Unravelling the role of Zinc in the resistance to Necrotrophic fungi in *Arabidopsis thaliana* and *Noccaea caerulescens*

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UNRAVELLING THE ROLE OF ZINC IN THE RESISTANCE TO NECROTROPHIC FUNGI IN *ARABIDOPSIS THALIANA* AND *NOCCAEA CAERULESCENS*

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Urn unrolled the length of a scroll. Brilliant flowers glowed in the golden light.
"Orinjcrates' On the Nature of Plants," said Didactylos. "Six hundred plants and their uses . . ."
"They're beautiful," whispered Brutha.
"Yes, that is one of the uses of plants," said Didactylos. "And one which old Orinjcrates neglected to notice, too.
Terry Pratchett in Small Gods, 1992

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ABSTRACT

Zinc is an essential plant micronutrient but it can be toxic in elevated concentrations. Most of the plants adapted to metalliferous soils exclude metals from their tissues. However, some plants so-called hyperaccumulators are able to tolerate and accumulate extremely high metal concentrations in their shoots. Amongst the most accepted hypothesis to explain the acquisition of this trait is that heavy metals protect the plant against pathogens and herbivores (metal/elemental defence hypothesis). In fact, there is evidence that Zn and other heavy metals may be toxic for the attacking pathogens at high concentrations in plant. Metals have been accounted even for improving plant disease resistance below hyperaccumulation levels, thus being a possible explanation for the acquisition of the trait (defensive enhancement hypothesis). Nonetheless, other organic compounds related to the plant immune system, have been reported to be up or under regulated in response to Zn, thus enhancing the stress response (joint effects hypothesis) or saving metabolic cost to the plant (trade-off hypothesis).

In this thesis the metal defence hypothesis was tested in metal and non-metal hyperaccumulating species inoculated with necrotrophic fungi. Both direct metal toxicity and indirect metal-induced enhancement of organic defences were considered. For this purpose, three pathosystems were established with plants growing at different Zn concentrations. The pathosystems were: *N. caerulescens* infected with *Alternaria brassicicola* and *A. thaliana* infected with *Alternaria* or *Botrytis cinerea*. In the case of *A. thaliana*, *npr*1, *pad*1, *coi*1 and *etr*1, four stress response defective mutants, were also used. Moreover, natural occurring populations of the genus *Noccaea* were located in the Pyrenees. Plants were identified and their Zn and Cd accumulation ability characterized with a view to future field experiments.

Zn improved *Noccaea* resistance to *Alternaria*, leading to a trade-off between the metal and some of the organic-based defences. Although SA, JA, ABA and the expression of SA, JA and JA/Et pathways marker genes were evenly induced by *Alternaria* 24 hours after the inoculation, remarkably, one week later Zn and the hormone signalling showed a negative correlation. A trade-off was also reported between leaf glucosinolates and Zn, but, in contrast, one week after the inoculation glucosinolates concentration increased in the infected leaves with higher Zn concentration. Therefore, there were evidences to support the trade-off hypothesis in the case of the stress signalling pathways one week after the inoculation, but not for glucosinolates.

A.thaliana WT Zn-treated plants were more resistant to *Alternaria* attack, but the Zn effect was not clear against *Botrytis*. *A. thaliana* WT and the stress signalling defective mutants concentrated similar leaf Zn values, that were accounted to be potentially toxic for *Alternaria*, but not for *Botrytis*. Noteworthy, Zn amplified the expression of *PR1* and *BGL2*, genes related to the SA signalling pathway, and of *CHIB* and *PDF1.2*, genes from the JA/Et signalling pathway in response to *A. brassicicola* infection, where specially *PDF1.2* expression was greatly boosted. However, changes in the endogenous concentrations of SA, JA, ABA and ACC, were not reported to be responsible for the differences in the resistance to *Alternaria*. Zn-treated *A. thaliana* stress response defective mutants were not more resistant to none of the pathogens when Zn was applied or even more susceptible to *Botrytis*. As Zn did not compensate their metabolic defects, it is suggested that the plant organic defences activated by the enhanced Zn levels against *Alternaria* played a more important role than Zn as a direct inorganic defence. Taken all together, our results gave support to the joint effects hypothesis, applied to non-hyperaccumulators, in the *A. thaliana* - *A. brassicicola* pathosystem.

