion binding	PER4_PRX4__Peroxidase superfamily protein
At3g21720	[GO:0046872] metal ion binding	ICL__isocitrate lyase
At1g14550	[GO:0020037] heme binding	Peroxidase superfamily protein
At3g26830	[GO:0020037] heme binding	CYP71B15_PAD3__Cytochrome P450 superfamily protei
At1g26420	[GO:0050660] flavin adenine dinucleotide binding	AtBBE7__FAD-binding Berberine family protein
At1g26380	[GO:0050660] flavin adenine dinucleotide binding	AtBBE3_FOX_FOX1__FAD-binding Berberine family pr
At2g30770	[GO:0005506] iron ion binding	CYP71A13__cytochrome P450, family 71, subfamily A, p
At2g32020	[GO:0009737] response to abscisic acid	Acyl-CoA N-acyltransferases (NAT) superfamily protein

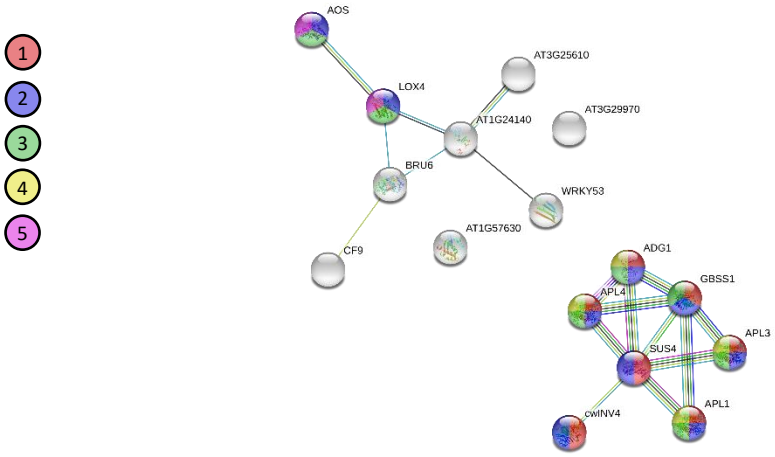

Fig. 4.5.13. Enrichment analysis of genes commonly regulated in response to 30 min of Cd treatment, NO and *F. oxysporum*. 6 selected genes have been analyzed with StringDB (https://string-db.org/), considering 5 genes and 5 genes in 1st and 2nd shells respectively as maximun number of interactors to show. Pathways represented significantly in this analysis are indicated in different colors according to KEGG Pathways: 1. Starch and sucrose metabolism; 2. Metabolic pathways; 3. Biosyntesis and secondary metabolites; 4. Amino sugar nucleotide sugar metabolism; 5. Alpha-Linolenic acid metabolism.

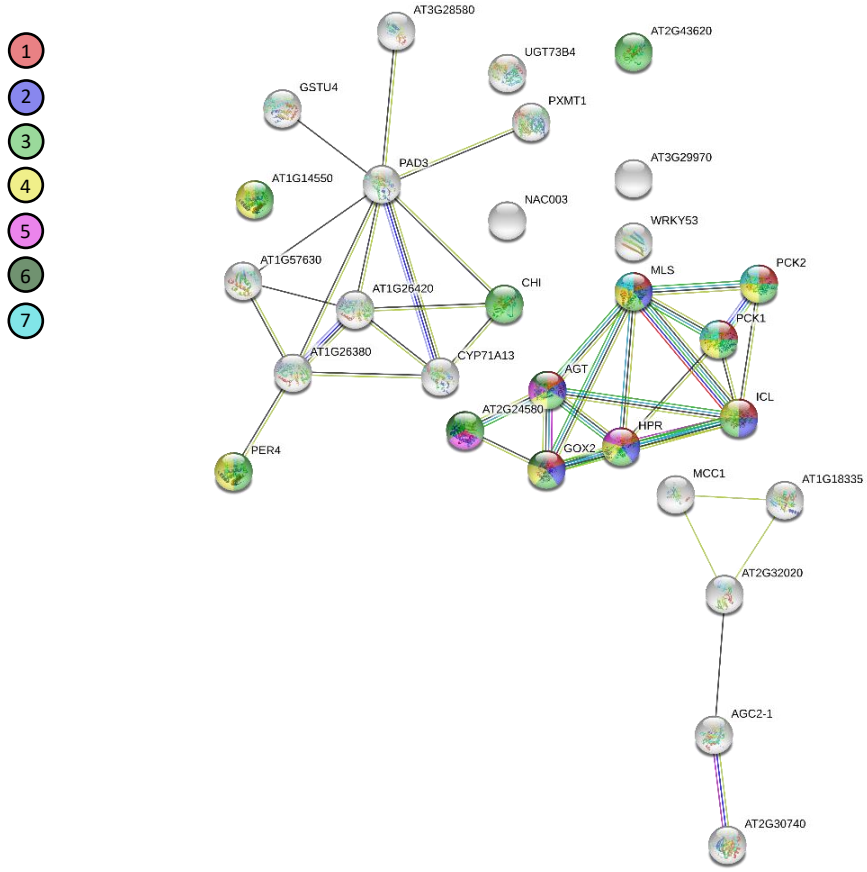

Fig. 4.5.14. Enrichment analysis of genes commonly regulated in response to 24 h of Cd treatment, NO and *F. oxysporum*. 19 selected genes have been analyzed with stringDB (https://string-db.org/), considering 5 genes and 5 genes in 1st and 2nd shells respectively as maximun number of interactors to show. Pathways represented significantly in this analysis are indicated in different colors according to KEGG Pathways: 1. Carbon metabolism; 2. Glyoxylate and dicarboxylate metabolism; 3. Metabolic pathways; 4. Biosynthesis of secondary metabolites; 5. Glycine, serine and threonine metabolism; 6. Peroxisome; 7. Pyruvate metabolism.

In conclusion, we obtained a cluster of genes regulated by Cd, which are related with NO and fungi/*F. oxysporum* and that may explain Arabidopsis enhanced resistance to *F. oxysporum* pre-treated with Cd. These genes are related with important signalling pathways in plant response to pathogens, such as MAPK, hormone signalling, secondary metabolism or pathogen-related proteins. We cannot rule out function of other signalling molecules such as reactive oxygen species and induction of antioxidant sytem by NO. Further work by different approaches such as, biochemical and genetic will show the specific mechanism(s) responsible(s) for the Cd-dependent induced resistance to *F. oxysporum* in our conditions.

4.5.4. Acknowledgements

This research was supported by grants EX12-BIO296, from Junta de Andalucía and PGC2018-098372-B-100 from the Ministry of Science, Innovation and Universities together and the European Regional Development Fund co-funding (MCIU/AEI/ERDF). LC T-C was supported by fellowships for Academic Staff from the Spanish Ministry of Education, Culture and Sports (FPU14/00622 and FPU17/04303, respectively). *Fusarium oxysporum* isolates were kindly donated by Prof. Di Pietro (University of Cordoba, Spain). None of the authors have any conflict of interest to declare.

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5. General discussion

5. General discussion

Nitric oxide (NO) is a small gaseous free radical, which plays a key role in the physiology of most living organisms. NO was first described in the mid-20th century as an intermediate of the nitrate denitrification pathway in the bacterium *Thiobacillus denitrificans* (Baalsrud and Baalsrud, 1954). Although its production in plants was first described 40 years ago in herbicide-treated soybean leaves (Klepper, 1979), the role of NO was not described until 20 years later. The first studies to describe its production in plants were virtually forgotten until the NO molecule was discovered to be responsible for the vasodilatation of vascular smooth muscles in mammalian systems and to be of vital importance in cardiovascular diseases (Furchgott and Zawadzki, 1980; Ignarro *et al*., 1987; Moncada and Higgs, 2006), discoveries that boosted research into the plant kingdom (Brouquisse, 2019). Although the key role played by NO in plant physiology has been extensively studied over the last 20 years, some issues such as NO metabolism and signal transduction mechanisms have not been fully elucidated (León and Costa-Broseta., 2019).

Nitric oxide synthase (NOS) has not been identified or characterized in plants (Astier *et al*., 2018); the study of NO metabolism and its role in plant physiology calls for biochemical techniques involving NO donors/scavengers and/or genetic strategies using available NO metabolism mutants in order to mimic a shortage or overproduction of NO in plants. In this study, we used two different experimental systems to investigate the endogenous role of NO in plant responses to stress:

1) Four well described NO metabolism mutants: *nia1 nia2*, which is affected in nitrate reductase activity, a source of NO (Yamasaki and Sakihama, 2000; Rockel, 2002). *argh1-1*, which lacks the arginase gene, leading to an increase in L-arginine, the NOS-l substrate (Flores *et al*., 2008). *Atnoa1* mutant, with no *NOA1* gene, which encodes a cGTPase protein, showing lower levels of NO under certain conditions (Moreau *et al*., 2008); and *nox1/cue1* mutant which is altered in a chloroplast phosphoenolpyruvate/phosphate translocator and generally shows higher levels of NO (Streatfield *et al*., 1999; He *et al*., 2004).

2) Biochemical technique involving treatment with NO donors (GSNO/SNAP), scavengers (cPTIO) and NO-production inhibitors (Aminogunidine), when necessary.

Our group has been working on stress caused by heavy metals, particularly cadmium (Cd), for over twenty years. We firstly showed that long-term treatment with Cd induces oxidative stress and senescence in pea plants (Sandalio *et al*., 2001; McCarthy *et al*., 2001; Romero-Puertas *et al*.,

2002; Romero-Puertas *et al*., 2004), while NO production decreases (Corpas *et al*., 2004; Rodríguez-Serrano *et al*., 2009), with a concomitant increase in ethylene (Rodríguez-Serrano *et al*., 2009). Meanwhile, several studies of different species highlighted the production of NO in response to heavy metal stress in plants and its role as a protector molecule when applied to plants exogenously (Terrón-Camero *et al*., 2019). However, as the variability of treatment, species, age and experimental conditions made it difficult to reach precise conclusions, we carried out an initial bibliographic search for up-to-date information on heavy metal stress and nitric oxide. Using bioinformatics analysis, we also investigated NO production in response to Cd and whether NO activity and function depend on NO being supplied exogenously or produced endogenously in the plant itself. Most studies show that exogenous applications of NO prior to or during heavy metal treatment negatively correlate with ROS production and oxidative damage, resulting in improved plant fitness, as indicated by increased root length, biomass and chlorophyll content (Terrón-Camero *et al*., 2019; Fig.4.1.2. and Fig.4.1.3) as compared to non-treated plants. Although a reduction in ROS production and oxidative damage were observed in plants supplemented with NO, following bioinformatic analysis, obtained two different groups related to antioxidant systems, which were not always found to increase (Fig.4.1.2 and Fig.4.1.3). This controversial findings relating to the antioxidant system could be partly explained by the timing of treatment, as antioxidants can change over time (Samsatly el al., 2018; Hasanuzzaman *et al*., 2019), and/or by the specific enzyme analyzed, as not all enzymes have a similar sensitivity to stress (Mittler *et al*., 2002; Emamverdian *et al*., 2015; Hasanuzzaman *et al*., 2019).

Over 80% of the studies analyzed show an increase in NO in response to heavy metal stress, especially under short-term treatment conditions. However, the function of endogenous NO in plant responses to heavy metal stress is unclear. NO is involved in reducing root growth in plant responses to stress caused by heavy metals, particularly Cd; it is also involved in Cd uptake, suggesting that NO is capable of regulating different transporters, mainly for transporting Fe (Besson-Bard *et al*., 2009; Lee and Hwang, 2015; Bahmani *et al*., 2019; Terrón-Camero *et al*., 2019). To determine the role of endogenous NO in plant responses to Cd stress, we analyzed four NO metabolism mutants: two overproducers (*nox1* and *argh1-1*) and two low NO-producing mutants (*Atnoa1* and *nia1 nia2*). The decrease in root length due to Cd stress was not found to correlate with NO levels in the mutants, as *argh1-1* and *Atnoa1* were more affected and *nia1 nia2* and *nox1* were less affected than WT plants, with root length actually observed to change even under control conditions. As a signalling molecule, given its cytoprotective or cytotoxic properties depending on its concentration (Lamattina *et al*., 2003), NO needs to be at an optimum level and

precisely regulated under control and stress conditions (Kolbert, 2016). Thus, when not strictly regulated, different levels of NO may lead to the same phenotype due to the complexity of NO metabolism, whose role can even change over time. For example, *Phytogb1* silencing, leading to higher NO levels, and overexpression, leading to lower NO levels, in *Solanum lycopersicum* and *Medicago truncatula*, which showed a similar pattern of symbiosis as measured by the percentage of arbuscular mycorrhiza and nodules, respectively (Martínez-Medina *et al*., 2019; Berger *et al*., 2020). This phenotype could be explained by the different functions of NO depending on the timing of interaction. As high NO levels stimulate plant defence responses, while low levels affect nodule development, both an excess and shortage of NO can inhibit nodule establishment (Berger *et al*., 2020).

With regard to heavy metal uptake, we found that down-regulation of the Fe and non-specifically Cd transporter, *IRT1* observed in WT seedlings in response to Cd is altered in all the mutants analysed, suggesting that NO is involved in regulating *IRT1* and thus in Cd uptake. Interestingly, *nia1 nia2* mutants were found to have a modified Cd translocation rate, with virtually no Cd accumulation observed in the roots. This should encourage further research to determine the role of nitrate reductase (NR) in Cd and possibly Fe translocation. Certain aquaporins such as NIP, and other transporters such as NRAMP and CAX, involved in Fe and Cd uptake and translocation, may be regulated by NO (Terrón-Camero *et al*., 2019; Bahmani *et al*., 2019). Under Fe deficiency conditions, the lack of long-distance iron signalling has been reported to depress ethylene synthesis and signalling, as well as GSNOR expression and activity, leading to a reduction in GSNO content (García *et al*., 2018). All these processes cause the up-regulation of Fe acquisition genes in a complex cycle in which sucrose and auxin also appear to be involved (Lin *et al*., 2016).

Contrary to our findings on exogenous NO supply, we found a positive correlation between endogenous NO production and oxidative lipid damage. The NO overproducers, *nox1* and *argh1-1*, showed higher levels of lipid peroxidation than WT, while *nia1 nia2*, which does not increase NO levels in plant responses to Cd, showed no increase in lipid peroxidation. These phenotypes were reverted by aminoguanidine, a NO production inhibitor of NOS, and by a NO donor (GSNO), respectively, which also correlates NO overproduction with oxidative damage. These findings suggest that, although an initial increase in NO can be produced in plant responses to Cd stress, probably indicating that NO plays a signalling role, NO levels must be regulated at a later stage in order to prevent further oxidative damage.

Interestingly, the peroxisomal antioxidant catalase was found to be protected in *nia1 nia2* mutant responses to Cd stress. Contrary to what occurs in WT, the activity and expression of this enzyme were maintained in *nia1 nia2*. NO-dependent post-translational regulation of peroxisomal antioxidant- and ROS-producing enzymes has been described in pea plant responses to Cd (Ortega-Galisteo *et al*., 2012; Sandalio *et al*., 2019), suggesting that NO regulates ROS levels inside peroxisomes. Although NO, as well as RNS such as GSNO and $ONOO^-$, are localized in peroxisomes (Ortega-Galisteo *et al*., 2012; Corpas *et al* 2017), the role of NO in peroxisomal metabolism remains little understood. These organelles, whose metabolism and dynamics are modified with regard to their morphology, numbers and speed, are capable of rapidly sensing changes in their environment (Rodríguez-Serrano *et al*., 2009; Sinclair *et al*., 2009; Hu *et al*., 2012; Kao and Bartel, 2018), suggesting that peroxisomes may constitute an important decision-making platform in the cell (Sandalio and Romero-Puertas, 2015). Recently, our group described the key role played by peroxisomes in early plant responses to Cd. We showed that Cd produces time course-dependent changes in peroxisomal dynamics, starting with the formation of peroxisomal membrane extensions (peroxules) followed by peroxisome proliferation to finally return to normal morphology and numbers together with an increase in their speed of movement (Rodríguez-Serrano *et al*., 2016; Calero-Muñoz *et al*., 2019). While all these changes have been found to be regulated by ROS and RBOHs (Sinclair *et al.* 2009; Barton, Mathur and Mathur 2013; Rodríguez-Serrano *et al.* 2016), the precise role played by NO in peroxisomal dynamics under control and stress conditions remains little understood.

In this study, we observed an early increase in NO production in WT seedlings treated with Cd, which is similar to what happened in most of the cases analysed by Terrón-Camero *et al*. (2019). The results obtained with the treatment using the NO scavenger cPTIO suggest that NO regulates peroxisomal dynamics, particularly with regard to peroxule formation, in plant responses to Cd. We again used genetic and biochemical techniques to clarify the function of NO in peroxisomal dynamics under control and stress conditions. Our results suggest that NO is necessary for peroxule production in plant responses to Cd, while an excess of NO does not alter the percentage of peroxisomes involved in producing peroxules. Interestingly, although the peroxin PEX11a is essential for peroxule production (Rodríguez-Serrano *et al*., 2016), induction of the gene did not appear to be directly involved in this process. This suggests that peroxule production may require post-translational modifications (PTMs) of the PEX11a protein, whose dependence on NO needs to be verified in Arabidopsis. In yeast, redox changes in Cys and phosphorylation are involved in activating Pex11p protein and organelle division (Knoblach and

Rachubinski 2010; Schrader, Bonekamp and Islinger 2012; Farré, Mahalingam, Proietto and Subramani 2019).

In addition, we showed that peroxisomal proliferation, which is a well-described effect of Cd treatment in different species (Romero-Puertas *et al.* 1999; Rodríguez-Serrano *et al.* 2016; Calero-Muñoz *et al.* 2019; Chen, Li, Schulz, Furst and Chien 1995), is altered in *nia1 nia2* mutants. This phenotype is recovered when NO is exogenously supplied to the mutants, suggesting that it plays a positive role in peroxisomal proliferation. Thus far, while only the dependence of peroxisome proliferation on ROS has been described (López-Huertas *et al*., 2000), JA appears to be a negative regulator (Castillo *et al*., 2008). Further analysis of crosstalk between ROS, NO and JA is required to gain a deeper understanding of the regulation of peroxisome proliferation. Furthermore, NO appears to regulate the number of peroxisomes in the cell, and non-optimal levels of NO, which impairs normal peroxisome distribution, negatively affects the cellular redox metabolism in these mutants. The impairment of peroxisome numbers per cell in the NO-related mutants did not appear to be due to disturbances in the transcriptional regulation of peroxines involved in peroxisomal division but rather to other mechanism(s), a finding which requires further analysis.

The oxidative metabolism inside the organelle was also found to be altered in NO-related mutants. The percentage of peroxisomes with precipitates inside the organelle due to H_2O_2 or oxidized catalase, was higher under control conditions in *nia1 nia2* and *nox1* mutants as compared to WT. CAT, one of the principal antioxidants of peroxisomes, was found to follow a different PTM pattern in NO-related mutants. The *S*-nitrosylation and oxidization of CAT in *nia1 nia2* mutants were more marked under control conditions, with both these PTMs known to decrease CAT activity and stability, respectively (Romero-Puertas *et al*., 2002; Ortega-Galisteo *et al*., 2012). Interestingly, as reported for other proteins in sorghum and citrus plants under salt stress conditions, CAT *S*-nitrosylation appears to protect this enzyme against oxidation under Cd stress conditions (Tanou *et al*., 2014; Baena *et al*., 2017).

Finally, some peroxisome-dependent signalling in plant responses to Cd, mainly involved in the plant's acclimation to stress, was found to be altered in NO-related mutants, an alteration which clearly occurs in the presence of an excess of NO. All our findings together point to NO being a key regulator of peroxisomal, and thus also, plant responses to Cd stress, possibly leading to acclimation to the stress. In addition, peroxisomal metabolism is affected by NO under control

conditions, suggesting that optimum NO levels are required for normal peroxisomal function under control and stress conditions.

To gain a deeper understanding of the role of NO in plant responses to Cd, we searched available databases for possible NO-regulated genes, which were then compared to plant transcriptomic analyses previously carried out in our laboratory under Cd stress conditions. We found over thirteen thousand directly or indirectly NO-regulated/dependent genes. When comparing NO-dependent genes with Cd-regulated genes in our transcriptome, we found that almost 41% of the genes regulated following short-term treatment were common to both, NO and Cd, while this percentage fell to around 24% after long-term treatment. These results are in line with the notion that NO is mainly produced after short-term Cd treatment and that NO levels is restrained later on. The principal categories of genes found using gene ontology in plant responses to Cd were also observed when analysing, at the two time points used, NO-dependent genes such as response to stress and in signal transduction.