The plants from four non-metalliferous *Noccaea* populations in the Eastern Pyrenees were identified attending to morphological characters as *Noccaea brachypetala* and they were reported to be Zn and Cd hyperaccumulators. Moreover, plants form three populations differed in the rosette shape while growing hydroponically, suggesting underlying genetic differences that were patent in the heterogeneous expression patterns of metal transporter genes in response to Zn and Cd supply. The populations were fragile and of difficult accessibility, which limits their use for field studies in metal hyperaccumulation and defence.

RESUMEN

El zinc es un micronutriente esencial para las plantas, pero tóxico a concentraciones elevadas. La mayoría de las plantas adaptadas a suelos metalíferos excluyen el exceso de metales de sus tejidos. Sin embargo, algunas plantas denominadas hiperacumuladoras toleran y acumulan en parte aérea concentraciones de metales extremadamente altas. Una de las hipótesis más aceptadas que explica la adquisición de este rasgo relaciona una elevada concentración de metales con una mayor resistencia a patógenos y herbívoros (hipótesis de la defensa metálica/elemental). De hecho, existen evidencias de que altas concentraciones de metal en planta pueden ser tóxicas para microorganismos fitopatógenos, incluso a niveles por debajo del umbral de hiperacumulación (hipótesis de la mejora defensiva). No obstante, el Zn podría sobre o subregular compuestos orgánicos relacionados con el sistema inmune de la planta, acentuando la respuesta al estrés (hipótesis de los efectos conjuntos) o ahorrando costes metabólicos (hipótesis de la compensación).

En esta tesis se examinó la hipótesis de la defensa metálica en plantas hiperacumuladoras y no hiperacumuladoras inoculadas con hongos necrotróficos. Para ello se consideraron el efecto directo tóxico del Zn y la interacción con las defensas orgánicas, estableciéndose tres patosistemas con plantas creciendo en distintas concentraciones de Zn: *Noccaea caerulescens* inoculada con *Alternaria brassicicola* y *Arabidopsis thaliana* inoculada con *Alternaria* o *Botrytis* cinerea. En el caso de *Arabidopsis*, se emplearon cuatro mutantes con defectos metabólicos en la respuesta al estrés: *npr*1, *pad*1, *coi*1 y *etr*1. Además, se localizaron en los Pirineos poblaciones naturales de plantas del género *Noccaea*, que fueron identificadas y caracterizadas con vista a futuros experimentos de campo.

El Zn mejoró la resistencia de *Noccaea* a *Alternaria*, y se correlacionó negativamente con algunas de las defensas orgánicas. A pesar de que el SA, JA, ABA y las expresiones de varios genes marcadores de las rutas del SA, JA y JA/Et fueron inducidos por igual 24 horas tras la inoculación con *Alternaria*, una semana más tarde el Zn y las rutas de señalización de estrés se correlacionaron negativamente. Asimismo, se encontró una compensación entre el Zn y los glucosinolatos en hoja, pero, por el contrario, una semana después de la inoculación con *Alternaria* la concentración de glucosinolatos se vio incrementada en las hojas infectadas con mayor concentración de Zn. Por tanto, la hipótesis de la compensación se puso en evidencia para las rutas de señalización de estrés tras una semana de infección, pero no para glucosinolatos.

Las plantas de *A. thaliana* silvestres tratadas con Zn fueron más resistentes a *Alternaria*, pero no claramente a *Botrytis*. *Arabidopsis* silvestre y los mutantes de las rutas de señalización de estrés acumularon concentraciones similares de Zn en hoja, descritas como tóxicas para *Alternaria*, pero no para *Botrytis*. El Zn interaccionó con las defensas orgánicas y amplificó la expresión de los genes relacionados con las rutas de señalización del SA (*PR1* y *BGL2*) y del JA/Et (*CHIB* y *PDF1.2*), especialmente *PDF1.2*, en respuesta a la infección por *Alternaria*. Sin embargo, los cambios en las concentraciones de SA, JA, ABA y ACC no fueron responsables de las diferencias en la resistencia a *Alternaria*. Los mutantes tratados con Zn no fueron más resistentes a ninguno de los patógenos o incluso más susceptibles a *Botrytis*. Como el Zn no compensó su defecto metabólico, se sugiere que las defensas orgánicas activadas por el Zn tuvieron un papel más importante contra *Alternaria* que el Zn como defensa inorgánica. Los resultados obtenidos en el patosistema *A. thaliana* - *A. brassicicola* apoyaron la hipótesis de los efectos conjuntos, aplicada a plantas no hiperacumuladoras.

Las plantas de tres poblaciones de suelos no metalíferos de los Pirineos hiperacumularon Zn y Cd y fueron identificadas como *Noccaea brachypetala* según características morfológicas. Además, creciendo en hidroponía, presentaron diferencias fenotípicas, lo cual sugiere que existen diferencias genéticas subyacentes puestas de manifiesto en la expresión de genes de trasportadores de metales en respuesta a Zn y Cd exógenos. El uso de estas poblaciones para estudios de campo se ve limitado por su fragilidad y dificultad de acceso.

CONTENTS

ABBREVIATION LIST

ACC	1-Aminocyclopropane-1-carboxylic acid
ABA	Abscisic acid
CHIB	Basic endochitinase
COI1	Coronatine-insensitive protein 1
BGL2	Beta-1,3-glucanase 2
Et	Ethylene
ETR1	Ethylene receptor 1
GSs	Glucosinolates
HR	Hypersensitive response
JA	Jasmonic acid
LOX2	Lypoxigenase 2
NPR1	Non-expressor of pathogenesis related genes 1
PAD1	Phytoalexin deficient 1
PR1	Pathogenesis related 1
PDF1.2	Plant defensine 1.2
SA	Salicylic acid
SAR	Systemic acquired resistance
VSP2	Vegetative storage protein 2
WT	Wild-type

Part I

INTRODUCTION

1 INTRODUCTION

Studies on plant defences against pathogens and herbivores most commonly comprise the assessment of plants organic-based compounds, such as hormones or peptides. However, in crop managenent, traditionally used inorganic-based formulations, mainly containing sulphur, copper or zinc, have been proven to be effective against pathogens, although they are no exempt from causing a negative impact on the environment (Tweedy 1981; Eijsackers et al. 2005; Zubrod et al. 2015). Inside the plant, as part of the defence mechanisms, heavy metals have been proposed to have a role as inorganic defences, acting directly and/or interplaying with other metals and/or the organic defenses (Coleman et al. 2005; Poschenrieder et al. 2006; Cheruiyot et al. 2015). Knowing the physiological mechanisms through which plants protect themselves against disease is relevant in order to design improved approaches to disease management. Here, deepening into the particular case of zinc against necrotrophic fungi in metal hyperaccumulating and non-hyperaccumulating plants, four main aspects are covered. First, it is discussed the role of Zn in plants, paying attention to the singular case of metal hyperaccumulation. Second, at the interaction of pathogens and plants, it is addressed the concept of disease and its effects in plant productivity. Third, a wider section considers the influence of mineral nutrition in plant disease, with focus on the case of the metal defence hypothesis and delving into the role of the organic defences. Fourth, the particular actors that integrate the models of study in this thesis are presented. Figure 1.1 shows a layout of the topics included in the theoretical frame.

1.1 THE ESSENTIAL ROLE OF ZINC

Zinc is an essential element for all organisms. It is required for the activity of more than 300 enzymes distributed throughout all six groups of reaction types (McCall et al. 2000). In most enzymes, Zn is integrated in the enzyme structure. Most plant Zn enzymes contain one Zn atom, e.g. carbonic anhydrase; exception is alcohol deshydrogenase which contains two Zn atoms, one with a structural function and another with a catalytical function (Marschner 2012). An example of Zn-activated enzyme is the leaf Zn pyrophosphatase.

In several plant species, the activities of Zn-containing enzymes such as carbonic anhydrase, alcohol dehydrogenase and Zn/Cu superoxide dismutase are good indicators of the plant Zn nutrition. The activity of these enzymes strongly decreases with Zn deficiency and