Interestingly, the biotic stress response category covers a group of highly regulated genes which were shared by both NO and Cd treatment. Responses to abiotic and biotic stress have been reported to share signalling pathways with signatures, in particular, found to be common to both plant responses to heavy metal stress and different pathogens involving mitogen-activated protein kinases (MAPKs) and phytohormones such as, JA, SA and ET (Opdenakker *et al*., 2012). Thus, *Medicago sativa* and Arabidopsis seedlings treated with high levels of Cu or Cd activate different MAPKs, which, in turn, activate various cellular signalling mechanisms (Jonak *et al*., 2004; Suzuki *et al*., 2001). MAPKs have been detected in tomato plants infected with *Fusarium oxysporum*, with MAPK-5 capable of interacting with other proteins, such as the WRKY transcription factor protein family (Aamir *et al*., 2018). In addition, several studies have shown that ROS are among the signalling messengers produced in response to biotic and abiotic stresses that activate MAPK pathways. Both ROS and MAPK molecules are capable of inter-regulation, although the precise mechanisms involved in this process are little known (Jalmi and Sinha, 2015). Other studies show that high levels of Cu, Ni and Cd in *A. thaliana*, *Thlaspi* and *Phaseolus coccineus* plants respectively, induce JA in response to multiple biotic stresses (Freema *et al*., 2005; Maksymiec *et al*., 2005; Cabot *et al*., 2013; Morkunas *et al*., 2018). On the other hand, high levels of reduced glutathione appear to be a key factor in both metal tolerance and pathogen resistance (Maksymiec *et al*., 2005, Freeman *et al*., 2005). NO is also one of the early signalling molecules involved in plant defences, which was one of the first functions assigned to NO. Although NO is known to play a key role in the development of programmed cell death in incompatible interactions

(Delledonne *et al*., 1998; Durner *et al*., 1998), its role in other interactions is unclear (Molina-Moya *et al*., 2019; Martínez-Medina *et al*., 2019).

Some years ago, our group began to study interactions between tomato and Arabidopsis plants and *Fusarium oxysporum*, an abundant soil-borne pathogen that causes vascular wilt in a wide range of plants, as well as considerable losses in over a hundred crop species (Gordon, 2017; Laurence *et al*., 2012; Leslie and; Ma *et al*., 2013; Rana *et al*., 2017; Summerell, 2006). The role of NO in this specific interaction is unclear. Digging deeper using our transcriptomic analysis, given that some genes regulated by Cd and NO were found to be specifically associated with responses to fungi, particularly *Fusarium oxysporum*, we initially analysed the production and possible function of NO in infected Arabidopsis plants by using NO metabolism mutants. We also investigated possible defences against *F. oxysporum* through pre-treatment with Cd and looked for NO-regulated genes in response to Cd, which could explain why plants share survival strategies under both biotic and abiotic stress conditions.

How NO functions in plants infected by pathogenic fungi appears to be dependent on either the necrotrophic or the biotrophic character of the pathogen associated with different spatio-temporal descriptions of NO production (Martínez-Medina *et al*., 2019). Necrotrophic fungi appear to produce an adverse situation whereby high levels of NO may trigger plant cell death and expand the infection, while low amounts of NO could act as key signalling molecules that induce plant immune responses to the fungus (Floryszak-Wieczorek *et al*., 2007; Turrión-Gomez and Benito, 2011). On the other hand, hypersensitive response-dependent cell death is one of the principal strategies for preventing biotrophic fungal growth (Govrin and Levine, 2000), while a continuous rapid increase in NO production is a key step in plant resistance to these pathogenic fungi (Schlicht and Kombrink, 2013; Piterková *et al*., 2009; Qiao *et al*., 2015). In tomato and Arabidopsis plants infected with the hemi-biotrophic pathogen *Fusarium oxysporum*, an early peak in NO, possibly involved in defence responses, has been observed (Martínez-Medina *et al*., 2019; Gupta *et al*., 2014). As the infection progresses, the tomato *Phytogb1* gene has been reported to be repressed, thus triggering a further increase in NO and cell death, which is characteristic of necrotrophic behaviour (Martínez-Medina *et al*., 2019). However, the precise function of NO in plant interactions with root fungal pathogens remains largely unexplored.

We used genetic and biochemical techniques to gain a deeper insight into the role of NO in Arabidopsis-*Fusarium oxysporum* interactions. The genetic approach involved using two NO-related mutants, the nitrate reductase-null mutant *nia1 nia2* and the NO overproducer *nox1*.

Strikingly, we found that the lack of nitrate reductase makes plants more susceptible to *Fusarium oxysporum*, although the NO burst in response to the fungus was similar in WT and *nia1 nia2*. On the other hand, although the survival pattern observed in the NO overproducer *nox1* was similar to that of WT plants, 5% of *nox1* plants resisted the fungus. Interestingly, both *nia1 nia2* and *nox1* were more susceptible to a virulent strain of *Pseudomonas syringae* due to disabled SA-dependent resistance (R) gene-mediated protection against pathogens (Durrant and Dong 2004; Vitor *et al*., 2013; Yun *et al*., 2016), a susceptibility which can be reversed by exogenous applications of NO in *nia1 nia2* mutants (Vitor *et al*., 2013). In *nox1* plants, both salicylic acid (SA) synthesis and signalling are suppressed, thus indicating their susceptibility to virulent non-host pathogens (Yun *et al*., 2016).

We analysed various well-described plant defence responses to the fungus such as peroxidase activity (Turra *et al*., 2015), phenol content, H_2O_2 production (Berendsen *et al*., 2012; Stringlis *et al*., 2018), as well as iron-related gene expression and activity (Schmidt *et al*., 2000; Martínez-Medina *et al*., 2017). All the parameters analysed were affected in a similar way in both NO-related mutants, suggesting that these parameters were not a key factor in *nia1 nia2* susceptibility. However, the induction of defence genes, especially NO-dependent pathogenesis-related (PR) genes, which probably enables some *nox1* plants to resist the fungus, occurred earlier in both NO-related mutants respect to WT plants (Durner *et al*., 1998). However, the percentage of surviving plants may not be very high, because, despite being a signalling molecule, NO initially activates defence responses, while, at the later necrotrophic stage, the fungus may use NO to kill off cells. However, in the *nia1 nia2* double mutant, this early induction of PR genes appears to be insufficient, probably due to the higher fungal burden observed in *nia1 nia2* plants. Given all these results, it is possible to suggest that *nia1 nia2* susceptibility is related to the infection process, particularly penetration by the fungus. We then examined cellulose content, an essential component of the plant cell wall and the first barrier against pathogens, in WT and NO-related mutants (Kesten *et al*., 2017). Using microscopic and spectrophotometric analyses, we observed a decrease in cellulose content in *nia1 nia2* mutants as compared to WT and *nox1*, suggesting that disturbances in cellulose content could partially explain the increased susceptibility of *nia1 nia2* mutants. Preliminary results showed a recovery in cellulose content in *nia1 nia2* plants supplemented with NO donors, thus corroborating that NO plays an important role in regulating cell wall cellulose. To investigate genes possibly responsible for impaired cellulose content in *nia1 nia2* mutants, we carried out a bioinformatics analysis of a transcriptome available for these mutants (Gibbs *et al*., 2014). Interestingly, the cell wall, in which around 7% of the differentially

regulated genes are localized, is a feature which differs significantly in *nia1 nia2* with respect to WT. We again looked for genes in this category related with fungus, where we found that cellulose synthase A4 (CESA4) met both requirements. The enzymatic complex cellulose synthase (CSC), whose correct conformation is crucial for cellulose microfiber production and the cell wall, is comprised of the subunits CESA4, CESA7 and CESA8 (Hill *et al*., 2014; Kumar *et al*., 2018). Databases search showed an inhibition of *CESA4* transcripts in plant responses to Phytophthora and Botrytis (http://bar.utoronto.ca/eplant/). Accordingly, we found an repression of *CESA4* in WT plants following infection with *Fusarium oxysporum*. Repression of cellulose synthesis in plants appears to be triggered by fungal pathogens in addition to an increase in fungal cellulases, thus weakening the first anti-fungal barrier in plants (Zhang *et al*., 2016; Kesten *et al*., 2017). An opposite trend was observed in *nia1 nia2* mutants, with a two-fold induction of *CESA4*, which was over ten-fold higher than gene expression in WT plants after 4 d of infection, suggesting that *CESA4* expression is altered in *nia1 nia2* responses to *Fusarium oxysporum*. A similar response to *CESA4* was observed in the *MYB46* transcription factor, which has been reported to regulate *CESA4* (Kim *et al*., 2014). However, under control conditions, *CES4A* expression was similar in WT and *nia1 nia2* mutants, suggesting that changes in cellulose content under normal conditions may be post-translationally regulated in *nia1 nia2*, whose activity and interactions with other accessory proteins could be affected (Kumar *et al*., 2018). We used GPS-SNO bioinformatics software to make predictions, which showed three Cys residues from CESA4 as possible *S*-nitrosylation targets, with Cys517 showing the highest statistical score. Further research is needed to determine whether *S*-nitrosylation occurs in CESA4 and this PTM's functionality. Interestingly, *CESA4* gene expression in *nox1* mutants under control conditions was half that observed in WT, suggesting again that NO-dependent transcriptional regulation occurred. Different levels of NO have been reported to affect cellulose synthesis and content in *Solanum lycopersicum* roots by modifying *SlCESA1-3* transcript expression (Taylor, 2008; Correa-Aragunde *et al*., 2008). However, the way in which NO and NR regulate cellulose content and its function in plant responses to *Fusarium oxysporum*, a key element in plant survival, require further research.

In order to determine other shared functions of NO in plant response to Cd and in Arabidopsis-Fusarium interactions, we pre-treated plants with Cd for 24 h, which were then infected with the fungus. 50% of the plants pre-treated with the metal were found to survive the infection, with death occurring in the other 50%, later than in control plants. This protection mechanism could be partly due to NO-dependent signalling which takes place during the early plant responses to Cd. In fact, genes regulated in plants exposed to Cd after 30 min of treatment

and NO-dependent, which play a role in anti-fungal plant defences, are associated with flavonoids, secondary metabolites and hormones. For example, probiotic root-associated microbes that activate iron-deficiency responses during colonization have been reported to stimulate scopoletin in a MYB72-dependent manner, thus enhancing plant growth and protection (Noorbakhsh and Taheri, 2016). Other members of this transcription factor family such as MYB75 have been identified in plant responses to Verticillium associated with ethylene production (Pantelides *et al*., 2010). In addition, genes involved in octadecanoid and phenylpropanoid pathways are activated by pre-treatment with NO, resulting in cell wall defence responses via callose deposition, phenolic compound accumulation and lignification at early time points following infection with the necrotrophic fungus *Rhizoctonia solani* (Stringlis *et al*., 2018). Under our experimental conditions, phenols, acting as secondary metabolites that benefit the host plant, were found to be altered in NO-dependent mutants under control conditions and following infection. This suggests that NO is involved in phenol production and that the increase in these metabolites enhances plant resistance. Indeed, NO supplementation has been shown to increase phenol content in tomato-*Rhizoctonia solani* interactions (Noorbakhsh and Taheri, 2016). Enrichment analyses of microarray data after 24 h of Cd treatment showed that genes common to both NO and the fungus are involved in the MAPK signalling pathway and plant pathogen interactions, given that NO is generally involved in crosstalk between different signalling pathways. However, further analysis is necessary in order to clarify the mechanisms underlying Cd protection against Fusarium under our experimental conditions.

Other studies show that pre-treatment with heavy metals, through different protection mechanisms, protects against subsequent infection (Morkunas *et al*., 2018). Most of these studies show that heavy metal protection is related to ROS metabolism and/or the induction of defence-signalling pathways. Thus, P450 genes are commonly found in Arabidopsis responses to Cu, as well as *Alternaria alternata* and *Alternaria brassicicola*, suggesting that ROS signals are used by heavy metals to enhance plant resistance to fungi (Narusaka *et al*., 2004). The induction of resistance to *Fusarium oxysporum* in *Triticum aestivum* by pre-treatment with Cd is related to GSH-induced glutathionylation, which protects proteins against oxidative damage (Mohapatra *et al*., 2016). ROS production and cell death decreased in *Cajanus cajan* pre-treated with Cd and further infected with *Fusarium incarnatum*, although this is not always related to an increase in the antioxidant system (Satapathy *et al* 2012). In Arabidopsis plants, increased resistance to *Botrytis cinerea* following pre-treatment with Cd or Cu is reported to be exclusively caused by an induction of defence genes such as *PDF1.2* (Cabot *et al*., 2012). A combination of both ROS

metabolism and defence responses have been involved in heavy metal protection in most cases. Thus, Chmielowska *et al*. (2010) have reported that Cu-dependent protection of pepper plants against *Verticillium dahlia* is due to a combination of an increase in defence genes such as PR1 and β-1,3-glucanase, as well as ROS metabolism, which increases prolinę oxidase (POX) activity and the phenolic-dependent peroxidase gene CAPO. Arasimowicz-Jelonek *et al* (2014) produced similar results with potato plants pre-treated with Al and further infected with Phytophthora when PR proteins, phenylalanine ammonia lyase (PAL), H_2O_2, SA and *S*-nitrosothiols were induced. Also, *Vitis vinifera* pre-treated with Mn induces resistance to *Uncinula necato* due to an induction of SA and ABA, POX and defence proteins such as PAL, PR and NBS-LRR proteins (Yao *et al*., 2012).

In conclusion, all the findings described above suggest that NO is involved in regulating antioxidant capacity, as well as peroxisomal-dependent signalling and dynamics in plant responses to Cd stress, which are key processes in plant acclimation and survival. NO is also involved in plant cellulose content in an NR-dependent manner, which is an essential plant defence barrier, and in regulating defence genes essential for plant survival. Interestingly, gene regulation in plant responses to Cd stress was found to increase plant defences against *Fusarium oxysporum*, suggesting the presence of mechanisms common to both abiotic and biotic stresses, which requires further analysis (Fig. 5.1).

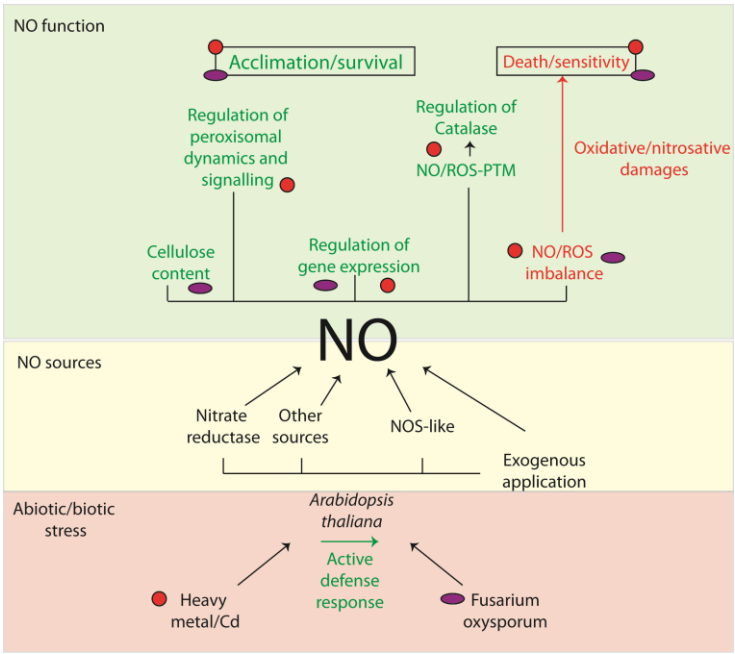

Figure 5.1. Summary of NO functions in *A. thaliana* under Cd and *Fusarium oxysporum* infection. The figure shows the plant's main sources of NO in cadmium stress and *Fusarium oxyporum* infection and its potential roles. In green are represented the beneficial functions for the plant and in red the possible harmful functions. The red mark indicates the effects of NO studied under stress by heavy metals, and the purple mark represents the same effects of NO under stress Fusarium oxyporum.

6. Conclusions

6. Conclusions

1. Bioinformatics analysis of different reports demonstrate that exogenous nitric oxide attenuates damages in plants exposed to Cd, mainly by alleviating oxidative damage. Several evidences showed an early endogenous NO production in plants exposed to Cd, which may trigger signalling pathways involving Ca, hormones, MAPKs and diverse TFs. At later stages however, it appears that plant needs to keep NO level under control to avoid further Cd uptake and root growth inhibition.

2. Cd induces oxidative stress due to the increase in ROS and RNS and the decrease of antioxidant defences, leading to losses in seedling fitness. An excess of NO in *nox1* and *argh1-1* mutants, promotes toxicity symptoms, while low NO levels in *nia1 nia2* mutants improved antioxidant defences, which are crucial to prevent damages, suggesting the existence of mechanisms to control endogenous NO levels after long Cd treatment.

3. NO participates in the fast plant response to Cd by contributing to peroxule production and peroxisome proliferation. Additionally, peroxisome number and distribution, as well as ROS metabolism in these organelles, are altered depending on the levels of NO, probably as result of NO/ROS-dependent posttranslational modifications of proteins including enzymatic antioxidants. Therefore, NO is required for optimal peroxisomal function and signalling under control and stress conditions.

4. Our results suggest that the NO level needs to be optimized and strictly regulated for appropriate plant response to *Fusarium oxysporum* infection. Furthermore, nitrate reductase is involved in proper cell wall assembling, through post-transcriptional and/or post-translational CESA4 and MYB46 regulation, which makes *nia1 nia2* mutants highly susceptible to *Fusarium oxysporum* infection and shows the importance of cell wall in plant defense.

5. Meta-analysis of Cd-dependent transcriptomes have revealed both NO dependent and independent genes, which are shared with plant pathogen responses, in particular with plant response to fungal infection. This may explain why pre-treatment with Cd has a priming effect improving plant survival against *Fusarium oxysporum* infection.

7. General references

7. General references

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8. Annexes

Annex 8.1
8.1. Reactive oxygen and nitrogen species as key indicators of plant responses to Cd stress

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Highlights

- **Reactive oxygen and nitrogen species (ROS/RNS) have a dual role in plant responses to Cd stress.**
- **An excess of ROS and RNS induce oxidative stress in plants under Cd treatment.**
- **ROS and RNS in low concentrations, act as signalling molecules, capable of orchestrating plant responses to Cd stress.**

ABSTRACT

Although cadmium (Cd), an extremely toxic non-essential heavy metal, has no biological function, it is capable of entering plant roots. Cd not only presents a problem for plants, which have developed specific Cd detection, transport and detoxification mechanisms, but also for humans as it can enter the food chain. After entering the root, Cd can be loaded into the xylem and then into the leaves and fruits, thus constituting a major environmental and health hazard worldwide. Understanding the mechanisms involved in plant responses to Cd stress would facilitate the production of crops with a lower Cd uptake and accumulation capacity as well as plants with greater Cd uptake potential for phytoremediation. One of the most common symptoms of Cd toxicity is the induction of oxidative stress in plants, which have developed various strategies to avoid this toxicity, including the early production of reactive oxygen and nitrogen species (ROS and RNS) with signalling functions. This review focuses on the dual role of ROS and RNS in plant responses to Cd stress: in low concentrations, as signalling molecules capable of orchestrating plant responses on the one hand, and at higher concentrations, as oxidative stress inducers on the other.

Key words: Cadmium; H_2O_2; Nitric oxide; Reactive oxygen species; Reactive nitrogen species; Signalling.

Published in Environmental and Experimental Botany Volume 161, May 2019, Pages 107-119 https://doi.org/10.1016/j.envexpbot.2018.10.012.

8.1.1. Introduction

Many natural chemical elements, most of which are essential to life, are present in the earth's crust. Others, such as the potentially toxic heavy metals mercury (Hg), aluminium (Al), lead (Pb) and cadmium (Cd), constitute a major environmental problem when they accumulate (Chen *et al*., 2006). Various anthropogenic activities such as emissions caused by metal mines and industries, coal combustion, solid incineration and direct deposits, especially phosphates, origin Cd and other types of heavy metal pollution (Clemens *et al*., 2013; Clemens and Ma, 2016).

Despite its oxidation state of "+2" and presence only in inorganic form, the bioavailability of Cd is highly dependent on soil structure, organic matter and pH (Clemens and Ma, 2016). Although, to our knowledge, Cd has no biological function, it is able to enter the food chain through plant roots via the cortical tissue and subsequently translocate to above-ground tissues (Shahid *et al*., 2017). Though mainly retained in roots, Cd, whose content generally decreases between roots and seeds, can be rapidly loaded into the xylem and leaves (UNEP, 2008). Cd has been found in most food samples analyzed, such as cereals, vegetables, nuts and tubers, with concentrations ranging from 0.01 to 0.05 mg/kg dry weight (Clemens *et al*., 2013). Cd uptake by plants not only severely limits plant productivity (Sharma and Dietz, 2009) but is also considered a major health risk to humans. There is overall agreement on the highly dominant role played by food intake in chronic exposure to Cd, which is one of the most toxic substances released into the environment (FAO/WHO, 2001; UNEP, 2008; Clemens *et al*., 2013).

A better understanding of molecular mechanisms involved in plant responses to Cd and plant uptake, Cd root retention, translocation and grain loading would facilitate the development of both low Cd-accumulating crops to provide food with lower Cd content and high Cd-accumulating plants for use in phytoremediation to extract Cd from soil and to restore vegetation cover (Clemens and Ma, 2016; Sanz-Fernández *et al*., 2017). Even at low concentrations, Cd is highly toxic to plants, which can produce severe effects due to its solubility and mobility (Pinto *et al*., 2004). Much Cd toxicity is related to its similarity to essential elements such as Fe, Mn, Zn and Ca that enable Cd to first enter the plant and then to replace these elements in proteins (Verbruggen *et al*., 2009).

As demonstrated in numerous studies, oxidative stress is one of the mechanisms that cause metal toxicity in plants (Cuypers *et al*., 2016; Romero-Puertas *et al*., 2012; Schützendübel and Polle, 2002). It is produced by an imbalance in the production and removal of reactive oxygen and nitrogen species (ROS and RNS), leading to an excess in these molecules which damages nucleic acids, lipids and proteins (Sandalio *et al*., 2012). In recent years, however, low levels of ROS and RNS have been recognized to play a key role in signalling networks governing plant stress

responses (Foyer and Noctor, 2005; Romero-Puertas *et al*., 2012). In this review, we discuss plant responses to Cd, with a particular focus on the role of ROS/RNS and their dual function in relation to ROS/RNS-dependent damage and signalling.

8.1.2. Cadmium toxicity and plant coping mechanisms

The following signs of Cd-induced toxicity can be found in most of the heavy metals described previously (Clemens, 2006; Schützendübel and Polle, 2002; Sharma *et al*., 2016): 1) a macro- and micro-nutrient imbalance, which was one of the first symptoms of Cd stress to be described, particularly in Fe and Ca (Gupta *et al*., 2017; Loix *et al*., 2017; Sandalio *et al*., 2001) a redox status imbalance causing oxidative stress (see paragraph 8.1.4.2.), mainly due to an imbalance in antioxidant defences, the triggering of Fenton reactions through the displacement of Cu and Fe ions from metalloproteins and respiratory chain impairment (Cuypers *et al*., 2016; Dixit *et al*., 2001; Romero-Puertas *et al*., 1999; Sandalio *et al*., 2001; Schützendübel *et al*., 2001; Sharma and Dietz, 2009; Valko *et al*., 2005); and 3) enzyme inactivation caused by both Cd binding to Cys residues (Schützendübel and Polle, 2002) and direct displacement of essential metals by Cd at specific structural and/or functional sites; this occurs with calmodulin when Ca is replaced by Cd, thus inhibiting calmodulin-dependent phosphodiesterase activity in radish and the photosystem II reaction centre, which prevents PSII photoactivation (Clemens, 2006; Faller *et al*., 2005); Zn-binding molecules are also good candidates for Cd given their chemical similarities (Clemens, 2006).

Although the imbalance in nutrient uptake and distribution has been described in a wide range of species such as wheat, pea and sunflower (Rizwan *et al*., 2016; Rodríguez-Serrano *et al*., 2009; Sandalio *et al*., 2001; Shukla *et al*., 2003), responses to Cd toxicity varied among genotypes and in terms of the Cd stress applied (Rizwan *et al*., 2016). The decrease in different nutrients under Cd stress is most likely due to direct competition for transporters during uptake or to transporter inhibition to avoid metal entry, as occurs with IRT1, an iron transporter partly responsible for Cd entry into root cells (Connolly, 2002; Connolly *et al*., 2003; Rizwan *et al*., 2016). Interestingly, it has been shown recently that NADPH oxidases may have an important role in regulating the entering and the root-to-shoot nutrient transport in Arabidopsis response to Cd although the mechanism is still unknown (Gupta *et al*., 2017). Deficiency in different nutrients, such as Fe, Mn and P, may induce chlorosis, one of the visible and most characteristic symptoms of Cd stress (Liu *et al*., 2016). A decreased nutrient content, inhibition of NO_3^- assimilation and photosynthetic process damage lead to a characteristic decrease in the growth of plant aerial tissues and roots under Cd stress (Rodríguez-Serrano *et al*., 2009; Sandalio *et al*., 2001; Watanabe *et al*., 2010). Growth may also be inhibited by a decrease in chorophyll content, the transpiration rate and water use efficiency that are usually observed in plants under Cd stress (Faller *et al*., 2005;

Qian *et al*., 2009; Sandalio *et al*., 2001). All symptoms of Cd toxicity ultimately reduce crop growth and yield and sometimes lead to genotoxicity, resulting in plant death. The severity of toxicity, however, depends on the growth stage of the plant, metal concentration, length of treatment and the plant's coping mechanisms.

Once Cd has entered the cell, the best characterized plant response is the synthesis of phytochelatins (PCs), a family of Cys-rich peptides enzymatically produced for the purposes of heavy metal chelation and sequestration (Cobbett and Meagher, 2002; Mendoza-Cózatl *et al*., 2010). Before PCs synthesis, sulfate assimilation and Cys and GSH biosynthesis need to be upregulated (Clemens, 2006). Furthermore, depending on the species, PC synthase activity may be regulated at both the transcriptional and posttranslational level in response to heavy metals with higher Cd ion affinity and with lower affinity for other metal ions such as Cu (Vatamaniuk *et al*., 1999). Cd-PC complexes are drawn into the vacuole through ABC transporters (Vögeli-Lange and Wagner, 1990), Cd/proton antiporters (Berezin *et al*., 2008; Korenkov *et al*., 2007) and V-ATPase and V-PPase activity (Sharma *et al*., 2016). Even the volume and form of the vacuole, which plays a key role in Cd detoxification, changes in response to heavy metal stress (Sharma *et al*., 2016). Another characteristic and oft-described plant response to Cd is the up-regulation of stress proteins, such as heat shock and antioxidant proteins, and advances in omic techniques have enabled researchers to investigate global sets of genes, proteins and metabolites that respond to Cd stress (see paragraph 3). Recently, microRNAs (miRNAs), which negatively regulate specific target mRNAs, such as TFs and stress response-related genes at the post-transcriptional level, have been also found to be involved in mechanisms of heavy metal plant tolerance (Cuypers *et al*., 2016; Ding *et al*., 2011).

On the other hand, plants have developed root-level extracellular strategies to avoid Cd entry into the cytosol. It is worth noting the plant-microorganism interactions with bacterial strains present in the rhizosphere of some hyperaccumulators, such as *A. halleri*, which are capable of decreasing Cd concentrations in shoots (Farinati *et al*., 2011), and also with mycorrhizal fungi (Courbot *et al*., 2004; Janousková *et al*., 2006; Jentschke *et al*., 1999). Mycorrhized plants are more tolerant of heavy metals due to the ability of fungus to retain metals in roots and to prevent their translocation to aerial parts (Ferrol *et al*., 2016; Göhre and Paszkowski, 2006); it has also been suggested that the beneficial effect of microorganisms on plants may be partly attributable to the metabolism of the microbial antioxidant defence (Azcón *et al*., 2010). Other major strategies to prevent Cd from entering the cell are: 1) Cd immobilization by cellular and extracellular carbohydrates (Clemens and Ma, 2016); 2) metal accumulation in the plant cell wall, the first barrier, which can retain low concentrations of Cd effectively in the Arabidopsis plant (Loix *et al*., 2017; Memon and Schröder, 2009; Van Belleghem *et al*., 2007); 3) changes in permeability to

prevent heavy metal transport throughout the plasma membrane and induction of metal ion exit flux by transporters such as ATPases, NRAMPs and CDFs and ZIPs (Clemens *et al*., 2013); 4) physical avoidance of contaminated soil; this has recently been demonstrated in relation to the Cd-phobic mutant MRC-22 which is capable of arresting growth in the Cd-affected primary root and of inducing a number of lateral roots located in the Cd-free zone (Watanabe *et al*., 2010).

Hyperaccumulators, such as *Arabidopsis halleri*, *Thlaspi praecox*, *Thlaspi caerulescens* and *Sedum alfredii*, are an interesting group of plants, which accumulate high concentrations of Cd without showing any toxicity symptoms (Verbruggen *et al*., 2009; Zhao *et al*., 2006). Transcript analysis of *A. halleri* and *A. thaliana* plants has shown that a number of metal homeostasis genes, such as HMA and ZIP gene family members, as well as stress genes, may be the key for Cd hyperaccumulation (Becher *et al*., 2004; Weber *et al*., 2006). Zhang and Qiu (2007) have also shown that peroxidases may play a pivotal role in metal hyperaccumulation.

8.1.3. Use of omic technologies to investigate plant responses to cadmium stress

Omic technologies, which can investigate multiple molecules simultaneously, are primarily used for the universal detection of genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) as well as for biomarker detection. Numerous studies using omic techniques have been carried out in recent years in order to provide an overview of plant responses to Cd at the transcription, proteome and metabolome level. The results of transcriptomic and proteomic analyses carried out over the last six years are presented in Table 8.1.1. and Suppl. Table S8.1.1., respectively; while a smaller number of metabolomic studies are presented in Table 8.1.2. (a scheme is also presented in Fig. 8.1.1.). Previous omic analyses are described in other reviews (Dupae *et al*., 2014; Romero-Puertas *et al*., 2012; Villiers *et al*., 2011). Despite the enormous amount of data available, the diverse range of experimental conditions, such as the concentration of the metal used, timing, cultivation method and age of plant, makes it rather difficult to gain a comprehensive understanding of global plant responses to Cd (Fig. 8.1.1.; Table 8.1.1.; Table 8.1.2., Suppl Table S8.1.1.; Dupae *et al*., 2014; Villiers *et al*., 2011). Nonetheless, while taking into account the different types of analysis employed, certain general conclusions can be drawn.

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Table 8.1.1. A summary of transcriptomic studies of plant response to Cd treatment in the last five years.

Plant system/Treatment	Technique	Transcriptomic regulated by Cadmium
Sedum alfredii. **Shoot** **100 µM/5 µM Cd: 8 days**	RNA-Seq	110 up and 123 down: cell wall modification, metal translocation and remobilizati than that in NHE shoots.
Populus x canescens **Plant** **200 µM Cd:20 days**	Affymetrix array	505 up and 336 downr: 85 GO groups highlight anion, nucleoside phosphate and response to stress, regulation of cellular process, catalytic activity and biologic
Elodea nuttallii **Shoot** **500/5000 µg L^{-1} : 24 h**	RNA-Seq	54 up and 158 down: metabolism of energy reserves, ion transport and transport transduction, homeostasis, cellular import, transport facilities, detoxific
Arabidopsis thaliana **Plant** **Mutants Cd: 21 days**	ATH1 chip Affymetrix	204 up and 333 down: ROS metabolism, ion transport, heat shock, and temp. str metabolic functions, detoxification, stress response, growth and development, t storage, protein synthesis, and DNA repair.
Salix matsudana **Root and leaf** **50 µM Cd: 10 h**	RNA-Seq	912 up and 669 down in leaf, 448 up and 459 down root: biotic stress and involve secondary metabolism, signalling pathways and cell wall metabolis
Zea mays **Root** **200 mg/L Cd: 0, 12 24 and 48 h**	RNA-Seq	867 induced, 1500 repressed in B73 and 2, 852 induced, 3, 248 repressed in Mo1 115 involve in stress and defense responses proteins, transporters, as well as trans
Boehmeria nivea **Plant** **100 mg/kg Cd: 20 days**	RNA-Seq	155 genes: stress-responsive, antioxidant systems and GSH metabolism-rel
Miscanthus sacchariflorus **Root** **100 µM Cd: 24h**	RNA-Seq	345 up and 336 down: metabolic pathway, starch and sucrose and biosynthesis metabolites and metal ion transporters
Oryza sativa **Root** **100 µM Cd: 1h**	RNA-Seq	1772 : hormone signalling, transcriptional regulation (NACs and WRKYs), tra upregulated. ZRT/IRT-like proteins are downregulated. 104 are specifically respo pressures.

Table 8.1.1. Continuation A summary of transcriptomic studies of plant response to Cd treatment in the last five years.

Plant system/Treatment	Technique	Transcriptomic regulated by Cadmium
***Brassica rapa* Root** **10 mg/kg Cd: 28 days**	cDNA lib. RNA –Seq	797 ROS related genes
***Hordeum vulgare* Shoot/root** **80 µM Cd: 5 days**	RNA-Seq	Not analized
***Triticum polonicum* Root** **40 µM Cd: 5 days**	RNA-Seq	96 down and 667 up-regulated involve in glutathione metabolism, oxidation-reducti carbohydrate metabolism, metal transport, nitrate metabolism, metal chelat
***Arabidopsis thaliana* Root/shoot** **2 µM Cd: 7 days**	ATH1 chip Affymetrix	Col 0 698 up 736 down ; Bur-0 1090 up- and 722 down: systemic acquired resistance resistance, response to jasmonic and salicylic acid, defence incompatible interactio response to hypoxia, response to ROS and toxic substance, cell wall biogenesis, and metabolic process, glucosinolate biosynthetic process
***Vicia sativa* Roots/stems/leaves** **5/50 µM Cd: 24h**	RNA-Seq	69 (L3) and 28 (ZM) with 5 uM Cd, while in the Cd treatment, 1036 (L3)and 335(Z µM: cell wall, stress response, the glutathione pathway, metal transporters and transcr
***Brassica napus* Leaf** **50/ 200 µM Cd: 30 days**	cDNA lib. RNA –Seq	680 up and 1422 down: photosynthesis proteins, thiamine metabolism, sulfur me endoplasmic reticulum, ribosome, flavonoid biosynthesis, degradation of aromatic c galactose metabolism, ubiquinone and other terpenoid-quinone biosynthesis and pla interaction
***Arabidopsis halleri* Root/Shoot** **5 µM Cd: 10 days**	RNA-Seq	More than1000: secondary metabolism, phenylpropanoids and glutathione, cel

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Table 8.1.2. A summary of metabolomic studies of plant response to Cd treatment in different plant species.

Plant system/Treatment	Technique	Metabolitesproduced by Cadmium
Silene cucubalus **Cell** **150 µM Cd: 3 days**	NMR spectroscopy	25: energy production and TCA cycle
Arabidopsis thaliana **Cell** **0,5, 2, 5, 20, 50, 200 µM Cd: 1 day**	LCQ-Duo IT	13: PC and GSH
Arabidopsis thaliana **Cell** **50, 200 400 µM Cd: 1 to 48h**	LC/ESI-MS	10: PCs; Thiols (GSH)
Brassica napus **Phloem/xylem** **75 µM Cd: 1h, 7 and 14 days**	HPLC/MS	7: phytochelatin synthesis; Thiols (GSH); sugars
Arabidopsis thaliana **Cell** **5, 50 or 200 µM Cd: 1, 4, 8, 11, 24h**	ESI-MS (LC/MS)	14: GSH levels, and PC (most diverse when Cd doses are increased) 14 metabolites
Solanum lycopersicum **Leaf** **20, 100 µM Cd: 90 days**	H NMR, HPLC-PDA;	28: Cd detoxification and antioxidant defense and decrease inisoprenoid molecule
Glycine max **Microsome** **100 µM Cd: 3 days**	(CE)/MS	32: 18 with changes, Amino acids associated with heavy metal detoxification processes, ATP biosynthesis, secondary metabolites in response to heavy metal, glutathione biosynthesis, proline cultivars
Arabidopsis thaliana **Seedling** **5 and 50 µM Cd: 14 days**	GC–TOF–MS	80: Antioxidants; Aminoacid poliamines ; a-ketoglutarate and oxaloacetate

Table 8.1.2. Continuation A summary of metabolomic studies of plant response to Cd treatment in different plant species.

Plant system/Treatment	Technique	Metabolitesproduced by Cadmium	R
***Brassica juncea* Leaf** **25, 50, 100 µM Cd: 21 days**	Mass Spectrometry: Q-TOF Setting	47: glutathione homeostasis phytochelatins purine metabolism ; Calvin cycle; plant tricarboxylic acid cycle (TCA); glycolysis hormones/signalling molecules.	(D'Alessa
***Solanum lycopersicum* Root** **25 µM micorrized or not**	UPLC/Q-TOF	23 non mycorryze 32 mycorrize; hormones; Phenolic compounds; glutamate derivatives; phytochelatin; fructan inulins; melatonin intermediate; quinones/quinols	(Kum
***Raphanus sativus L.* Radish taproot** **400 mg L^{-1} Cd: 72h**	GC-MS	33: Galactose metabolism; Starch and sucrose metabolism; Aminoacyl-tRNA biosynthesis; Oxidative phosphorylation	(Wan
***Oryza sativa* Plant** **10, 50, 100, 1000 µM Cd: 3 days**	MCR-ALS	54: secundary metabolites, aminoacid syntesis and metabolite, abc transporter, glicerolipid metabolism, carbom metabolisem, Glyoxylate and dicarboxylate metabolism, Aminoacyl-tRNA biosynthesis	(Navarro-
***Arabidopsis halleri* Root/Shoot** **5 µM Cd:10 days**	RP HPLC	15: flavonols, hydroxycinnamic acids	(Cors

The results of transcriptomic analyses suggest that plant responses to Cd are more dependent on dosage timing than exposure period. In addition, plant tissue was found to be regulated differentially (Harada *et al*., 2010; Herbette *et al*., 2006; Ogawa *et al*., 2009; Table 8.1.1.), which is similar to the finding from proteomic analysis (Suppl. Table S8.1.1., Dupae *et al*., 2014). Short-term responses are mainly associated with signalling pathways by hormone metabolism and transcription factor regulation (Table 8.1.1.); long-term exposure responses are associated with up-regulation of genes related to biotic responses, detoxification and oxidative stress, protein metabolism, including PTMs and proteolytic activity, as well as transporters; and down-regulation of genes related to photosynthesis and the carbon metabolism (Table 8.1.1.). Similarities between transcriptomic and proteomic analyses, especially following long-term treatment, are linked to the following gene/protein groups: 1) detoxification, including the removal of toxic compounds and molecules such as ROS and damaged proteins; 2) defence, such as pathogenesis-related proteins and heat shock proteins; 3) photosynthesis, which causes overall inhibition probably to avoid oxidative damage; and 4) energy remobilization and reductive power (Fig. 8.1.1.; Table 8.1.1.; Suppl. Table S8.1.1.; Dupae *et al*., 2014; Romero-Puertas *et al*., 2012; Villiers *et al*., 2011).

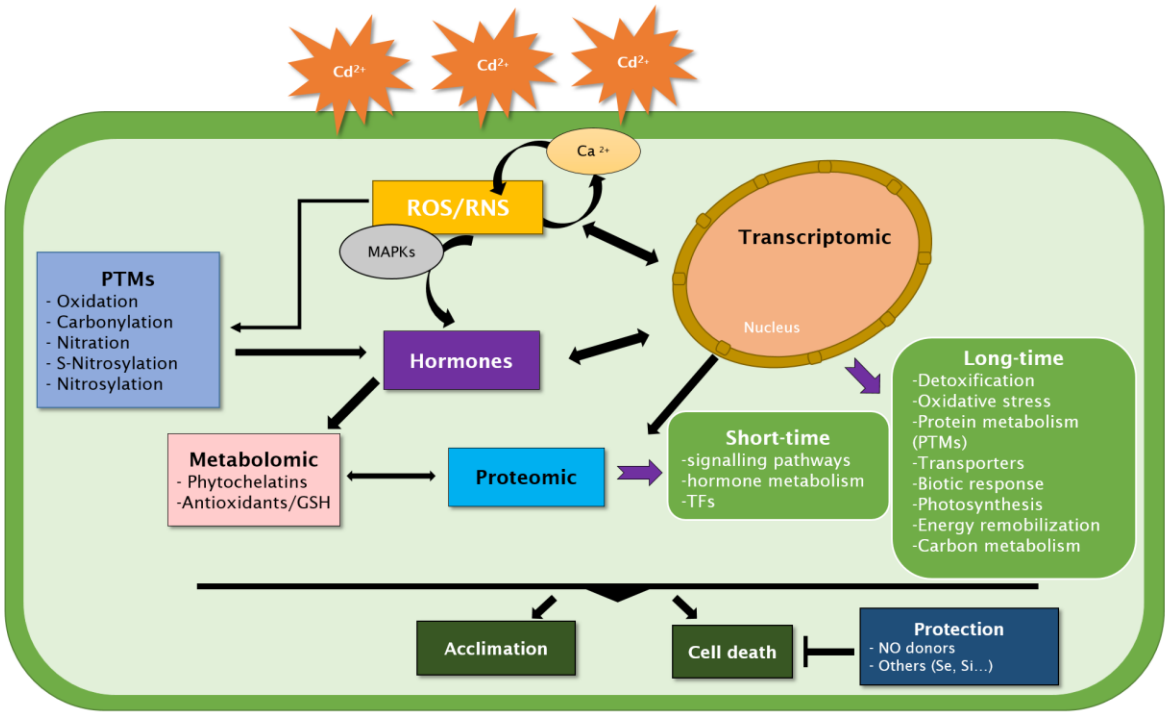

Fig. 8.1.1. General plant responses to Cd treatment: Different omic analyses showed that short-term treatment induced mainly signalling pathways involving hormone metabolism and transcription factors (TFs), while long-term treatment induced mainly restorative metabolism, transport and energy and carbon metabolism reorganization. Treatment timing and severity, and plant species characteristic will lead to acclimation or cell death of the plant, giving some protection different molecules including nitric oxide (NO).