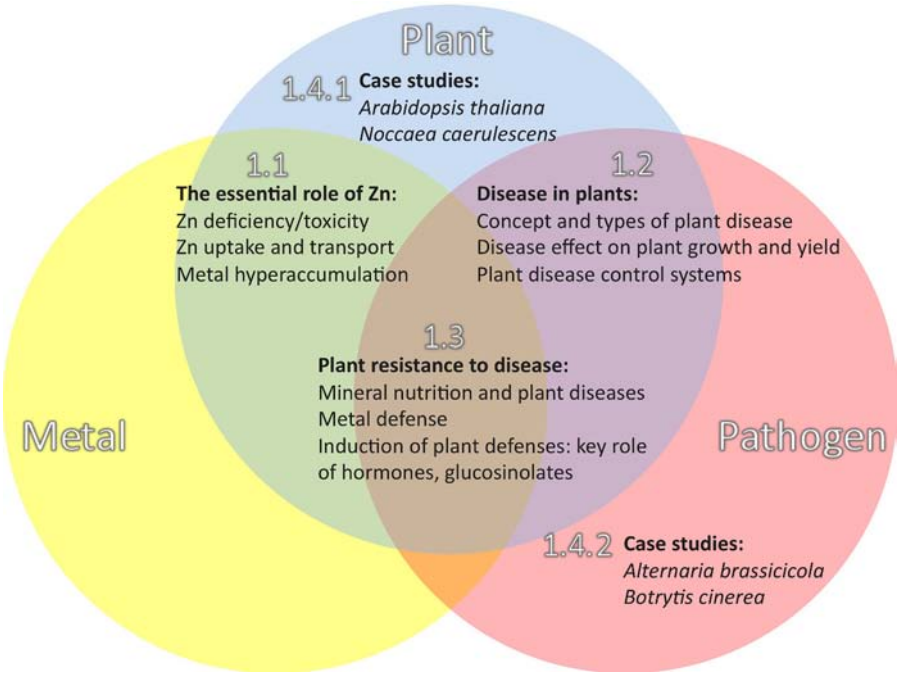


Figure 1.1: Layout of the theoretical frame regarding the role of Zn in the resistance to pathogens in plants.

therefore the enzyme activity can be used as a specific marker of the Zn status of the plant.

Zinc plays an essential role in nucleic acid metabolism, in ribosome structure and functioning and, consequently, in protein synthesis, and in the carbohydrate metabolism. Zinc-finger proteins, the largest class of Zn-binding proteins are important factors regulating transcription (Broadley et al. 2007).

Growth of plants with insufficient Zn supply is severely reduced and several of the deficiency symptoms resemble auxin deficiency. Therefore, Zn is thought to be involved in auxin metabolism. However, the exact mechanisms of auxin- Zn interaction are still not established (Marschner 2012).

1.1.1 *Zn deficiency and toxicity*

Zinc ranges second after iron in quantitative micronutrient requirements in plants. Zinc availability in soils largely depends on the kind of clay minerals, pH and organic matter. Zinc deficiency is considered the most important and widespread micronutrient deficiency problem worldwide. In Turkey, India, and China about 50% of the arable land is affected by Zn deficiency. Even higher percentage values are reported for Iran (60%) and Pakistan (70%). (Alloway 2009). Also soils in the Mediterranean area can be prone to Zn deficiency especially in areas with high carbonate and phosphorus contents (Rashid and Ryan 2004).

Sufficient leaf tissue concentrations for crop plant performance usually range from 20-30 to 70-80 mg/kg dry weight. Concentrations between 15 to 20 mg/kg are considered in the critical range; although the Zn deficiency level depends largely on phosphorus availability. Adequate P/Zn ratios for most crop plants lay between 15 and 180 with an optimum of 65 (Bergmann 1993).

Zinc toxicity may occur in contaminated soils because of mining and smelting activities or due to long–term application of sewage sludge, manures, or Zn-rich municipal waste compost. Locally high soil Zn concentrations can be found under galvanized structures (Fava et al. 2002), e.g. electric power towers. In most plants, Zn toxicity occurs when leaf Zn concentrations reach 300 to 1000 mg/kg dry weight with a typical diagnostic value of 500 mg/kg (Chaney 1993). Zinc toxicity reduces root growth, impairs photosynthesis and photoassimilate translocation, and alters ion homeostasis (Ruano et al. 1987, 1988). Phosphorus and or iron deficiency can be secondary stresses induced by excess Zn (White et al. 1979). Zinc interferes both in the uptake and translocation process of Fe (Olsen 1972). At least in part, this can be attributed to the fact that both metal ions share common transport systems and binding molecules, especially organic acids and nicotianamine. In fact, maintenance of both Zn and Fe homeostasis is regulated at the uptake and transport level as well as by subcellular compartmentation and chelation (Lin and Aarts 2012).

1.1.2 *Zn uptake and transport*

Zinc uptake and transport in plants is governed by multiple transport systems that must cooperate in a coordinated way to achieve adequate Zn availability for biological processes and Zn homeostasis despite changes in the supply from the growth substrate. Metal chelators and metal transporters in cell membranes ensure the correct Zn trafficking in the plant. Best characterized are the transport systems in *Arabidopsis thaliana* and *Oryza sativa* (Colangelo and Guerinot 2006; Sinclair and Krämer 2012; Takahashi et al. 2012).