Suppl. Table S1: A summary of transcriptomic studies of plant response to Cd treatment in the last ten years.

Plant system/Treatment	Technique	Protein regulated by Cadmium
***Glycine max* Leaf β-aminobutyric 100 µM Cd 5days**	2DE LTQ XL Orbitrap MS and MALDI-TOF	17 :Oxygen-evolving enhancer protein 1, ibulose bisphosphate carboxylase smal carbonic anhydrase, methionine synthase, and glycine dehydr
***Oryza sativa* Root/Leaf SNP 100 µM Cd: 8 days**	2DE MALDI-TOF/ TOF-MS	16 leaf and 25 root: photosyntesis, carbohidrate metabolism, nitrogen metabolism oxydative stress response, signal trasduction and cell divi
***Glycine max* Microsome 100 µM Cd: 3 days**	2DE LC-MS/MS	13 and 11 differentially expressed proteins in the Enrei and Harosoy cultivars: de biosynthesis
***Caesalpinia peltophoroides* Leaf 32 mg/L Cd: 4, 72 h**	2D-PAGE ExPASy TagIdent	83: stress resistance to stress, detoxification, degradation, antioxidant, transp photosynthesis, electron transport, biosynthesis reactions, and transcr
***Glycine max* Leaf 100 µM Cd: 3 days**	2DE LTQ XL /MS	71: metabolism, energy production, glycolysis, TCA cycle, photosynthesis related enzymes.
***Populus spp* Leaf 20 µM Cd: 2, 14 days**	2D LC MALDI-LR TOF/MS	46 short term and 67 long term: response to biotic and abiotic, carbohydrate metab and amino acid synthesis, ATP synthesis and proton transport transcription and tra carbohydrate metabolism, photosynthesis, glycolysis associated proteins; lignin an division, ascorbate metabolism.
***Hordeum vulgare* Grain Zhenong8 and W6nk2 in soils**	2D MALDI-TOF-TOF	17: Protease inhibitor, storage protein, response to stress, carbohydrate metabo synthesis,Signal, protease inhibitor, response to stress, antiviral protein, an
***Brassica juncea* Leaf 25, 50, 100 µM Cd: 21 days**	2DE nanoHPLC-MS/MS	94: photosystem components and the alteration of metabolic enzymes, ATP sy anhydrase, and enzymes involved in antioxidant responses (glutathione and phyto Calvin cycle.
***Solanum torvum* Root 50 µM Cd: 24h**	2DGE MALDI-TOF MS	8: phytohormone synthesis, defence responses, energy metabolism, and cyt

Suppl. Table S1. Continuation: A summary of transcriptomic studies of plant response to Cd treatment in the last ten years

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Plant system/Treatment	Technique	Protein regulated by Cadmium
***Kandelia candel* Root 100, 200, 400,800 µM Cd: 3 days**	2DE MALDI-TOF/TOF	53: proteins involved in oxidative response, including antioxidant enzymes, enzy biosynthesis, enzymes in TCA and PPP cycles
***Arabidopsis thaliana* Cell 50, 100, 200, or 300 µM Cd:24, 48, 72, 96, 120 h**	2D DIGE MS/MS	110 proteins involve in cell wall biosynthesis, protein folding, and degradation ca homeostasis, and anti-oxidative processes
***Populus tremula* Leaf 20 µM Cd: 56 days**	2DE MALDI MS/MS	73: glutamine and sugar metabolism, protein folding and proteolysis, protein bio biotic stressand abiotic stress, fatty acid metabolism and mitochondrial protei
***Oryza sativa* Grain 0.1 mmol L^{-1}: 5,10,15,25 days**	2D MALDI-TOF/TOF MS	14 proteins in D23: nutrient accumulation, including storage of proteins and starc 38: photosynthesis, protein synthesis, molecular chaperones, and ROS clea
***Nicotiana tabacum* Leaf 50 µM Cd: 5 days**	2DE MALDI-TOF-TOF	18: metabolism, photosynthesis, stress response, signal transduction, protein sy transport and cell structure.
***Populus nigra* Leaf 50 µM Cd: 1h**	2DE LC–MS/MS	21: stress response related to photosynthetic carbon metabolism and e
***Gossypiun spp* Leaf 500 µM Cd: 24 h**	2DE MALDI-TOF–TOF	21: synthesis and regulation, photosynthesis/CO_2 assimilation, energy and carbo homeostasis, cell rescue/defence, chaperones and stress related protein
***Spinacia oleracea* Leaf 50 mg/g Cd: 7 days**	2DE-MALDI TOF/TOF MS	53: signal transduction, protein synthesis, stress response and defence, photosy division, energy generation, transport, secondary metabolism, ar
***Helianthus annuus* Leaf 50 or 700mg Cd: 45 days**	2D DIGE nESI-LC–MS/ MS	18: involve in energy, defence and photosynthesis

Suppl. Table S1. Continuation: A summary of transcriptomic studies of plant response to Cd treatment in the last ten years

Plant system/Treatment	Technique	Protein regulated by Cadmium
***Populus yunnanensis* Leaf 100 µM Cd: 4, 8, 12 days**	2DE MALDI-TOF/ TOF-MS	83: response to stress, material metabolite, photosynthesis and energy metabol antioxidant activity, protein kinase, and predicted prote
***Eichhornia crassipes/Pistia stratiotes* Plant 100 mg/L 2, 3, 5 days**	2DE MALDI- TOF-MS/MS	59: Metabolism process, Photosyntesis, Growth and development, defence resp transcription and traslation,protein posttranslation modification, ion transporter a oxydation and reduction process and biosintesis and degrad
***Brassica napus* Leaf Ala 100, 500µM Cd: 15 days**	2DE MASCOT	34: CO_2 assimilation/ photosynthesis, protein synthesis/ regulation, stress relate dehydration of damaged proteins, catalysis, defence related proteins, redox home
***Cakile maritima* Leaf 25, 50, 100µM Cd: 21 days**	2DE HPLC-MS MicroTOF-Q	36: thiol compound anabolism, including glutathione and phytochelatin homeostas and Calvin cycle , triacylglycerol biosynthesis and photorespiration, carbonic an photosystem integrity and metabolic activity, glutathione–ascorbate homeostasis, t and biogenesis of PSII.
***Sorghum bicolor* Leaf 100, 150µM Cd: 5 days**	2DE MALDITOF/ TOF MS	33: carbohydrate metabolism, transcriptional regulation, translation an
***Oryza sativa* Roo 200 µM Cd: 3, 5,7 days**	2DE LC–MS/MS	8: plant energy metabolism, tricarboxylic acid cycle, glycolysis and
***Cucumis sativus* Leaf/root Se 50 µM Cd: 7 days**	2DE MS/MS	26: response to stress, metabolism, photosynthesis, storage and
***Oryza sativa* Cell Si 30 µM 12h or 5 days**	iTRAQ-based	100: carbohydrate and energy metabolisms, defence and stress response, biogene protein processes, amino acid and fatty acid metabolisms, signalling
***Amaranthus hybridus* Root 60 mg/kg Cd: 90 days**	2DE MALDI-TOF-TOF	28 : energy metabolism, protein metabolism, stress and defence, and signal t microRNAs, cell walls, and other structural componen
***Cucumis sativus* Leaf SNP 100 µM Cd:10 days**	Itraq LS-ESI MS/MS	377, 297 and 432 DEPs: binding, catalytic activity, structural molecule activity, t process, response to stimulus

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Suppl. Table S1. Continuation: A summary of transcriptomic studies of plant response to Cd treatment in the last ten years

Plant system/Treatment	Technique	Protein regulated by Cadmium
***Raphanus sativus* Root 50 µM Cd: 12h**	2DE MS.	91 up and 66 down-regulated control vs Cd10 , while 340 up- and 286 down in Cd ROS scavenging, cell transport and signal transductio
***Oryza sativa* Grain N fertilizers 4 genotypes**	2DE MALDI-TOF-TOF MS	N treatment vs control, 28 and 17 protein species were up-accumulated wherea (R8097) were down-accumulated: metabolism, detoxification, signal tran
***Sedum alfredii* Leaf 40 or 200 µM Cd: 15days**	LC MS/MS Triple TOF 5600 plus	248 in HP leaves than in NHP leaves. With Cd 13 proteins in HP leaves and 33 pro Associated with vacuolar sequestration, cell wall/membrane modification
***Crocus sativus* Leaf 20 mg kg^{-1} Cd: 30 days**	2DE MALDI-TOF-TOF-MS	94 up regulated and 64 down-regulated Metabolism, signal transduction, stress a photosynthesis; transport, protein synthesis
***Medicago sativa* Root H_2 100 µM Cd: 24, 72, or 96 h**	Itraq-8 plex	92: defence and response to stress, sulfur compound metabolic, aminoacid and pro and energy metabolic, secundary metabolic, oxidation-reduction, and me
***Arabidopsis thaliana* Leaf 100 mmol L^{-1} Cd: 7 weeks**	2D DIGE ESI LC-MS -MS	32 disease/defence, energy and metabolism
***Medicago sativa* Cell walls/Plant 10 mg ·kg^{-1} Cd: 2 month**	2D DIGE	179: cell wall proteins and 30 proteins in the soluble fraction are involved in cel response, carbohydrate metabolism and promotion of the lignific
***Amaranthus gangeticus* Leaf/stem/root 1.82 mg·kg^{-1}/11.79 mg·kg^{-1} Cd; 35 days**	2DE MALDI-TOF-TOF	286 (leaf) 307 (stem) 324 (root) in Pen vs C and 28 protein (leaf) 278 (stem) 19 treatment ; 145 (leaf) 363 (stem) 358 (root) in Pen vs C and 143 (leaf) 262 (stem) 2 in high treatment: metabolism, energy, protein synthesis, transporter, cell stru metabolism.
***Brachypodium distachyon* Leaf 500 µM Cd: 2,4 days**	2D DIGE MALDI-TOF-TOF-MS	78 up 38 down (2 days) 96 up 17 down (4 days) photosynthesis/respiration, ener stress/defence/detoxification , protein folding and degradation, and amin
***Microsorum pteropus* Leaf/root 500 µM Cd: 7 days**	2DE MALDI-TOF/TOF	8 in Root: antioxidants and regulation of energy metabolism, 20 in Leaf : photo cellular metabolism

Comparative metabolomic analysis of plant responses following Cd stress highlight effects similar to those found by transcriptomic and proteomic analyses, with different metabolites mainly involved in the TCA cycle, energy production and antioxidants (Table 8.1.2; Villiers *et al*., 2011). It is also worth noting metabolites such as PCs and GSH, with various analyses showing a very large number of PCs produced by Cd stress (Table 8.1.2.). When metabolite and proteomic studies, carried out under similar material conditions, are compared, the main response to Cd stress appears to be activation of nitrogen, carbon and sulfur metabolisms in order to trigger PCs and to minimize the effects of the metal (Sarry *et al*., 2006).

8.1.4. Reactive oxygen species metabolism in response to cadmium

The term reactive oxygen species (ROS), which are produced by aerobic metabolism, refers to species derived from the reduction of oxygen (Halliwell and Gutteridge, 2015) and made up of both free radicals and non-radical compounds. The most important free radicals are superoxide ($O_2^{\cdot-}$), hydroxyl (·OH), alkoxyl (RO^{-}), peroxyl (ROO^{-}) and hydroperoxyl ($HO_2\cdot$); the principal non-radical compounds are hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^{-}$). ROS steady-state levels are regulated by the interplay between different ROS-producing and ROS-scavenging mechanisms. The ROS-scavenging mechanism involves enzymatic antioxidants, such as superoxide dismutase (SOD), which remove $O_2^{\cdot-}$ radicals, as well as catalases (CATs), peroxidases (POXs) and peroxiredoxins, which decompose H_2O_2 (Sandalio *et al*., 2009), and non-enzymatic compounds such as glutathione (GSH), ascorbate (ASC), tocopherol, flavonoids, phenols and proline (Ellouzi *et al*., 2013; Hasanuzzaman *et al*., 2017; Hossain *et al*., 2017; Halliwell and Gutteridge, 2015). An excess of ROS is dangerous mainly due to reactions with lipid proteins and nucleic acids, which severely damage the cell (Chmielowska-Bąk *et al*., 2017). This explains why both enzymatic and non-enzymatic antioxidants play a coordinating role in regulating ROS accumulation in different subcellular compartments. An example of this coordination is the ASC-GSH cycle composed of ascorbate peroxidase (APX), monodehydroascorbate peroxidase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), as well as the reduced and oxidized forms of ascorbate (ASC/DHA) and glutathione (GSH/GSSG) in addition to NADPH, produced by several dehydrogenases such as those involved in the pentose phosphate cycle (Hossain *et al*., 2017). However, ROS also play an important role as signalling molecules in various processes including tropism, cell death and cell responses to biotic and abiotic stress (Mittler *et al*., 2011).

8.1.4.1. Cadmium-induced sources of Reactive oxygen species

As Cd, a divalent cation, is not involved in cellular redox reactions, it is unable to produce ROS directly. However, an enormous amount of information from biochemical and transcriptomic studies demonstrate that oxidative stress is one of the first consequences of Cd toxicity in plants and other organisms (Yu *et al*., 2017; Gupta *et al*., 2017; Sandalio *et al*., 2001). ROS are not only produced in the cell by high electron flow rates along the mitochondrial electron-transport chain and chloroplast, but also in peroxisomes, plasma membrane, nuclei and the apoplast (Fig. 8.1.2; Sandalio *et al*., 2009; Halliwell and Gutteridge, 2015). Various sources of ROS have been found to be induced by Cd treatment. NADPH oxidases (*NOX*s), also called RBOHs (respiratory burst oxidase homologs), from plasma membrane, are considered one of the first Cd-induced sources of ROS production, as observed in different plant species using hystochemical/cytochemical techniques and *rboh* Arabidopsis mutants (Garnier *et al*., 2006; Gupta *et al*., 2017; Horemans *et al*., 2007; Olmos *et al*., 2003; Ortega-Villasante *et al*., 2007; Romero-Puertas *et al*., 2004). *NOX* catalyzes the production of $O_2^{\cdot -}$, which can be converted into H_2O_2, either spontaneously or in a SOD-catalysed reaction. Ten RBOH-encoding genes (RBOH A-J) have been described in Arabidopsis plants, with low Cd concentrations inducing *RBOHF* up-regulation (Horemans *et al*., 2007). In the same plant species exposed to higher Cd concentrations, analysis of H_2O_2 content in WT and *rboh* mutants suggests that each RBOH behaves differently with respect to H_2O_2 production in response to Cd, with RBOHC being the most involved and RBOHF not contributing significantly to ROS production (Gupta *et al*., 2017). Although a large number of studies report that NADPH oxidases play a predominant role in ROS production, some authors have questioned this finding and point to mitochondria as the main source of ROS under Cd toxicity conditions (Heyno *et al*., 2008). These authors suggest that ROS accumulation in response to Cd observed in the plasma membrane could be due to mitochondrial ROS diffusion. However, crosstalk between mitochondria and RBOHC has recently been reported in Arabidopsis plants exposed to moderate Cd concentrations. Alternative oxidase 1 (*AOX1*) has been shown to activate or maintain mitochondrial signalling pathways regulating cellular antioxidative defences at the post-transcriptional level under moderate Cd exposure. These pathways require ethylene, either directly or indirectly via *RBOHC*, to regulate *AOX1* expression (Keunen *et al*., 2015). In tobacco cell cultures, several waves of ROS have been reported, with NADPH oxidase from plasma membrane being the first wave, followed by the mitochondria, probably associated with semi-ubiquinone radicals, and a final third wave linked to membrane lipid peroxidation (Garnier *et al*., 2006). ROS production in mitochondria takes place in complex I and complex II, although aconitase, present in the mitochondrial matrix, can also be involved (Rasmusson *et al*., 2008). In addition, peroxisomes contribute to ROS production under Cd toxicity conditions, as shown by the

accumulation of H_2O_2 in these organelles. Peroxisomes contain the following sources of $O_2^{\cdot-}$: xanthine oxidase (XOD), uricase (UO), sulfite oxidase (SO) and electron transport chains associated with the membrane (Sandalio and Romero-Puertas, 2015) and the sources of H_2O_2: glycolate oxidase (GOX) and acyl-CoA oxidase (ACX) (Sandalio and Romero-Puertas, 2015). GOX activity has been proven to increase in pea plants in response to Cd (Romero-Puertas *et al*., 1999, 2004). Time course analyses of H_2O_2 and $O_2^{\cdot-}$ production in *Glycine max* have revealed several waves of ROS production in plants exposed to Cd and have identified GOX as a source of ROS (Pérez-Chaca *et al*., 2014). A close relationship between GOX from photorespiration and RBOHs after long Cd treatment periods has also been reported in Arabidopsis plants (Gupta *et al*., 2017). Chloroplasts have been shown to contribute to ROS production in protoplasts from Arabidopsis (Bi *et al*., 2009).

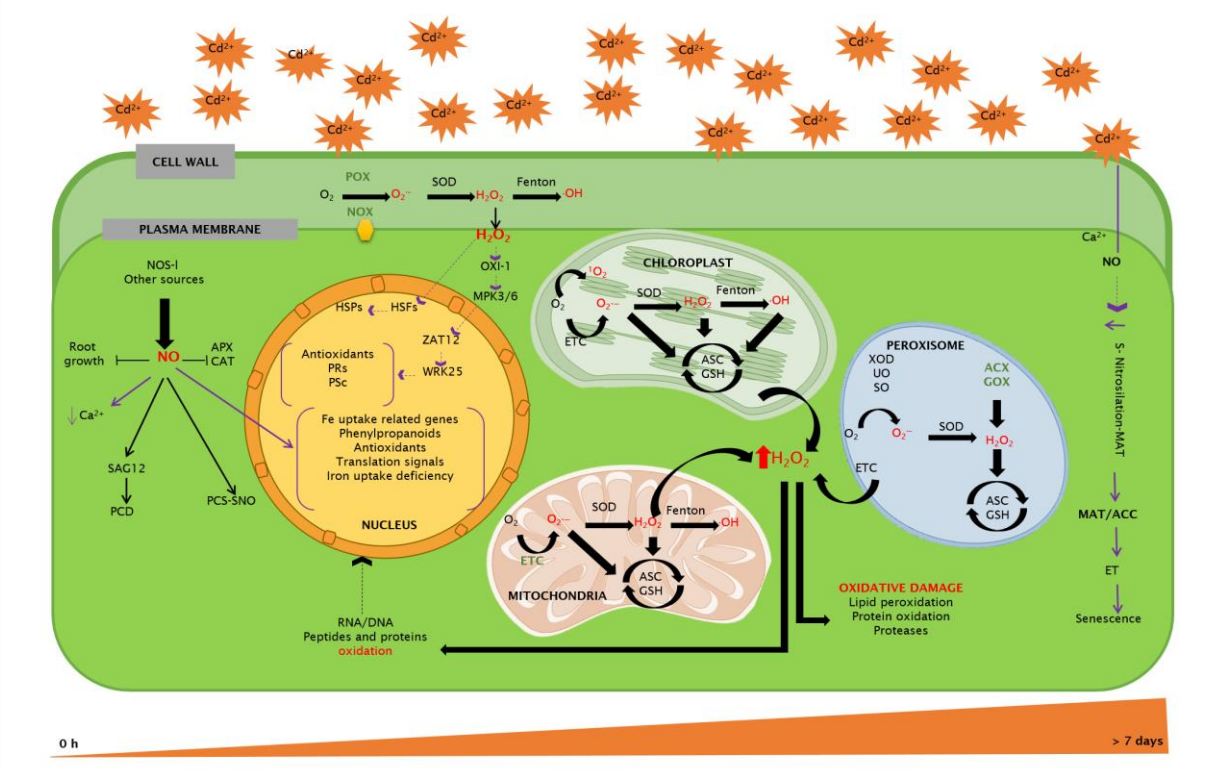

Fig. 8.1.2. Sources and function of ROS/RNS in plant response to Cd stress. Different sources of ROS have been shown to produce ROS after Cd treatment, mainly NADPH oxidases and peroxidases as the first barrier; peroxisomal glycolate oxidase (GOX) and acyl-CoA oxidase (ACX); and mitochondrial complex I and II. Low levels of H_2O_2 lead to a MAPK-dependent cascade inducing different TFs families, such as ZAT and WRKY probably regulating antioxidant systems of the cell. On the other hand, high levels of H_2O_2 lead to oxidative damage, such as lipid peroxidation and carbonylation. Main NO source described in response to Cd is nitric oxide synthase-like (NOS-l) and NO has been shown to be linked to Cd and Ca entry in the cell, regulation of oxidative metabolism and root growth inhibition.

Cd-dependent H_2O_2 and $O_2^{\cdot-}$ accumulation in tonoplasts from bundle sheet cells has also been observed in pea leaves, although the source has yet to be identified (Romero-Puertas *et al*., 2004). ROS accumulation has been detected in vascular tissues from Cd-treated pea plants by confocal electron microscopy and ROS cytochemistry (Rodríguez-Serrano *et al*., 2006, 2009;

Romero-Puertas *et al*., 2004); this is associated with lignification, a process which is particularly active in these tissues under metal toxicity conditions (Loix *et al*., 2017; Rodríguez-Serrano *et al*., 2009; Schützendübel *et al*., 2001).

With regard to the Cd-dependent regulation of ROS production, several studies suggest that a first level of regulation is carried out by Ca^{2+} and protein phosphorylation/dephosphorylation (Chmielowska-Bąk *et al*., 2014; Romero-Puertas *et al*., 2007). Cd-dependent ROS production is also regulated by phospholipases and phosphatidylinositol-triphosphate (IP3; Garnier *et al*., 2006; Yakimova *et al*., 2006).

8.1.4.2. Cadmium-induced oxidative stress and tolerance mechanisms

Numerous studies have shown that Cd toxicity is directly related to ROS over-accumulation, whose effect on different molecules promotes oxidative damage to proteins, lipids and nucleic acids (Fig. 8.1.2; Chmielowska-Bąk *et al*., 2017; Sandalio *et al*., 2012). One of the most common oxidative protein modifications is carbonyl derivative formation which gives rise to protein carbonylation, an irreversible modification that leads to protein functionality loss and promotes protein degradation (Gallego *et al*., 2012; Romero-Puertas *et al*., 2002; Halliwell and Gutteridge, 2015). The posttranslational modification carbonylation, whose protein targets have been identified in several plant species, is one of the most commonly used parameters to analyze oxidative stress in biological systems (Camejo *et al*., 2015; Liu *et al*., 2012; Nguyen and Donaldson, 2005; Møller *et al*., 2017). In pea plants exposed to Cd, most of the carbonylated peroxisomal proteins have been identified as antioxidants such as CAT, Mn-SOD and GR (Romero-Puertas *et al*., 2002). Carbonylated proteins, which are typically non-functional, are more prone to be degraded by several proteases in order to prevent further damage to other macromolecules (Pena *et al*., 2011; Romero-Puertas *et al*., 2002; Møller *et al*., 2017). Recent advances indicate that carbonylation is not only a random mechanism but also a selective protein oxidation process (Pena *et al*., 2012).

Lipid peroxidation, another common marker of oxidative stress, which can be detected by malondialdehyde content, affects membrane structure and functionality (Halliwell and Gutteridge, 2015). The structure of nucleic acids DNA and RNA, major targets of oxidative stress, can be affected by 8-hydroxyguanosine (8-OHG), one of their oxidative markers, which is involved in damage repair and gene expression and causes mutations (Chmielowska-Bąk *et al*., 2017; Halliwell and Gutterdige, 2015). 8-OHG has recently been reported to affect mRNA and regulate gene translation (Chmielowska-Bąk *et al*., 2017). Post-transcriptional gene regulation during oxidative stress responses in plants is a complex process, which affects RNA-binding proteins, splicing, RNA editing and stability, among others (Van Ruyskensvelde *et al*., 2018). These

symptoms of oxidative damage in response to Cd stress have been demonstrated in numerous plant species (Cuypers *et al*., 2016; Gupta *et al*., 2017; Sandalio *et al*., 2001).

One of the consequences of plant cell exposure to Cd is the drastic consumption of GSH for both Cd sequestration and PC synthesis, which limits the level of GSH necessary to maintain the cellular redox balance and leads to ROS accumulation (Romero-Puertas *et al*., 2007). Time-course analyses carried out in different organs of Arabidopsis plants have revealed that Cd-exposed roots develop a rapid phytochelatin response after 2 h, which involves a drastic reduction in GSH, although an increase in GSH, FeSOD, AsA and CAT content has been observed after 24 h (Jozefczak *et al*., 2014). On the other hand, leaves show a delayed response to Cd, probably to protect them against GSH depletion and oxidation (Jozefczak *et al*., 2014). In addition, Cd-dependent ROS over-accumulation induces high levels of GSSG which, in turn, promotes glutathionylation, a pivotal post-translational regulation mechanism of certain enzymes, such as GST and GR (del Buono *et al*., 2014), as well as chloroplastic 2-Cys Prx and mitochondrial Prx IIF (Calderón *et al*., 2017), which reduces enzymatic activity. However, little information exists on the contribution of this protein modification to Cd responses in plants.

Studies of different plant species have demonstrated that the severity of oxidative damage in response to Cd can vary depending on the species, metal concentration and treatment period (Keunen *et al*., 2015; Rizwan *et al*., 2016; Sandalio *et al*., 2001). Significant differences can be observed between different cultivars (León *et al*., 2002; Shamsi *et al*, 2014). A feature common to all these studies is the Cd-induced disturbances in antioxidative defences, including the thioredoxin superfamily (thioredoxins, Trx; NADPH dependent thioredoxin reductases, NTR and glutaredoxins, Grx) that are disulfide reductases, with a key role for in anti-oxidant defence. The increase in which is associated with the tolerance of various plant species to Cd stress (Ashrafzadeh and Leung, 2017). Thus, the expression levels of genes encoding Cu/Zn-SOD, GST1, PODs, TrxR2, PrxR, FER3 and NDPK are higher in high Cd accumulator pak choi cultivar seedlings (Baiyewuyueman) than those in low Cd accumulator cultivar seedlings (Kuishanaijiaoheiy; Yu *et al*., 2017). Transgenic plants over-expressing antioxidants have been used to analyze their role in plant responses to Cd. Thus, over-expression of the *E. coli* glutathione reductase gene (*gor*) renders Nicothiana plants more Cd tolerant than WT plants, while Mn-SOD over-expression did not improve tolerance to Cd (Poage *et al*., 2011). However, overexpression of the Cu/Zn SOD gene from *Sedum alfredii* (a Cd/Zn/Pb co-hyperaccumulator) in transgenic Arabidopsis plants enhanced Cd tolerance by improving antioxidative defence capacity through enhancement of SOD, glutarredoxin and peroxidase activity (Li *et al*., 2017). Furthermore, transgenic Arabidopsis plants over-expressing gene *HvAPX1* from barley were found to be more Cd stress tolerant (Xu *et al*., 2008). Peroxiredoxins also play an important role in protecting cells against Cd toxicity, as

observed with *AtPrxII F*, regenerated by mitochondrial thioredoxin Trx h2 and glutaredoxins. *AtPrxII F* showed an up-regulation in response to Cd and the reduced growth in *KO-AtPrxII F* Arabidopsis seedlings in the presence of the metal as compared to WT (Finkemeier *et al*., 2005). Glutaredoxins, which are GSH-dependent thioredoxins, whose expression is induced in response to Cd stress in plants, may also act as antioxidants in pea plants (Smiri *et al*., 2011) and thioredoxins appears to play a role in preventing protein disulfide bridge formation in the cytoplasm of *Brassica napus* leaves (Ali *et al*., 2015).

However, in other plant species, such as pepper, the highest Cd tolerance of different cultivars is more dependent on NADPH availability than on antioxidant capacity (León *et al*., 2002), and soybean resistance to Cd stress has also been shown to depend on its NADPH production capacity (Pérez-Chaca *et al*., 2014).

In several species, aldo-keto reductases (AKRs) have been shown to enhance tolerance of various abiotic stresses by scavenging toxic aldehydes. *IbAKR*-overexpressing tobacco plants show higher tolerance to Cd stress than WT due to their ability to scavenge malondialdehyde (MDA) and methylglyoxal (MG), enhanced proline content and superoxide dismutase activity, as well as up-regulation of genes encoding ASC-GSH cycle components (Huo *et al*., 2018). Proline, an osmolite that makes a significant contribution to Cd tolerance, can act as a ROS quencher, chelates heavy metals and is also part of the redox signalling pathway (Signorelli *et al*., 2014; Zouari *et al*., 2016). The tolerance of different mustard cultivars to Cd is also related to proline accumulation (Asgher *et al*., 2013).

8.1.4.3. H_2O_2-dependent signalling in plant responses to cadmium

Despite the chemical identity of reactive oxygen species, such as $O_2^{\cdot-}$, $^{\cdot}OH$ and $^1O_2$, and the induction of a specific imprint on the transcriptome response by the subcellular origin of ROS (Rosenwasser *et al*., 2013), H_2O_2-dependent signalling is the best known in-plant response to stress, particularly Cd, mainly due to its stability (Petrov and Van Breusegem, 2012). It has been suggested that the complex network that converts H_2O_2 signals into biological data is evolutionarily conserved (Vandenbroucke *et al*., 2008). Thus, the H_2O_2-dependent network in response to Cd includes a first step composed of sensors, such as the mitogen-activated protein kinase (MAPK AtMPK3/6) cascade and calmodulin (Fig. 8.1.2; Cuypers *et al*., 2016; Jonak *et al*., 2004; Liu *et al*., 2010; Opdenakker *et al*., 2012). In addition, OXI1 kinase has been shown to be a central player in metal-induced oxidative stress responses acting upstream of MAPK (Opdenakker *et al*., 2012; Rentel *et al*., 2004). MAPK cascades are able to regulate different transcription factor (TF) families and consequently also downstream gene expression. Thus, the WRKY, NAC, bZIP, DREB, ZAT and MYB TF families constitute intermediate links in the H_2O_2-dependent signalling

network (Table 8.1.1; Petrov and Van Breusegem, 2012). In particular, induction of *ZAT12* and *WRKY25* has been observed in Arabidopsis plants under Cd and Cu stress (Opdenakker *et al*., 2012), with *WRKY25* appearing to act downstream from *ZAT12* (Rizhsky *et al*., 2004). Although much information is available on transcriptomic changes in plant responses to Cd stress (Table 8.1.1), information on the downstream targets of these TFs remains scarce. ZAT12 is involved in regulating antioxidant systems in Arabidopsis plants under H_2O_2 stress (Rizhsky *et al*., 2004), suggesting that this TF plays a regulatory role in detoxification and oxidative stress-related proteins observed using transcriptomic and proteomic analyses (Table 8.1.1). On the other hand, redox changes in TF Cys residues cannot be ruled out and may directly regulate nuclear gene expression (Dietz, 2014; Suzuki *et al*., 2012); good examples of these residues, which have been shown to respond to oxidative stress in both animals and plants, are heat shock factors (HSFs) (Petrov and Van Breusegem, 2012). Using both transcriptomic and proteomic analyses, heat shock proteins have been shown to be one of the main categories of protein found in plants under Cd stress conditions (Table 8.1.1 and Table 8.1.2; Fig. 8.1.1.), and it has been suggested that HSFs are upstream sensors of H_2O_2 beyond ZAT and WRKY TFs (Miller *et al*., 2008).

Extensive crosstalk between H_2O_2 and phytohormones, of which one of the most studied is ethylene (ET), has been detected in plants under Cd stress. Thus, the ET signalling pathway and the ET responsive factors ERF2 and ERF5 are regulated in early plant responses to Cd (Herbette *et al*., 2006; Weber *et al*., 2006); in late responses, ET levels have also been shown to increase which is probably due to induction of Cd-dependent senescence (Rodríguez-Serrano *et al*., 2009). In addition, ACS2 (1-aminocyclopropane-1-carboxylatesynthase 2) and ACS6 appear to be responsible for the increase in ET in Arabidopsis plants exposed to Cd; both isoforms can be phosphorylated by MPK3 and MPK6, suggesting that there is a connection between ROS production and ET in response to Cd (Cuypers *et al*., 2016; Schellingen *et al*., 2015). Also, the production or regulation of phytohormone biosynthesis and/or signalling pathways has been observed in plant responses to Cd stress (Cuypers *et al*., 2016). Jasmonic acid (JA) biosynthesis is upregulated in rice and Arabidopsis plants (Ogawa *et al*. 2009); this phytohormone can regulate genes associated with GSH and PC synthesis as part of the plant response to Cd (Xiang and Oliver, 1998). However, the increase in JA may contribute to metal toxicity, as MeJa has been shown to be able to activate lipoxigenase, H_2O_2 production and lipid peroxidation (Maksymiec *et al*., 2005; Rodríguez-Serrano *et al*., 2009; Wang and Wu, 2005). Salicylic acid (SA) is another important signalling element in plants, and SA-dependent toxicity under metal stress has been directly linked to H_2O_2 concentrations (Tao *et al*., 2013). Nevertheless, the link between SA and H_2O_2 varies depending on the experimental conditions and species; SA can alleviate Cd-induced damage in barley roots (Metwally *et al*., 2003) and can prevent oxidative damage and cell death by inhibiting

ROS production (Zhang and Chen, 2011). SA concentrations have also been shown to behave differently in the leaves and roots of Cd-treated pea plants (Rodríguez-Serrano *et al*., 2009). Although some initial research has been carried out, much remains to be done to fully understand the relationship between H_2O_2 and the different phytohormones involved in plant responses to Cd stress.

8.1.5. Reactive nitrogen species metabolism in response to Cadmium

Reactive nitrogen species (RNS), such as nitric oxide (NO), *S*-nitrosothiols (SNOs), higher nitrogen oxides (*Nox*), dinitrosyl iron complexes and peroxynitrite ($ONOO^-$), are produced as a result of normal metabolism. However, an excess of RNS can be dangerous for the plant mainly due to reaction with lipids, proteins and nucleic acids, giving rise to membrane leakage, enzyme inactivation and DNA damage or mutation, which can severely damage the cell (Neill *et al*., 2008; Romero-Puertas and Sandalio, 2016). Nevertheless, low levels of NO can regulate a plethora of plant cellular processes ranging from development and plant response to stress (Besson-Bard *et al*., 2009; León *et al*., 2014; Romero-Puertas and Sandalio, 2016; Yu *et al*., 2014). An increasing number of studies also suggest that NO plays a key role in plant responses to Cd stress.

8.1.5.1. Nitric oxide production under cadmium stress

Up to eleven different NO-producing mechanisms have been described in plants. These can be summarised with respect to the following two main pathways: the oxidative arginine- and hydroxylamine-dependent pathway and the reductive nitrate-dependent pathway (del Río, 2011; Fröhlich and Durner, 2011; Gupta *et al*., 2011; Mur *et al*., 2013). One of the most effective reductive pathways for NO production is the pathway involving nitrate reductase (NR); which is capable of reducing nitrite under certain conditions (Rockel, 2002). Nitrite:NO reductase in root apoplasts, xanthine oxidoreductase and mitochondrial nitrite reduction via cytochrome c oxidase/reductase are also involved in reductive pathways (Godber *et al*., 2000; Stöhr *et al*., 2001; Stoimenova *et al*., 2007; Wang *et al*., 2010). Arginine-dependent nitric oxide synthase-like activity (NOS-l) has been observed in oxidative pathways (Moreau *et al*., 2010).

It is difficult to draw general conclusions concerning NO production under Cd treatment conditions, which is dependent on dose, timing and species. NO induction was observed in 3-week-old Arabidopsis roots after 7 h of Cd treatment (200 µM), while in leaves, this was detected after 96 h (50 µM Cd), suggesting that Cd needs to be transported from roots to leaves (Besson-Bard *et al*., 2009). An increase in NO after Cd treatment was also observed in the roots of different plant species such as wheat, brassica, pea, barley, soybean, lupinus, tobacco and poplar (Balestrazzi *et al*., 2009; Bartha *et al*., 2005; Groppa *et al*., 2008a; Mahmood *et al*., 2009; Pérez-

Chaca *et al*., 2014; Valentovičová *et al*., 2010) as well as in Arabidopsis and soybean culture cells (De Michele *et al*., 2009; Kopyra *et al*., 2006). In barley roots, NO was observed in the pericycle, parenchymatic stelar cells and protophloem companion cells (Valentovičová *et al*., 2010). However, NO was observed to decrease following Cd stress in rice roots after 1 d (Xiong *et al*., 2009), and in pea roots and leaves after 14 d (Rodríguez-Serrano *et al*., 2006; 2009). In Arabidopsis, NO gradually decreased as Cd concentrations, from 25 µM to 100 µM, and exposure time, from 1 to 5 d, were increased (Gupta *et al*., 2017). The decrease in NO over longer treatment times may be due to early induction of senescence caused by excess ROS and ET (Lehotai *et al*., 2011; McCarthy *et al*., 2001; Rodríguez-Serrano *et al*., 2009; Romero-Puertas *et al*., 2002); and to an inhibition of NOS-l activity caused by Ca-deficiency (Rodríguez-Serrano *et al*., 2009). Interestingly, NO homeostasis in response to Cd in *rboh* mutants has been shown to differ from WT plants suggesting an unkwon ROS-dependent NO regulation mechanism (Gupta *et al*., 2017). Most of these studies point to NOS-l activity as the main source of NO induction in response to the heavy metal (Fig. 8.1.2.).

8.1.5.2. Role of nitric oxide under Cd stress

NO is a highly reactive molecule that can diffuse through biological membranes, although the mechanisms involved in the functioning of NO are not well understood. NO is now well known to be capable of regulating different processes by activating secondary messengers and by inducing gene transcription (Besson-Bard *et al*., 2008; Gaupels *et al*., 2011; Palmieri *et al*., 2008). In addition, a crucial feature of NO's mode of action is the direct modification of proteins through covalent post-translational modifications (PTMs), which enable biological processes in the cell to be regulated (Martínez-Ruiz *et al*., 2011).

8.1.5.2.1. NO as a protector molecule

Numerous studies have shown that exogenous applications of NO protect plants against Cd-induced damage. NO is capable of alleviating toxicity in species such as *Lupinus luteus*, *Oryza sativa*, *Medicago truncatula*, *Helianthus annuus*, *Triticum sativum*, *Cicer arietinum* and Arabidopsis (Flores *et al*., 2008; Hsu and Kao, 2004; Kopyra and Gwóźdź, 2003; Kumari *et al*., 2010; Laspina *et al*., 2005; Méndez *et al*., 2016; Singh *et al*., 2008; Xiong *et al*., 2010; Xu *et al*., 2010). It can directly scavenge certain ROS such as $O_2^{\cdot-}$, as the half-life of NO, which rapidly interacts with other molecules, lasts only a few seconds and generates a certain number of RNS that may also be toxic at high concentrations (Kopyra and Gwóźdź, 2003; Neill *et al*., 2008). Most of these studies point to the NO-dependent regulation of antioxidant systems as the main cause of these defences (Arasimowicz-Jelonek *et al*., 2011). Thus, NO prevents a Cd-induced increase in SOD from *Helianthus annus* and restores CAT activity decrease by Cd treatment (Laspina *et al*.,

2005). In addition, NO induces gene expression associated with the ASC-GSH cycle in Cd-treated pea plants (Romero-Puertas *et al*., 2007) despite its effect on CAT and APX activity in Arabidopsis culture cells (De Michele *et al*., 2009). Furthermore, NO signalling is associated with the accumulation of antioxidant enzymes, glutathione (GSH) and phosphatidic acid, which increases Cd tolerance in rice (Yang *et al*., 2016). Hasan *et al*., (2016) also suggest that GSH increases tomato tolerance to Cd stress not only by promoting Cd chelation and sequestration, but also by stimulating NO, SNOs and the antioxidant system through a redox-dependent mechanism.

Several studies have also highlighted other possible effects of protection by NO, such as the reduction in metal accumulation by NO priming and the restriction of Cd translocation from roots to shoots in ryegrass seedlings and rice (He *et al*., 2014; Singh and Shah, 2014; Wang *et al*., 2013; Xiong *et al*., 2009). Exogenous NO also raises Cd tolerance in rice plants by increasing hemicelluloses and pectin content in the root cell wall, by decreasing Cd accumulation in the cellular soluble fraction in rice leaves (Xiong *et al*., 2009) and by maintaining the auxin equilibrium (Xu *et al*., 2010). NO indirectly contributes to plant protection against Cd toxicity through *Bacillus amyloliquefaciens* acting as a signalling molecule downstream of auxins (Chen *et al*., 2017). It is important to note that, depending on its level, NO has both cytoprotective and cytotoxic properties (Beligni and Lamattina, 2001). Sodium nitroprusside (SNP) pretreatment is also reported to stimulate ROS-mediated Cd cytotoxicity in *B. juncea* (Verma *et al*., 2013).