Plasma membrane transporters of the ZIP family participate in Zn uptake into the cell (zinc and iron transporter like; ZRT/IRT). In *A. thaliana*, AtZIP2, AtIRT1 and AtIRT3 are responsible for Zn^{2+} uptake into the cell; in rice OsZIP4, OsZIP5, OsZIP8 and OsIRT1 have been identified as plasma membrane located Zn uptake transporters (Ricachenevsky et al. 2015).

Uptake into the vacuole is mediated by proteins of the zinc-induced-facilitator family, AtZIF1, AtZIF2 and VIT, the vacuolar iron–transporter family, AtVIT1 and OsVIT1 and OsVIT2. Moreover, heavy metal associated proteins (HMA) and metal-tolerance-proteins (MTP) contribute to Zn efflux from cytoplasm to apoplast or uptake into the vacuoles (Ricachenevsky et al. 2015). Both cytoplasmic efflux and

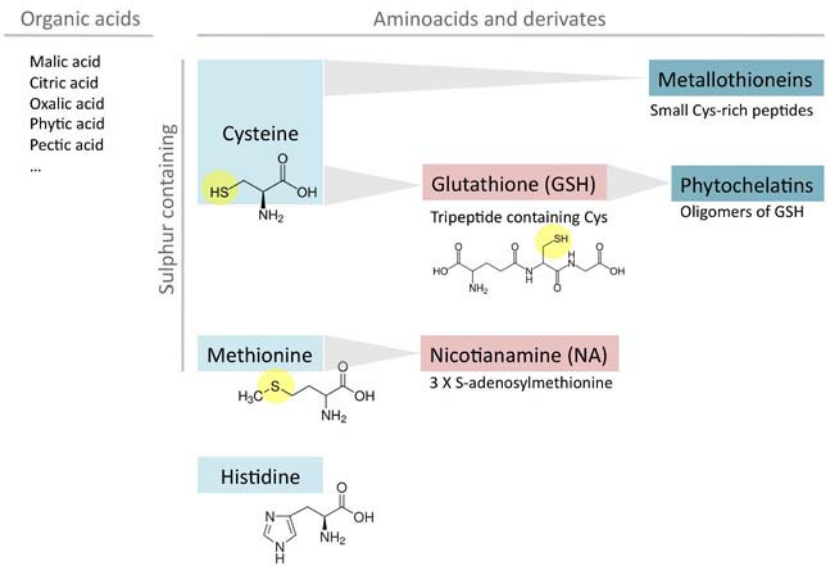


Figure 1.2: Principal classes of metal chelators in plants.

vacuolar influx transporters play a role in metal detoxification (Hall 2002).

Recent investigations revealed that ZIP1p, ZIP3p and ZIP4p are more expressed in the pericycle that in the epidermal cells of *Arabidopsis* implying their possible role in Zn uptake and transport from root endodermal cells to xylem cells (Almira 2015). Also the Zn transporting P-type ATPase AtHMA2 and AtHMA4 are mainly expressed in the vascular tissue and seem responsible for Zn transport from roots to shoots (Park and Ahn 2014).

The transcription factors bZIP19 and bZIP23 (basic region leucine zipper) have been identified to be involved in the response of *Arabidopsis* to Zn deficiency. These transcription factors target a small group of 16 genes, 8 of which belong to the ZIP family. Moreover, they target NAS genes coding for enzymes responsible for the biosynthesis of nicotianamine (NA) an important chelator of Zn and Fe in plants (Assunção et al. 2010, 2013). NA-complexed Zn seems to be a major transport form of Zn in the phloem (Clemens et al. 2013). Phloem transport of Zn is an important process for efficient use of Zn, especially in the reproductive stage and the fruit and seed development. Other metal chelators are shown in figure 1.2.

1.1.3 *Metal hyperaccumulation*

In soils with high availability of metals (e.g. Zn, Cd, Ni, Mn, Al), or non-metallic trace elements (e.g. As, Se), most adapted species try

to restrict the uptake and to exclude excess ions at least from their photosynthetic tissues. Some species, however, have evolved a different strategy: (hyper)accumulating large, potentially toxic, concentrations of these elements in their shoots Baker (1981). Table 1.1 gives a few examples of trace element concentrations in shoots of some hyperaccumulating species along with an indicative range for non-hyperaccumulators, taking *Brassica napus* and *Agrostis* sp. or other grasses as references for less efficient and highly efficient shoot excluders, respectively (Poschenrieder et al. 2014). Huge differences in leaf concentrations between hyperaccumulators and non-hyperaccumulators are evident.