8.1.5.2.2. NO-dependent signalling in plant response to Cd

Changes in plant gene expression in response to NO and in NO-altered mutants involve the principal categories of cellular metabolism such as signal transduction, defence, cell death, transport, basic metabolism, ROS production and even degradation processes (Besson-Bard *et al*., 2009; Gibbs *et al*., 2014). In a recent analysis of CATMA arrays and the role of NO in regulating plant responses to Cd, more than forty genes, mainly associated with iron homeostasis, nitrogen assimilation, metabolic proteolysis and root growth, were detected (Fig. 8.1.2; Besson-Bard *et al*., 2009). Various studies have highlighted the role of NO in promoting root growth reduction in response to Cd (Besson-Bard *et al*., 2009; Groppa *et al*., 2008b; Valentovičová *et al*., 2010). Two studies have demonstrated the NO-dependent induction of *LeIRT1*, a major plant root iron transporter (Graziano and Lamattina, 2007; Jin *et al*., 2009). Under Cd stress, the iron uptake system is repressed in order to prevent Cd accumulation in the plant (Connolly, 2002; Connolly *et al*., 2003); in the presence of the nitric oxide synthase inhibitor NOS-l, this repressive mechanism is enhanced in Arabidopsis roots, thus suggesting that NO may promote Cd accumulation (Besson-Bard *et al*., 2009). Similar results have been observed in tomato plants under different nitrogen growth conditions (Luo *et al*., 2012). Heme oxygenase 1 (HO1) has been shown to benefit

Arabidopsis plants under Cd stress by diminishing NO production and thus improving Fe homeostasis (Han *et al*., 2014; Neill *et al*., 2002), which is similar to the results obtained by gibberellin treatment that reduce NO in response to Cd (Zhu *et al*., 2012). The contribution of NO to Cd accumulation may partly explain NO-dependent root-growth inhibition in response to Cd stress (Besson-Bard *et al*., 2009). On the other hand, NO-dependent repression of auxin accumulation and signalling under Cd stress has been shown to be involved in inhibiting Arabidopsis root meristem growth (Yuan and Huang, 2016).

Treatment with a NOS inhibitor has been shown to offset the decrease in the accumulation of Ca, which is another element affected by the presence of Cd in the medium, thus suggesting that NO is part of this process, although the mechanism involved remains unclear (Besson-Bard *et al*., 2009). Interactions between NO and Ca under Cd stress have also been reported in pea plants, in which Ca prevents Cd effects from inhibiting NOS-l-dependent NO production (Rodríguez-Serrano *et al*., 2009). In addition, a brief study has highlighted the NO-dependent nature of signalling proteins such as 1-aminocyclopropane-1-carboxylic acid synthase (ACS), mitogen-activated protein kinase2 (MAPKK2) and various TFs induced in response to Cd in soybean (Chmielowska-Bak *et al*., 2013).

8.1.5.2.3. Nitric oxide-dependent post translational modifications in plant response to cadmium

It has been well established that NO is capable of regulating a diverse range of biological processes in plants by directly modifying proteins through the PTMs *S*-nitrosylation, metal nitrosylation and nitration (Romero-Puertas and Sandalio, 2016). Over 1.000 proteins have been shown to be targets of *S*-nitrosylation, which involves the covalent binding of a NO group to a protein cysteine residue (Hu *et al*., 2015; Kovacs and Lindermayr, 2013) while less targets of nitration, which involves adding a nitrite group to a Tyr residue, and of nitrosylation in plants have been identified (Vandelle and Delledonne, 2011). Although less research has been carried out on NO-dependent PTMs in plant responses to Cd stress, the results obtained point to a regulation of antioxidant and ROS production systems (Ortega-Galisteo *et al*., 2012) as well as Cd sequestration by PCs (De Michele *et al*., 2009). A study have reported a decrease in CAT *S*-nitrosylation in pea peroxisomes under Cd stress (Ortega-Galisteo *et al*., 2012), which may account for the slight increase in CAT activity observed previously (Romero-Puertas *et al*., 1999). NO is also able to regulate H_2O_2 production in peroxisomes under Cd stress by changing the *S*-nitrosylation pattern of glycolate oxidase (GOX; Ortega-Galisteo *et al*., 2012). Although a number of antioxidant and ROS-producing enzymes have been shown to be targets of nitration, to our knowledge, nitrated proteins in plants affected by Cd stress have not been identified. Three main nitrated protein bands

have been observed in soybean roots after 6 h and 24 h of Cd stress, although no differences were found in total nitrated protein content, suggesting that this PTM modulates Cd-induced responses but does not cause major changes in the pattern of protein nitration (Pérez-Chaca *et al*., 2014).

8.1.6. Conclusion.

In recent years, an enormous amount of data relating to plant responses to Cd stress have been obtained using omic techniques, although the extremely broad diversity of parameters used, ranging from metal concentration and length of treatment to culture system and tissue analyzed, as well as the diversity of tolerance in different species and even cultivars make it difficult to reach general conclusions. The features common to plant responses to Cd are oxidative stress and the induction of protection systems, such as antioxidants and heat shock proteins, to restore protein acitivity, to prevent oxidative damage and normally to respond rapidly and to promote high tolerance of oxidative stress caused by Cd. Accordingly, initial ROS and RNS production results in signalling patways that trigger plant responses to Cd stress, and particular attention need to be paid to the mechanisms underlying specific ROS- and RNS-dependent responses to this type of stress. Further study of ROS/RNS crosstalk and other signalling components, such as hormones and TFs, would help to unravel the mechanisms underlying plant responses to Cd, their specific characteristics and the features common to different species.

8.1.7. References

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Anexed 8.2

8.2.1. Supplenmental Table S8.1.3

Suppl. Table S8.1.3: metal stress has been shown. A literature serch in different databases (https://www.ncbi.nlm.nih.gov/p https://apps.webofknowledge.com/UA) related to heavy metals in plants and NO was conducted. The search was narrowed down to the l endogenous NO production as a response to metal application are shown. The code of each paper appears in the first column and Fig. 4.1.1 Main conditions used in each paper have been summarized as metal used (Metal); time of the treatment (Timing); Species; age of the plant a and method used; main results and the proposed role.

Code	Metal	Timing	Species	Age	Tissue	NO and $ONOO^-$ prod. /method/ source	Results
Al_1	$AlCl_3$ (30 µM)	3-24h	*Triticum aestivum* cv Yang-5 Jian-864	3d	seedling	+NO (3h Jian 864) +NO (12h Jian 5) (DAF-FM DA) NR	-root length/ cell viability +lipid peroxidation/ carbonyl content +$O_2^{\cdot-}$/H_2O_2/ $HO^{\cdot}$ +SOD/ CAT/ GR/ POD +LOX +NR/ NOS-l activity +callose
Al_2	$AlCl_3$ (30 µM)	3-24h	*Triticum aestivum* cv.Yang-5 Jian-864	3d	seedling	+NO (DAF-FM DA)	-root length +antiox. capacity +H_2O_2/ $O_2^{\cdot}$ -Pro/ GSH/ DHA/ GSSG +DPPH +ASC/ APX/ GR/ GPX/ GST/ MDHAR/ D =γ-ECS
Al_3	$AlCl_3$ (30 µM)	24h	*Triticum aestivum* cv.Yang-5	3d	roots	+NO (DAF-FM DA)	-root length/ cell viability +Al uptake +malate eflux =*TaALMT1* +pectins and hemicelluloses -methylsterification pectin +Al content in pectin +PME
As_1	Na_2HAsO_4 (25-50 µM)	4-24h	*Oryza sativa* cv. No. 3	4d	roots	-NO (DAF-2DA)	-root length +As uptake +lipid peroxidation/ root oxidazibility +H_2O_2/ $O_2^{\cdot-}$ -NO_2^- +SOD/ CAT/ APX/ GPX

Annex 8.3.

As_2	KH_2AsO_4 (0.1-1 mM)	7d	*A. thaliana*	14d	roots	+NO (DAF-FM DA)	-root length/ biomass/ cell viability +lipid peroxidation +$O_2^{\cdot-}$ =NADPH oxidase -CAT +GR =GOX/ ICDH/ G6PDH/ 6PGDH +GSNOR activity -GSSG/ GSH -GSNO +protein tyrosine nitration
As_3	Na_2HAsO_4 (25-50 µM)	1-5d	*A. thaliana AtrbohC*	21d	leaves roots	-NO (DAF-2)	-root length/ biomass +As in leaves after 1d (WT) -As in leaves and roots (*AtrbohC*) after 5 +H_2O_2 +ASC/ DHA +GSH/ GSSG +lipid peroxidation -CAT +GR/ GOX
As_4	Na_2HAsO_4 (50 µM)	15d	*Pisum sativum* cv. Azad P-1	15d	leaves	-NO (Griess reagent)	-biomass +As uptake -Chl/ photosynthesis -DES activity -H_2S -NR +Cys +lipid peroxidation +$O_2^{\cdot-}$/ H_2O_2/ $HO^{\cdot}$ -APX/ GR/ MDHAR/ DHAR -GSH/ ASC +GSSG/ DHA
As_5	Na_2HAsO_4 (1.5 mg L^{-1})	24h	*Pistia stratiotes*	Adult	leaves	+NO (DAF-2DA)	+As uptake +H_2O_2/ $O_2^{\cdot-}$ -Chl/ photosynthesis/ photorespiratory ra =glucose/ starch -glycerate/ gly/ ser/ sucrose +nocturnal respiration/ mitoch. respiratio -trichomes damage in membranes, protoplast and mesophyll cells

As_6	$NaAsO_2$ (25 µM)	7d	*Oryza sativa* cv. Sarjoo52	10d	roots	-NO (DAF-FM DA)	-root length/ biomass -Chl +H_2O_2 +lipid peroxidation +SOD/ CAT/ GPX/ APX/ GR +NR activity -SA +*OsIRT1/ OsYSL2*
As_7	$NaAsO_2$ (25 µM)	4-12d	*Oryza sativa* cv. indica IC-115730	12d	roots	+NO (DAF-FM DA)	-root length/ cell viability +As uptake -biomass +H_2O_2/ O_2^{-} +JA +DEGs
As_8	$NaAsO_2$ (0.1-10 mg/L)	3h	*Vicia faba* (Qingpi)	35d	leaves (guard cells)	+NO (DAF-FM DA)	-cell viability +ROS +Ca^{2+} +NR
As_9	Na_2HAsO_4 (100 µM)	24h	*Spirodela intermedi*a W. Koch	3d	plant	+NO (DAF-FM DA)	+H_2O_2/ O_2^{-} +As uptake +lipid peroxidation -cell viability +SOD/ CAT/ POX +NR activity
As_10	$NaAsO_2$ (150 µM)	48h	*Oryza sativa* var. Pusa Basmati (PB1)	7d	roots shoot	+NO (Griess reagent)	-root length/ biomass/ lateral roots -germination -NO_2^{-} +As uptake and translocation -NiR activity -Chl/ protein content =carotenoids +cys/ Pro +lipid peroxidation +H_2O_2 +SOD +CAT/ APX/ GR -*OsPIN1a, 1b, 1c and 1d, OsPIN2, OsPI and OsPIN10b* expression +*NRT/ AMT/ NiR/ PHT/ KTP*

Annex 8.3.

As_11	KH_2AsO_4 (50 µM)	10d	*Pisum sativum* cv. Lincoln	20d	roots leaves	-NO (DAF-2) (roots) +NO (leaves) -$ONOO^-$ (APF) (roots)	+As uptake -biomass/ root length +lipid peroxidation/ protein carbonyl grou -GSH/ GSSG/ MDHAR/ DHAR +gly/ glutamic acid/ GABA content/ Pro +endopeptidase activity/ PC2/ PC3 =H_2O_2 =ASC/ HPR -SOD/ APX/ GOX +NADPH oxidase +GR/ CAT -G6PDH =6PGDH +NADP-ICDH/ NADP-ME -GSNOR
As_12	$NaAsO_4$ (25 µM)	4-8d	*Festuca arundinacea* cv. Arid3	21d	leaves	+NO (Hb)	-biomass +As uptake +lipid peroxidation +H_2O_2/ $O_2^{\cdot-}$ +SOD/ CAT =APX
As_13	$NaAsO_4$ (100 µM)	10d	*Oryza sativa* cv. Koman	21d	plants	-NO (DAF-2DA)	-root length/ biomass +secondary roots +$O_2^{\cdot-}$/ H_2O_2 +As uptake +APX -DHAR +DHA +ASC
As_14	$NaAsO_2$ (150 µM)	12d	*Oryza sativa*	12d	roots leaves	+NO (DAF-FM DA)	+As uptake +SOD/ CAT/ APX/ GR/ GST +H_2O_2/ $O_2^{\cdot-}$ -cell viability
-	$CdCl_2$ (150 µM)	3-12h	*Nicotiana tabacum* cv. Bright Yellow2	3d	cells	+NO (Hb)	+Cd uptake -cell viability

-	$CdCl_2$ (1 mM)	24h	*Hordeum vulgare*	72h	root tips	+NO in pericycle, parenchyma and protophloem (DAF-DA)	+NADPH diaphorase activity -root length
-	$CdCl_2$ (100 µM)	7d	*Oryza sativa* cv. Xiushui 11	7d	roots shoot	-NO (DAF-FM DA)	-Cd uptake -root length +cell wall Cd concentration +non protein thiol +protein thiol
-	$CdCl_2$ (100 µM)	1-7d	*A. thaliana*	14d	roots shoot	+NO (DAF-FM DA, Hb)	+caspase -root length/ biomass
-	$CdSO_4$ (50 µM)	5d	*A. thaliana hy1-100/ hy1-1/ ho2/ ho3/ ho4/ nia1/nia2 Atnoa1/ phya/ phyb* (KO and 35s)	5d	shoot roots	+NO depending on mutants (DAF-FM DA)	-root length +putrescina =spermidine -spermine -PAs content -DAO -PAO +Cd uptake
-	$CdCl_2$ (5-50 µM)	2-3d	*Nicotiana tabacum* cv. Yunyan 85	28d	roots shoot	+NO (DAF-FM)	+Cd uptake -lateral roots/ root length
-	$CdSO_4$ (9 mM)	24-72h	*Triticum aestivum* cv. Sommez-2001/ Quality	20d	leaves roots	=NO (NO assay kit, ENZO)	+spermine -putrescine/ spermidine +Cd uptake
-	Cd (75-150 µM)	12h	*A. thaliana*	5d	roots	+NO (DAF-2 DA)	-rooth length -Aux content -PIN1, 3 and 7 proteins

Annex 8.3.

-	$CdSO_4$ (0-5 ppm)	14d	*Zea mays* cv. White/ Yellow	0d	leaves	-NO (Griess reagent)	+Cd uptake -phenols -GSH
-	$CdCl_2$ (0-20 µM)	4d	*Solanum lycopersicum* cv. Ailsa craig/ *cnr*	4d	roots	+NO (DAF-FM DA)	-root length +Cd uptake +NR +*SINR* +*SBP10, SBP12a, SBP15*
Cd_1	$CdCl_2$ (5 µM)	1-25d	*Hordeum vulgare* cv. Weisuobuzhi/ Dong 17	10d	leaves roots	+NO (Hb)	-biomass +$O_2^{\cdot-}$/ H_2O_2 -Chl/ photosynthetic rate +POD/ APX -CAT/ SOD +*POD/ CAT/ APX/ SOD* +lipid peroxidation +NR/ NOS-l
Cd_2	$CdCl_2$ (5-200 µM)	2-7d	*Solanum nigrum*	5-7d	roots	+NO (DAF 2-DA)	+lateral roots +Cd uptake -biomass/ cell viability +Pro +SOD/ CAT +H_2O_2
Cd_3	$CdCl_2$ (89 µM)	2-24h	*Lupinus luteus*	3-14d	roots leaves	+NO (DAF-2DA) =$ONOO^-$ (Folic acid method)	+$O_2^{\cdot-}$/ H_2O_2 +NADPH oxidase -cell viability +Cd uptake

Cd_4	$CdCl_2$ (5-200 µM)	0.5-96h	*Brassica juncea* cv. Varuna T-59	15d	seedling	+NO (24h) -NO (+24h) (Hb)	+Cd uptake -root length/ biomass/ water content +lipid peroxidation -Chl +Pro +photosynthetic pigments +Non protein thiols -H_2O_2 +SOD/ APX/ CAT
Cd_5	$CdCl_2$ (60 µM)	2d	*Matricaria chamomilla*	28d	shoot roots	+NO (DAF-FM DA)	+Cd uptake +GPX/ APX/ GR +GSH =GSSG +ASC/ PCs
Cd_6	$CdCl_2$ (15 µM)	1-6h	*Hordeum vulgare* cv. Slaven	4 cm roots	roots	+NO (DAF-2 DA)	-root length +root swelling +ROS
Cd_7	$CdCl_2$ (100 µM)	7d	*Trifolium repens*	14d	seedling	-NO (HbO_2)	-root length/ biomass -Chl +Cd uptake/ traslocation +H_2O_2 -CAT +lipid peroxidation +SOD (roots) -SOD (shoot) +APX/ GR/ GSH/ NPT +SA/ JA/ ET/ Pro
Cd_8	$CdCl_2$ (1 mM)	7d	*Cumumis sativus* cv. Marketer	28d	protoplast	+NO 4h (DAF-FM DA)	+ROS -cell viabilIty -microcallus

Annex 8.3.

Cd_9	$CdCl_2$ (100 µM)	14d	*Solanum lycopersicum*	first true leaf	roots	-NO (Griess reagent)	+H_2O_2/ $O_2^{\cdot-}$ +*SOD/ CAT/ APX/ GR* +Cd uptake +SNO -GSH/ ASC +PCs +*ERF1/ ERF2/ MYB1/ AIM1/ R2R3-MYB AN2 and others*
Cd_10	$CdCl_2$ (1.5 mM)	24h	*Vigna radiata* L. cv. BARIMung-2	5d	seedling	+NO (Griess reagent)	+Cd uptake/ Cd translocation +GSH/ GSSG/ DHA +PCs +lipid peroxidation +H_2O_2/$O_2^{\cdot-}$ +LOX -biomass/ root length -Pro/ ASC/ Chl -CAT +APX/ SOD/ GST/ GR/ GPX -DHAR/ MDHAR +putrecine/ spermidine/ spermine/ PAs
Cd_11	$CdCl_2$ (10 µM)	12h-7d	*Oryza sativa* cv. Japonica/ Zhonghua11	14d	roots shoot	+NO (6h) (DAF-FM DA)	-biomass -Chl/ photosynthesis +APX/ SOD/ GR +H_2O_2 +PAs/ PCs Gene studies
Cd_13	$CdCl_2$ (0-8 µM)	0-3d	*Oryza sativa* cv. Nageng 9108	0d	roots	+NO (DAF-2 DA)	-root length/ cell viability/ biomass +H_2O_2/ $O_2^{\cdot-}$ +lipid peroxidation +Cd uptake +NR/ NOS-l
Cd_14	$CdCl_2$ (5 mg/L)	3d	*Boehmeria nivea*	30d	roots shoot	=NO (Comercial reagent kit, Jiancheng)	-biomass +H_2O_2 =SNO -GSNOR/ GSH +GSSG -APX/ GR/ SOD +lipid peroxidation
Cd_15	$CdCl_2$ (50 µM)	1d	*A. thaliana irt1*	1cm roots	roots shoot	+NO (DAF-FM DA)	-root length +lipid peroxidation +Cd uptake

							-*IRT1*
Cd_16	$CdCl_2$ (100 µM)	6-24h	*Medicago sativa* cv. Victoria	5d	shoot roots	+NO (DAF-FM DA)	-biomass +lipid peroxidation +Cd uptake -SOD/ POD +APX -biomass
Cd_17	$CdCl_2$ (150 µM)	6d	*Triticum aestivum* cv. Wenmai	7d	leaves roots	=NO (Hb)	-biomass/ root length +Lipid peroxidation +$O_2^{\cdot-}$/ H_2O_2 +SOD/ POD =CAT/ APX -GR -ASC/ GSH/ carotenoids
Cd_18	$CdCl_2$ (0-5000 µM)	0-6d	*Cynodon dactylon*	21d	leaves	+NO (Hb)	+H_2O_2/ $O_2^{\cdot-}$ +SOD/ CAT/ POD/ GR -GSH +H_2S -biomass/ cell viability +lipid peroxidation
Cd_19	$CdCl_2$ (20 µM)	1-6h	*Hordeum vulgare* cv. Slaven	4cm roots	root tip	+NO (DAF-FM) +$ONOO^-$ (APF)	-H_2O_2 +$O_2^{\cdot-}$ -root length -Cd uptake
Cd_20	$CdCl_2$ (100 µM)	6-72h	*Sedum alfredii*	21d	roots	+NO (DAF-FM)	+NR/ NOS-l -cell viability +lipid peroxidation +H_2O_2/ $O_2^{\cdot-}$ +SOD/ GR -POD/ CAT/ APX +GSH/ ASC +GSNOR +γ-ECS =MDHAR/ DHAR +Cd uptake

Annex 8.3.

Cd_21	$CdCl_2$ (40 µM)	0-144h	*Glycine max*	10d	roots	+NO (pick 6h) (DAF-2 DA)	+Cd uptake +H_2O_2/ $O_2^{\cdot-}$ (72h) +SOD/ GOX -lipid peroxidation +carbonyl groups (pick 6h)/ N-tyr +CAT/ GST/ APX +*APX* +G6PDH/ 6PGDH/ ICDH/ NADPH +ME/ DHA/ GSSG (pick 6h) -GSH/ ASC +NR
Cd_22	$CdCl_2$ (25-100 µM)	1-5d	*A. thaliana rboh* mutants	21d	leaves roots	-NO (DAF-2 DA)	-biomass +H_2O_2 +lipid peroxidation +ASC/ DHA =GSH +GSSG +CAT/ GOX =GR/ *GR1/GR2*/ *CAT1*/ *CAT2/CAT3* +Cd uptake
Co_1	$CoCl_2$ (10–20 µM)	1-3d	*Oryza sativa* cv. Taichung Native 1/Indica	2d	seedling roots	=NO (DAF-FM DA)	+lateral roots -root length +HO activity +*OsHO1* =*OsHO2* =H_2O_2
Co_2	Co_3O_4 (0.025-1.0 mg/ml)	2h	*Solanum melongena* cv. Violetta lunga 2	7d	seedlings	+NO (DAF2-DA)	-root length and absence of root hairs +ROS localization around apical root meristem. +membrane potential +DNA damage -cell cycle
-	$K_2Cr_2O_7$ (3-120 µM)	7d	*Matricaria chamomilla*	49d	shoot roots	+NO (DAF2-DA)	-biomass +Cr uptake +APX/ GPX/ GSH/ GSSG -GR +phenols +ROS +Ca/ Fe/ Zn/ Cu

Cu_1	$CuSO_4$ (0-50 µM)	7d	*A. thaliana* *nox1*	14d	roots	=NO (DAF-FM)	-root length/ biomass/ cell viability -germination +$O_2^{\cdot-}$ -H_2O_2
Cu_2	$CuSO_4$ (0-25 µM)	7d	*A. thaliana* *nia1 nia2Atnoa1*	7d	seedling	+NO (DAF-FM DA) NR	-root length/ biomass/ cell viability +ROS
Cu_3	$CuSO_4$ (0-750 µM)	0-4d	*Hordeum vulgare*	3d	shoot	+NO (Hb)	-biomass -Chl +GSH/ ASC +H_2O_2 /$O_2^{\cdot-}$ +lipid peroxidation +NR/ NOS-l +SOD/ POD/ GR +Cu uptake
Cu_4	$CuSO_4$ (450 µM)	0-48h	*Hordeum* vulgare cv. Nude	3d	roots	+NO (12h) (DAF-FM DA)	-cell viability/ root length +lipid peroxidation +SOD/ CAT/ POD/ APX +NR +$O_2^{\cdot-}$/ H_2O_2
Cu_5	$CuSO_4$ (5-50 µM)	17d	*A. thaliana* (WT *nia1 nia2* *DR5::GUS*)	17d	shoot roots	+NO (root) (WT) -NO (*nia1 nia2*) (DAF-FM DA) NR	-biomass/ root length/ division cell -Aux
-	$MnCl_2$ (0-1000 µM)	7d	*Matricaria* *chamomilla*	49d	shoot roots	+NO (Cell ROX Deep Red reagent) -RNS	+Mn uptake +ROS +lipid peroxidation +$O_2^{\cdot-}$/ H_2O_2 =APX +GPX

Annex 8.3.

				7d			+GR +Mn uptake -biomass -root length +ROS/ H_2O_2 +lipid peroxidation
Ni_1	$NiSO_4$ (10-200 µM)	9d	*Oryza sativa* cv. Yang liang you 6	19d	shoot roots	+NO (NO-2-G kit, Comin)	-root length/ biomass/ cell viability +Ni uptake -Chl/ carotenoids +Pro +H_2O_2 +lipid peroxidation -SOD +CAT/ POD/ ASC/ *POD/ APX/ CAT/ GR* +GSH -*SOD*
Pb_1	$Pb(NO_3)_2$ (100 µM)	24h	*Pogonatherum crinitum*	5d	roots cells	+NO (HbO_2 and Griess regent)	NO production earlier than Pb accumula +NR activity +Pb uptake
Pb_2	$Pb(NO_3)_2$ (50-250 µM)	0-8h	*Triticum aestivum* cv. PBW 502	48h	roots	=NO (DAF-2DA and (Griess reagent)	-root length +lipid peroxidation +$O_2^{\cdot-}$/ H_2O_2/ HO -cell viability +CAT/ APX/ GPX/ GR/ SOD
Pb_3	$Pb(C_2H_3CO_2)_2$ (400 µM)	0-72h	*Prosopis farcta*	21d	shoot	+NO (until 12h) (Griess reagent)	+Pb uptake +ASC +H_2O_2 -biomass -phenolic acids =flavonoids/ polyamines/ *ADC* +Pro/ aa/ lignin content +CAT/ GPX/ APX/PAL +aconitase activity +*PAL*
Pb_4	$Pb(NO_3)$ (150 µM)	14d	*A. thaliana CFP-PTS1*	14d	seedlings	+NO (DAF-2) +$ONOO^-$ (APF)	-root length -CAT =GOX/ HPR +$O_2^{\cdot-}$

-	$ZnSO_4$ (0-300 µM)	7d	*Brassica juncea* cv. Indian mustard *B. napus* cv. oilseed rape	9d	roots shoot	+NO (DAF-FM) +$ONOO^-$ (APF)	+Zn uptake (*B. napus* roots). -biomass -lateral roots -$O_2^{\cdot-}$ (*B. juncea*) +$O_2^{\cdot-}$/ SOD (*B. napus*) -H_2O_2/ APX activity (*B. juncea*) +H_2O_2 and =APX (*B. napus*)
-	$ZnSO_4$ (200 µM)	4d	*Nicotiana tabaco* cv. Xanthi	35d	leaves	+NO (DAF2-DA)	+Zn uptake Lesions quite large +*NtBI-1, Ntrboh* and *NtSIPK* expression
Zn_1	$ZnSO_4$ (0.2-0.4 mM)	10d	*Solanum nigrum*	14d	roots	+NO (Hb)	-cell viability/ root length +Zn uptake -*SOD* (2-10d) +*SOD* (2-4d) +FeSOD2 (10d) +CAT1/ CAT2, cAPX,/ pAPX -SOD (2-4d) +APX/ CAT +*NOX* activity +$O_2^{\cdot-}$and H_2O_2 (- from 10d) +lipid peroxidation +lateral root +ferric-chelate reductase activity
Zn_2	ZnO NPs (250 mg/L)	3d	*Oryza sativa* cv. Jiafuzhan	3d	seedlings	+NO (DAF-2DA)	-root length/ biomass +Zn uptake -Chl +$O_2^{\cdot-}$/ H_2O_2 -lipid peroxidation +GSH +Cu/Zn-SOD/ Mn-SOD -CATa, CATb, APX/ POD
Zn_3	$ZnSO_4$ (10 mg/L)	1-10d	*Hydrilla verticilata*	14d	leaves	+NO (Hb)	+lipid peroxidation -Chl/ carotenoids +NP-SH =CAT +APX/ POD/ GR/ ASC/ SOD +DHA

Annex 8.3.

Zn_4	$ZnSO_4$ (3 mM)	6d	*Triticum aestivum* cv. Xihan 3	seeds	roots leaves	+NO (DAF-FM DA)	+Zn uptake/ Zn translocation -root length +lipid peroxidation +cell viability +CAT/ APX/ SOD/ GR -POD +NOS activity +H_2O_2 -$O_2^{\cdot-}$ -DAO and PAO

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8.2.2. Supplenmental Table S8.1.4

Suppl. Table S8.1.3: Summary of the studies where exogenous NO has been applied in plants against heavy metal stress. A (https://www.ncbi.nlm.nih.gov/pubmed/; https://www.scopus.com/search/; https://apps.webofknowledge.com/UA) related to heavy metals i was narrowed down to the last ten years. In this table studies related to exogenous NO application previously and/or during the metal app appears in the first column and Fig. 4.1.2, Suppl. Fig. S4.1.1 and Suppl. Fig. S4.1.2. Main conditions used in each paper have been sum treatment (Timing); NO donor; Species, age of the plant and tissue used; NO and/or ONOOdetection and method used; main results and the

Code	Metal	Timing	NO donor/ scavenger/ mutant	Species	Age	Tissue	NO or $ONOO^-$ production/ method/ source	Results
-	$AlCl_3$ (30 µM)	24h	cPTIO (30 µM)	*Triticum aestivum* cv.Yang-5	3d	Roots	-NO (DAF-FM DA)	+root length/ cell viability -Al uptake/ accumulation in cell wall =malate efflux =*TaALMT1* =hemiceluloses content -pectin methylesterase activity +methylesterification of pectin
Al_1	$AlCl_3$ (30 µM)	3-24h	SNP (250 µM) cPTIO (100 µM) Tungstate (100 µM)	*Triticum aestivum* cv. Yang-5/ Jian-864	3d	Seedlings	+NO 3h (Jian-864) +NO 12h Yang-5 (DAF-FM DA) NR	+root length -lipid peroxidation/ carbonyl content -H_2O_2/ $O_2^{\cdot-}$/ $HO^{\cdot}$ -LOX/ POD +SOD/ CAT/ GR -callose
Al_2	$AlCl_3$ (1 mM)	10d	SNP (2 mM)	*Artemisia annua*	60d	leaves roots	-	=root length/ biomass +photosynthesis/ stomatal conductanc internal CO_2 +Chl/ carbonic anhydrase activity -lipid peroxidation -H_2O_2/ $O_2^{\cdot-}$ +NR +CAT/ SOD +artemisinin/ POX
Al_3	$AlCl_3$ (30 µM)	3-24h	SNP (250 µM) cPTIO (100 µM) Tungstate (100 µM)	*Triticum aestivum* cv. Yang-5/ Jian-864	3d	seedlings	+NO (DAF-FM DA)	+root length =Pro -$O_2^{\cdot-}$

Annex 8.3.

			GSNO (0.5 mM)					+DPPH scavenging activity +ASC/ GSH/ APX/ DHAR/ MDHAR/ G -DHA/ GSSG +GST =γ-ECS
-	$NaAsO_2$ (0.1-10 mg/L)	3h	SNP (200 µM)	*Vicia faba* cv. Qingpi	35d	Leaves	+NO (DAF-FM DA) NR	+cell viability
As_1	$NaAs_1O_2$ (25 µM)	7d	SNP (25 µM)	*Oryza sativa* cv. Sarjoo 52	10d	Roots	=NO (DAF-FM DA)	+root length/ biomass -As uptake and translocation -H_2O_2/ lipid peroxidation +Chl -NR -SOD/ CAT/ GPX/ APX/ GR +SA *-OsLsi1/ OsIRO2/ OsYSL2* *+OsLsi2* *=OsFRDL1 and OsNRAMP5*
As_2	$NaAsO_2$ (150 µM)	48h	SNP (100 µM)	*Oryza sativa* cv. Pusa Basmati (PB 1)	7d	roots shoot	+NO (Griess reagent)	+germination/ root length/ lateral roots biomass -As uptake +Chl/ nutrients content =carotenoids -Pro/ cys -lipid peroxidation =H_2O_2 -SOD/ CAT/ APX/ GR +NO_2^-/ NiR activity *+OsPIN1/ OsPIN2/ OsPIN5c/ OsPIN1* *+NRT/ AMT/ NiR/ PHT/ KTP*
As_3	Na_2HAsO_4 (250-500 µM)	72h	SNP (0.25 mM)	*Triticum aestivum*	6d	Seedlings	-	+Chl/ Pro -lipid peroxidation -H_2O_2 -GSSG +APX/ MDHAR/ DHAR/ GR/ GPX/ CA +ASC/ GSH

As_4	Na_2HAsO_4 (50 µM)	7d	SNP (100 µM) PTIO (200 µM)	*Phaseolus vulgaris* cv. VL 63	7d	shoot roots	-	+root length/ biomass -root oxidation/ lipid peroxidation/ cell -As uptake +Chl/ carotenoids -SOD -H_2O_2 +APX/ DHAR/ GR/ POX =CAT
As_5	Na_2HAsO_4 (25-50 µM)	4-24h	SNP (25-50 µM)	*Oryza sativa* cv. No. 3	4d	Roots	+NO (DAF-2DA)	+root length -As uptake -lipid peroxidation/ root oxidizability -H_2O_2/ $O_2^{\cdot-}$ -SOD/ CAT/ APX/ GPX +NO_2^-
As_6	Na_3AsO_4 (25 µM)	4-8d	SNP (100 µM) PTIO (200 µM)	*Festuca arundinacea* cv. Arid3	21d	Leaves	+NO (Hb)	+biomass -lipid peroxidation -cell viability/ relative ion leakage -H_2O_2/ $O_2^{\cdot-}$ +SOD/ CAT/ APX (4d)
As_7	Na_2HAsO_4 (100 µM)	24h	SNP (50 µM)	*Spirodela intermedia* W. Koch	3d	Plant	+NO (DAF-FM DA) NR	-As uptake/ lipid peroxidation -H_2O_2/ $O_2^{\cdot-}$ +cell viability -SOD/ CAT/ POX -NR
As_8	Na_3AsO_4 (100 µM)	10d	SNP (100 µM) L-NAME (500 µM) cPTIO (200 µM)	*Oryza sativa* cv. Komal	21d	Roots	+NO (DAF-2DA)	+lateral roots/ root length/ biomass -H_2O_2/ $O_2^{\cdot-}$ -As uptake -APX +DHAR/ ASC +cell in G1 phase and -cells in G2 phase -NARs and PRBA
As_9	$NaAsO_2$ (50 µM)	5d	SNP (75 µM)	*Glicine max* cv C. JS 335	5d	roots	-	+germination/ root length/ biomass -As uptake -H_2O_2/ $O_2^{\cdot-}$ -lipid peroxidation +Pro/ sugar/ PCs

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As_10	$NaAsO_2$ (25 µM)	4-12d	SNP (30 µM)	*Oryza sativa* cv. Indica IC-115730	10d	Roots	+NO (DAF-FM DA)	+biomass/ root length -As uptake +cell viability -H_2O_2 +DEGs -JA
As_11	Na_2HAsO_4 (1.5 mg/L)	24h	SNP (0.1 mg/L)	*Pistia stratiotes*	adult	Leaves	+NO (DAF-2DA)	=As uptake -H_2O_2/ $O_2^{\cdot-}$ =photosynthesis +Chl -sucrose/ glucose +nocturnal and mitochondrial respirati -photorespiratory rate =trichomes normal integrity membrane
As_12	Na_2HAsO_4 (0-400 µM)	0-45d	SNP (100 µM)	*Vicia faba*	45d	shoot roots	-	+root lengh/ biomass/ germination +Chl/ carotenoids -lipid peroxidation/ phenolic compound +GA/ IAA/ ABA
-	$CdCl_2$ (100 µM)	10d	SNP (100 µM)	*Cucumis sativus*	15d	Leaves	-	-$O_2^{\cdot-}$/ H_2O_2 iTRAQ: 1691 proteins
-	$CdCl_2$ (100 µM)	6 -72h	cPTIO (50 µM) Tungstate (100 µM) L-NAME (100 µM)	*Sedum alfredii*	21d	Roots	-NO (DAF-FM DA)	-cell viability +lipid peroxidation +H_2O_2/ $O_2^{\cdot-}$ -SOD/ GSH +POD/ CAT/ APX -GR/ GSNOR/ γ-ECS =ASC/ MDHAR/ DHAR =Cd uptake

-	$CdCl_2$ (0.2 mM)	50d	SNP (0.05 mM)	*Nicotiana tabacum*	0d	Plant	-	+biomass +Chl +rubisco =rubisco activase
-	$CdCl_2$ (1 mM)	7d	L-NAME (0.2 mM) cPTIO (0.1 mM) GSNO (0.1 mM)	*Cumumis sativus* cv. Marketer	28d	Protoplasts	=NO (DAF-FM DA)	+cell viability -H_2O_2 +microcallus
-	$CdCl_2$ (20 µM)	1-6h	L-NAME (1-10 mM)	*Hordeum vulgare* cv. Slaven	4 cm roots	Roots	-NO (DAF-FM) +$ONOO^-$ (APF)	-root length -Cd uptake -H_2O_2 +$O_2^{\cdot-}$
-	$CdCl_2$ (25 µM)	15d	SNP (500-1000 µM)	*Pisum sativum* cv. Ran	15d	Leaves	-	+biomass/ leaf thickness +intercellular spaces in the mesophyll -length of guard cell/ perimeter of guar density of stomata -area of pavement cell/ perimeter of pa cell
-	$CdCl_2$ (0-8 µM)	0-3d	Thymol (0-40 µM) SNP (20 µM) cPTIO (20 µM)	*Oryza sativa*	0d	Roots	-NO (DAF-2 DA)	+root length/ cell viability -lipid peroxidation -H_2O_2 +$O_2^{\cdot-}$ -Cd uptake -NR
-	$CdCl_2$ (5-200 µM)	2-7d	IAA (10 nM) NPA (10 µM) TIBA (10 µM) GSNO (100 µM) cPTIO (200 µM)	*A. thaliana* *Solanum nigrum*	5-7d	roots	+NO (DAF-2 DA)	+Cd uptake +biomass/ lateral roots/ cell viability -H_2O_2 +Pro +SOD =CAT
-	$CdCl_2$ (100 µM)	0-7d	cPTIO (0.5 mM)	*A. thaliana*	14d	Seedlings	+NO (DAF-FM DA) (Hb)	+root length +Caspase 3

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-	$CdCl_2$ (1-50 µM)	3h	SNP (10 µM) L-NAME (0.5 mM) NaCl (10 mM)	*Nicotiana tabacum*	3 leaf stage	shoot roots	-NO (DAF-FM)	-Cd uptake +lateral roots
-	$CdSO_4$ (9 mM)	24-72h	SNP (100 µM) cPTIO (100 µM)	*Triticum aestivum* cv. Sommez Quality	20d	leaves roots	+NO (NO assay kit, ENZO)	+PAs -Cd uptake
Cd_1	$CdCl_2$ (1.5 mM)	48h	Putrescine (0.2 mM) SNP (1 mM)	*Vigna radiata* cv. Bari Mung-2	5d	Seedlings	+NO (Griess reagent)	+root length/ biomass -Cd uptake -lipid peroxidation -H_2O_2/ $O_2^{\cdot-}$ +Chl +SOD/ CAT/ APX/ DHAR/ GR/ ASC -MDHAR/ GPX/ DHA/ GSSG +GSH/ GST/ Pro/ PCs -LOX
Cd_2	$CdCl_2$ (150 µM)	10d	SNP (100 µM)	*Solanum lycopersicum*	40d	shoot roots	-	+root length/ biomass/ cell viability +Chl/ carotenoids/ photosynthesis +Pro/ Gly -H_2O_2 -lipid peroxidation +ASC/ GSH/ GSSG/ GST/ DHAR/ MD SOD/ CAT/ APX/ GR +flavonoids/ phenols/ mineral nutrients -Cd uptake
Cd_4	$CdCl_2$ (200 µM)	8d	SNP (100 µM)	*Oryza sativa*	21d	shoot roots	-	+root length/ biomass -lipid peroxidation -H_2O_2 -POD/ SOD/ APX/ GR/ ASC +CAT/ GSH +Chl +Cd uptake -Cd translocation

Cd_6	$CdCl_2$ (200 µM)	14d	SA (0.1 mM) SNP (0.25 mM)	*Arachis hypogaea* cv. Huayu 22/ Xiaobaisha	21d	roots shoot	-	+biomass/ root length -lipid peroxidation -$O_2^{\cdot-}$/ H_2O_2 +SOD/ POD/ CAT/ ASC +Chl/ photosynthesis +Cd uptake -Cd translocation Hormones studies
Cd_7	Cd (10 ppm)	1-3d	SNP (250 µM) cPTIO (100 µM)	*Cicer arietinum*	30d	Plant	-	+biomas -Cd uptake -lipid peroxidation -H_2O_2/ GSSG +CAT/ POX/ SOD/ ASC/ GSH/ APX/ G DHAR/ MDHAR
Cd_8	$CdCl_2$ (100 µM)	14d	SNP (50-400 µM)	*Lolium perenne*	14d	shoot roots	-	+biomass +Cd uptake -H_2O_2/ $O_2^{\cdot-}$ -lipid peroxidation +SOD/ POD/ CAT/ APX +Chl/ carotenoids
Cd_9	$CdCl_2$ (100 µM)	14d	SNP (0.1 mM) SA (0.2 mM)	*Lolium perenne*	21d	roots shoot	-	+biomass +Cd uptake -Pro/ lipid peroxidation -H_2O_2 +SOD/ POD/ CAT/ APX +Chl/ carotenoids/ photosynthesis Hormones studies
Cd_10	$CdCl_2$ (100 µM)	7d	SNP (30 µM) cPTIO (50 µM)	*Oryza sativa*	7d	roots shoot	-	+germination/ root length/ biomass -Cd uptake +Pro -lipid peroxidation -H_2O_2 +GPX/ SOD/ APX/ CAT

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Cd_11	$CdCl_2$ (750 µM)	0-6d	SNP (250 µM) NaHS (500 µM)	*Cynodon dactylon*	21d	Plant	+NO (HbO)	-biomass/ cell viability -H_2O_2/ $O_2.^-$ +SOD/ CAT/ POD/ GR/ GSH +H_2S -lipid peroxidation
Cd_12	$Cd(NO_3)_2$ (50 µM)	7d	SNP (50 µM) cPTIO (50 µM)	*Oryza sativa*	7d	roots shoot	+NO	+biomass/ cell viability -lipid peroxidation -H_2O_2/ $O_2^{\cdot-}$ -CAT/ SOD +Chl -Cd uptake
Cd_13	$CdCl_2$ (50-200 µM)	14d	SNP (250 µM)	*Arachis hypogaea*	14d	roots shoot	-	+biomass -$O_2^{\cdot-}$ -lipid peroxidation +SOD/ POD/ CAT/ ASC/ Pro =Cd uptake -Cd translocation +Chl/ carotenoids/ photosynthesis
Cd_14	$CdCl_2$ (100-150 µM)	14d	SNP (100 µM)	*Lolium perenne*	21d	shoot roots	-	+biomass/ root length -H_2O_2/ $O_2^{\cdot-}$ +Cd uptake/ Chl -Cd translocation -lipid peroxidation +SOD/ POD -CAT
Cd_15	$CdCl_2$ (50-200 µM)	1-4d	SNP (100 µM)	*Oriza sativa* cv. MSE9	5d	roots shoot	-	+root length/ biomass -H_2O_2 =lipid peroxidation -ASC/ GSH/ CAT +GR/ POX/ SOD
Cd_16	$CdCl_2$ (5-200 µM)	6-9h	SNP (0.01-20 mM) cPTIO (100 µM)	*Brassica juncea* cv. Varuna T-59	15d	roots leaves	+NO (Hb)	+root length/ leaf water content +Chl -H_2O_2 -SOD/ APX/ CAT -Pro/ non protein thiols/ lipid peroxidat

Cd_17	$CdCl_2$ (100 µM)	14d	SNP (100 µM)	*Lolium perenne*	21d	roots leaves	-	+biomass/ root length -lipid peroxidation $-O_2^{\cdot-}$ +SOD/ CAT/ POD/ ASC -Pro +Chl/ carotenoids
Cd_18	$CdCl_2$ (50 µM)	10-15d	SNP (100 µM) SO_4^{2-} (1 mM)	*Brassica juncea*	30d	roots leaves	-	+biomass +photosynthesis/ Chl -Cd uptake/ translocation $-H_2O_2$ -lipid peroxidation +SOD/ GR/ GSH =APX +rubisco/ thylakoid membrane +ATP-S +Cys
Cd_19	$CdCl_2$ (5 µM)	1-25d	SNP (250 µM)	*Hordeum vulgare* cv. Weisuobuzh/ Dong 17	10d	leaves roots	+NO (Hb)	+Chl/ photosinthetic rate -ROS/ lipid peroxidation +SOD/ CAT/ APX +*POD* =NR +NOS-l
Cd_21	$CdCl_2$ (5 mg/L)	3d	SNP (100 µM) cPTIO (100 µM)	*Boehmeria nivea*	30d	roots shoot	+NO	+*S*-NO +Biomass +GSH/ GSNOR -GSSG =GR -lipid peroxidation $+H_2O_2$ -APX/ SOD
Cd_22	$CdCl_2$ (10 µM)	12h-7d	SNAP (30 µM) cPTIO (30 µM)	*Oryza sativa* japonica cv. Zhonghua11	14d	Roots	+NO (DAF-FM DA) (6h)	+biomass $-H_2O_2$ +GSH/ APX/ SOD/ GR +phosphatidic acid -Cd leakage +photosynthesis/ Chl/ PCs +phosphatidic acid/ phospholipase D

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Cd_23	$CdCl_2$ (444.8 µM)	4d	SNP (100 mM)	*Typha angustifolia*	20d	leaves roots	-	+biomass/ cell viability -lipid peroxidation +Cd uptake -SOD/ CAT/ PCs -NPT +GSH/ ASC
Cd_24	$CdCl_2$ (100 µM)	6-24h	SNP (100 µM) cPTIO (200 µM)	*Medicago sativa* cv. Victoria	5d	shoot roots	+NO (3h) (DAF-FM DA	+biomass -lipid peroxidation/ Cd uptake +APX -POD/ SOD
Cd_25	$CdCl_2$ (60 µM)	2d	PTIO (60 µM) SNP (300 µM) GSNO (300 µM) DEA NONOate (300 µM)	*Matricaria chamomilla*	28d	shoot roots	+NO (DAF-FM DA)	+Cd uptake =GPX/ GR/ ASC +APX/ GSSG -GSH/ PCs
Cd_26	$CdCl_2$ (400-800 µM)	6d	SNP (200 µM)	*Sesamun inficum*	0d	Seedlings	-	+germination/ biomass/ root length +SOD/ CAT/ APX/ POX
Cd_28	$CdCl_2$ (50 µM)	1d	GSNO (50 µM) GA (5 µM)	*A. thaliana irt1*	1 cm roots	roots shoot	-NO (DAF-FM DA)	+root length -lipid peroxidation/ Cd uptake -*RGL1/ RGL2/ GAI/ RGA* -*IRT1*
Cd_29	$CdCl_2$ (100 µM)	7d	Hb (10 µM) cPTIO (200 µM)	*Oryza sativa* cv. Xiushui 11	7d	Seedlings	+NO (DAF-FM DA)	+biomass/ root length -Cd uptake/ PBT/ NPT +$CaCl_2$ +hemicellulose =cellulose content +pectin content

Cd_30	$CdCl_2$ (100 µM)	7d	SNP (50-400 µM) cPTIO (50 µM) L-NAME (50 µM)	*Trifolium repens*	14d	seedlings	+NO (HbO)	+biomass/ root length/ Chl -Cd uptake/ traslocation -H_2O_2 -lipid peroxidation +Pro +APX/ CAT/ SOD/ GR -GSH -SA/ ET +JA
Cd_32	$CdSO_4$ (50 µM)	21d	Over-expression of *NtHb1* *nia1* and *nia2*	*Nicotiana tabacum* *A. thaliana*	21d	seedlings roots	-NO (DAF-2DA)	-Cd uptake +biomass +*CAX3* +*CAX3/ ZIP1/ MTP1A/ NRAMP1/ IRT* *NtHMA-A*
Cd_33	$CdCl_2$ (50-500 mg/L)	40d	SNP (100 µM) L-NAME (100 µM)	*Festuca arundinacea*	40d	Plants	-	+biomass +Chl/ PSII =germination -Cd uptake (depend on tissue)/ transl =Photosynthesis
Cd_34	$CdSO_4$ (9 mM)	24-72h	SNP (50-200 µl)	*Triticum aestivum* cv. Sönmez-2001/ Quality	5d	Seedlings	-	-lipid peroxidation +CAT/ GST =APX +SOD (Sönmez) -SOD (Quality) +MDA (Quality) -MDA (Sönmez)
-	$CoCl_2$ (10-20 µM)	1-3d	SNP (500 µM) Hb (0.14 g L^{-1})	*Oryza sativa* cv. Taichung Native 1/ Indica type	2d	seedlings roots	+NO (DAF-FM DA)	-HO activity -*OsHO1*expression
-	$CoCl_2$ (0.1-100 µM)	28d	SNP (10-40 µM) BA (2.2 µM)	*Sideritis raeseri*	explant	Shoot	-	+shoot number and callus induction percentage

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-	$CuCl_2$ (50 µmol/L)	8-21d	SNP (100 µM) Hb (0.1%)	*Lycopersicon esculentum* cv. Meigui	14d	roots leaves	-	+biomass +Cu translocation/ Cu accumulation +pectates proteins
-	$CuSO_4$ (200 µM)	50d	SNP (0.05 mM)	*Nicotiana tabacum*	0d	plants	-	+biomass +Chl +rubisco content +rubisco activity +rubisco activase content
Cu_1	$CuSO_4$ (150-750 µM)	3h-4d	cPTIO (150 µM) Tungstate (150 µM) L-NAME (150 µM) SNP (200 µM)	*Hordeum vulgare* cv. Nude	3d	Seedlings	+NO (Hb) NR	+biomass +Chl/ rubisco activity =GSH/ ASC -H_2O_2 -NR =$O_2^{\cdot-}$ -NOS-l -lipid peroxidation +SOD/ CAT/ APX/ GR =POD/ GPX =Cu uptake
Cu_2	$CuSO_4$ (100 µM)	3d	SNP (200 µM) GSH (200 µM)	*Oryza sativa* cv. BR 11	14d	Seedlings	-	+lipid peroxidation +Pro +$O_2^{\cdot-}$/ H_2O_2 +CAT/ GPX/ DHAR/ GST/ ASC/ SOD/ MDHAR +PCs -Cu uptake +LOX
Cu_3	$CuSO_4$ (450 µM)	3h-1d	SNP (200 µM) c-PTIO (150 µM) Tungstate (150 µM)	*Hordeum vulgare* cv. Nude	3d	Roots	+NO (DAF-FM DA)	+root length/ cell viability -$O_2^{\cdot-}$/ H_2O_2 -lipid peroxidation +SOD/ POD/ APX -CAT

Cu_4	$CuCl_2$ (50 µM)	8d	SNP (100 µM) Hb (0.1%)	*Lycopersicon esculentum* cv. Meigui	21d	leaves roots	-	-biomass -SOD/ POD/ APX +CAT -H_2O_2 -lipid peroxidation -Cu uptake
Cu_5	$CuSO_4$ (5 50 µM)	14d	SNP (10 µM) cPTIO (50 µM) *nox1/ gsnor1-3/ nia1 nia2 vtc2-1/ vtc 2-3/ high miox4*	*A. thaliana*	14d	roots shoot	+NO (*nox1*, gsnor1-3) -NO (WT) (DAF-FM)	+biomass +cell viability in *nox1,gsnor* (25,50 µM +cell viability (SNP)
Cu_6	$CuSO_4$ (5-25 µM)	7d	cPTIO (500 µM) SNP (10 µM) *nia1 nia2noa1-2*	*A. thaliana*	7d	Seedlings	+NO (DAF-FM DA) NR	+biomass -cell viability
Cr_1	$K_2Cr_2O_7$ (1-10 mg/L)	12d	SNP (100 µM) L-NAME (100 µM)	*Festuca arundinacea schreb*	54d	Seedlings	-	+biomass +Cr uptake -$O_2^{\cdot-}$/ H_2O_2 -SOD +POD -lipid peroxidation =Pro +photosynthetic act. (Chl, *PSII,* quinor +translocation factors
Cr_2	$K_2Cr_2O_7$ (200-500 µM)	3-9d	SNP (200-500 µM)	*Zea mays* cv. Agrister	3-9d	roots cotiledons coleoptiles	-	+root length/ biomass -Cr uptake -lipid peroxidation +CAT/ SOD -GOX -NADP-ICDH / NADPME / G6PDH +6PGDH
Hg_1	$HgCl_2$ (60 µM)	3-7d	SNP (100-200 µM)	*Oryza sativa* cv. Zhonghua	7d	leaves roots	-	+biomass/ root length/ lateral roots -Hg uptake -$O_2^{\cdot-}$/ H_2O_2 -SOD/ CAT/ APX/ POD

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-	$MnCl_2$ (0-1000 µM)	7d	SNP (100-1000 µM)	*Matricaria chamomilla*	7d	shoot roots	+RNS	-H_2O_2 +biomass +root length -Mn uptake -Cat/ GPX +APX +ROS =GR +Fe
-	$MnCl_2$ (15 mM)	1-2d	SNP (100 µM) cPTIO(100 µM)	*Oryza sativa* cv. Pant-12	17d	leaves	-	-Mn uptake -lipid peroxidation -H_2O_2 +ASC =GSH/ APX -SOD/ GPX/ CAT/ DHAR/ GR
Ni_1	$NiCl_2$ (0.5 µM)	7d	SNP (0.2 mM)	*Eleusine coracana*	14d	shoot roots	-	+biomass / root and shoot length +Chl -$O_2^{\cdot-}$/ H_2O_2 -MDA/ LOX/ Pro +CAT/ SOD/ APX
Ni_2	$NiSO_4$ (10-200 µM)	9d	SNP (100-200 µM) cPTIO (200 µM)	*Oryza sativa* cv. Yangliangyou 6	19d	shoot roots	+NO (NO-2-G kit)	+biomass/ root length/ cell viability -Ni uptake +Chl/ carotenoids/ Pro -$O_2^{\cdot-}$/ H_2O_2 -lipid peroxidation/ LOX +ASC/ POD/ CAT +*CAT/ POD/ APX/ GR/ SOD*
Ni_3	$NiCl_2$ (0.5 mM)	10d	SNP (0.2 mM)	*Brassica napus* cv. PF	21d	Leaves	-	+biomass -lipid peroxidation/ Pro -Ni translocation -H_2O_2 +GPX/ APX/ CAT +Chl -LOX

Ni_4	$NiCl_2$ (1 mM)	30d	SNP (0.5 mM)	*Triticum aestivum* cv. Samma	30d	Plants	-	+biomass +carbonic anhydrase +SOD/ POD =CAT +Pro/ N, P, and K +Chl -lipid peroxidation
Ni_5	$NiCl_2$ (100 µM)	4d	SNP (100 µM)	*Triticum aestivum* cv. Yangmai 158	4d	Seedlings	-	-H_2O_2 =lipid peroxidation +POD/ APX/ SOD/ GR/ GST =CAT
Ni_6	$NiCl_2$ (0.2 µM)	4d	SNP (0.3 mM)	*Phaseolus vulgaris*	2d	roots leaves	-	+Ni uptake -SOD +CAT/ POD -Pro +Ca and Zn
-	Pb $(C_2H_3CO_2)_2$ (400 µM)	6h -3d	ASC (400 µM)	*Prosopis farcta*	21d	seedlings	+NO (except at 72h) (Griess reagent)	+flavonoids/ phenolic acids -lignin +*ADC* and *PAL* +CAT/ GPX/ APX +aconitase activity (24/48h) +H_2O_2 (6 and 12 h); -H_2O_2 from 24 to 48h and steady-state 72h.
-	Pb $(NO_3)_2$ (0-200 mg/kg)	20h	SNP (0.05-1 mM)	*Vigna unguiculata* cv. Walp.	3 leaf stage	roots leaves seeds	-	+biomass/ germination -Pb uptake and translocation +stomatal conductance +IAA, CKs and GA3 -ABA
-	$Pb(NO_3)_2$ (150 µM)	14d	DEA NONOate (2 mM) SIN-1 (2 mM)	*A. thaliana* CFP-PTS1	14d	Seedlings	-	-CAT

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-	$Pb(NO_3)_2$ (100 µM)	24h	SNP (5 mM) cPTIO (0.5 mM) L-NAME (0.3 mM) PBITU (0.3 mM) GSNO (3 mM)	*Pogonatherum crinitum*	5d	Roots	-NO (Hb/ Griess reagent)	-Pb accumulation -NR
Pb_1	$Pb(NO_3)_2$ (50-250 µM)	0-8h	SNP (100 µM)	*Triticum aestivum* cv. PBW 502	48h	Roots	+NO (DAF-2DA/ Griess reagent)	+root length/ cell viability -lipid peroxidation -conjugated dienes -$O_2^{\cdot-}$/ H_2O_2/ $HO^{\cdot}$ -SOD/ CAT/ APX/ GPX/ GR
Pb_2	$Pb(NO_3)_2$ (500 µM)	14d	SNP (50-400 µM)	*Lolium perenne*	21d	shoot roots	-	+biomass/ root length +Chl/ photosynthetic rate -translocation of Pb -$O_2^{\cdot-}$/ H_2O_2 -lipid peroxidation +SOD/ APX/ POD -CAT
Pb_3	$Pb(NO_3)_2$ (100 µM)	7d	SNP (0.5 mM) cPTIO (1 mM)	*A. thaliana*	0d	seedlings roots	-	+root length =Pb accumulation -H_2O_2 -lipid peroxidation -SOD/ CAT/ GR/ GPX/ POD
Pb_4	$Pb(NO_3)_2$ (200 mg/kg)	20h	SNP (0.05-1 mM)	*Vigna unguiculata* cv. Walp.	0d	Plants	-	+SOD/ CAT/ APX/ GR +Pro +Chl/ photosynthesis -lipid peroxidation
Pb_5	$Pb(NO_3)_2$ (100-500 µM)	15d	SNP (100-200 µM)	*Melissa officinalis*	15d	shoot roots	-	+biomass +Chl -lipid peroxidation +APX / CAT/ GPX

-	$ZnCl_2$ (400 µM)	2-10d	PTIO (0.2 mM) L-NAME (0.5 mM)	*Solanum nigrum*	10d	Roots	-NO (Hb)	-root length -Zn accumulation -H_2O_2/ $O_2^{\cdot-}$ -MDA (until 4d) +CAT/ APX -SOD -*NOX* activity
-	$ZnSO_4$ (3 mM)	6d	PTIO (250 µM)	*Triticum aestivum* cv. Xihan 3	seedlings	roots leaves	-NO (DAF-FM DA)	+cell viability -Zn accumulation -root length -H_2O_2 +$O_2^{\cdot-}$ =MDA/ DAO/ POD -CAT/ APX +SOD/ GR/ PAO/ NOS activity
-	$ZnSO_4$ (0.05 mM)	14d	SNP (0.5 mM)	*Triticum aestivum* cv. Kazakhstanskaya 10	4d	shoot roots	-	+biomass +photosynthesis +alternative oxidase (shoot) -alternative oxidase (roots) -lipid peroxidation
-	$ZnSO_4$ (100-500 µM)	14d	SNP (100-200 µM)	*Plantago major*	21d	leaves roots	-	+biomass +Chl -lipid peroxidation =H_2O_2 +peroxidase activity +CAT/ APX/ SOD
-	$ZnSO_4$ (200 µM)	4d	L-NAME (1 mM)	*Nicotiana tabacum* cv. xanthi	35d	Leaves	+NO (DAF-2 DA)	Small and delayed lesions =Zn accumulation in apoplast

Annex 8.3.

Zn_1	Zn ONPs (250 mg/L)	3d	SNP (10 μM) cPTIO (100 μM) *noa1* *noe1*	*Oryza sativa* cv. Jiafuzhan	3d	Seedlings	+NO (DAF-FM DA)	+root length/ biomass (10 μM) +Chl -Zn accumulation -$O_2^{\cdot-}$/ H_2O_2 =lipid peroxidation +GSH -SOD +*CAT/ APX/ POD (noe1)*
Zn_2	$ZnSO_4$ (500 μM)	10d	SNP (100 μM)	*Carthamus tinctorius* cv. Arak2811	21d	roots shoot	-	+biomass -translocation -lipid peroxidation -H_2O_2 -Pro +CAT/ APX/ GPX/ GSH/ ASC +GSH/GSSG ratio +α-tocopherol
Zn_3	$ZnSO_4$ (2 μM)	10-30d	GSNO (100 μM)	*Triticum aestivum* cv. Chinese Spring	30d	roots shoot	+NO (DAF-FM DA)	-biomass -Zn uptake =Zn accumulation =GSH =Chl +ASC
Zn_4	Zn^{2+} (10 mg/L)	1-4d	SNP (25-50 μM)	*Hydrilla verticillata*	10d	leaves	+NO (Hb)	-Zn uptake (4d) +Cu/ Fe/ Mn +lipid peroxidation/ carotenoids -Chl +SOD/ CAT/ APX/ ASC -POD/ DHA
Zn_5	$ZnSO_4$ (0.05-0.5 mM)	25d	SNP (0.1 mM)	*Zea mays* cv. DK 647 F1	35d	Roots	-	+root length/ cell viability +Chl +RWC -Pro

Zn_6	$ZnSO_4$ (500 µM)	10d	SNP (100 µM)	*Carthamus tinctorius* cv. Arak2811	21d	roots shoot	-	-biomass -Zn uptake -H_2O_2 -Pro/ MDA +Chl +CAT/ APX/ GPX/ ASC +α-tocopherol/ PCs

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Las plantas han desarrollado una serie de estrategias que le permiten adaptar su metabolismo frente a las condiciones cambiantes que las rodean Todo ello dispara procesos fundamentales en la planta, que conllevan una serie de respuestas eficientes frente al estrés, en las que la concentración y localización subcelular de RNS/ROS en los tejidos de la planta son esenciales para la función de estas moléculas. Por ello, las plantas han desarrollado una serie de mecanismos altamente regulados que les permiten producirlas y eliminarlas en su justa medida. Los peroxisomas tienen una función esencial en la homeostasis de ROS/RNS. Además, estos orgánulos pueden interaccionar con mitocondrias y cloroplastos, compartiendo rutas metabólicas y señalización pudiendo cumplir un papel de detoxificación de ROS, señalizador, sensor de ROS/RNS y de transporte de proteínas a otros orgánulos.

Se ha demostrado que el óxido nítrico (NO) es una molécula señal clave en la respuesta de la planta al estrés. Sin embargo, la función del NO en la respuesta de la planta al cadmio y en la interacción planta-hongo patogénico no está del todo definida y en el último caso, es casi desconocida. Nuestro trabajo mostró una posible relación entre la respuesta de la planta al Cd y al estrés biótico, que podría estar regulada por NO, lo que sugiere una conexión entre ambos tipos de respuesta al estrés.

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