Metal hyperaccumulating plants are able to accumulate in their shoots high concentrations of metals (e.g. zinc, cadmium, lead or nickel) that are toxic to the majority of plants species. The term was coined by Reeves in the 70ies of the last Century describing the extraordinarily high accumulation of Ni in a Caledonian tree species (van der Ent et al. 2013). Then the term has been spread to other metals and species, even including non-metallic elements such as selenium (Table 1.1). The criteria for considering a plant species as a metal hyperaccumulator not only include the condition of a high leaf metal concentration (examples for threshold are 10,000 in the case of Zn, 1,000 for Ni or 100 for Cd), but also the absence of toxicity symptoms at these high leaf concentrations, and high soil to shoot transfer factors (van der Ent et al. 2013).

The molecular, genetic and ecological characteristics of metal hyperaccumulation have been reviewed in the last years (Boyd 2004; Verbruggen et al. 2009; Krämer 2010; Rascio and Navari-Izzo 2011). At a molecular level, an increased expression of some of the transporters mentioned in the previous section has been related with the hyperaccumulation and hypertolerance trait in heavy metal hyperaccumulating species (Figure 1.3). Amongst them are the metal tolerance protein 1 (MTP1) and the heavy metal ATPases HMA3 and HMA4 (Verkleij 2008; Krämer 2010; Rascio and Navari-Izzo 2011; Park and Ahn 2014). These trasnporters have been used in several studies to evaluate the metal accumulation and tolerance in plants (Assunção et al. 2001) (Küpper and Kochian 2010).

The metal tolerance protein 1 (MTP1 = ZTP1 = ZAT) belongs to the cation diffusion facilitator family (CDFs) and it imports Zn into the vacuole of young leaves of both young and mature plants, where it is mainly chelated with organic acids. AtMTP1 silencing causes Zn hypersensitivity and a reduction in Zn concentrations in vegetative plant tissues (Desbrosses-Fonrouge et al. 2005; Krämer 2005; Küpper and Kochian 2010). MTP1 seems to be involved in Zn sequestration in dividing and expanding tissues and Zn accumulation (Mohr and Cahill 2003). In the heavy metal hyperaccumulator *N. caerulescens*, accessions with higher *MTP*1 expression show greater tolerance to

Table 1.1: Examples of elemental concentrations in leaves of excluders and hyperaccumulating species (recompiled from multiple sources by Poschenrieder et al. (2014)). [a] sufficient range; [b] on soil with high concentration; [c] on control (left) or polluted substrate (right); [d] under high (left) or low (right) pH; [e] soil pH $\leq$ 5.5.

Element	Species	Conc.(mg kg^{-1})
Zn	*Agrostis sp.*	25-75[a]
	Brassica napus	25 -70[a]
	Noccaea caerulescens	20000[b]
	Viola calaminaria	4500[b]
Ni	*Brassica napus*	20 -170[c]
	Alyssum bertolonii	13400[b]
	Berkheya codii	11600[b]
Mn	*Agrostis sp.*	50-100[a]
	Brassica napus	30 -150[a]
	Virotia neurophylla	31200[b]
Cd	*Agrostis sp.*	0.6 - 3.3[c]
	Brassica napus	0.4 - 7.0[c]
	Noccaea praecox	6000[b]
Al	*Agrostis sp.*	20-140[d]
	Brassica napus	160-240[d]
	Commelina sinensi (tea)	>1000[e]
Se	*grasses*	0.4-13[c]
	Brassica napus	0.4 -470[c]
	Biscutella laevigata	12000[b]

Zn than other with less expression of the gene. *MTP*1 orthologues are also highly expressed in other metal hyperaccumulator species such as *A. halleri* and *N. goesingense*. Nevertheless, MTP1 expression barely responds upon Zn exposure in *N. caerulescens*, suggesting that in hyperaccumulators, Zn rather induces a reduction of downregulation (Assunção et al. 2001; Becher et al. 2004; Dräger et al. 2004; Gustin et al. 2009).

*HMA*3 and *HMA*4 encode for P_{1B}-ATPases and an enhancement of its expression is regarded as a prerequisite for hyperaccumulation and hyperresistance in hyperaccumulators (Park and Ahn 2014). *AtHMA*3 is highly expressed in guard cells, hyathodes, vascular tissues and the root apex, where it is located in the vacuolar membrane. Overexpression of *AtHMA*3 renders the plants more tolerant to Zn, Cd, Co and Pb and increases accumulation of Cd, while a T-DNA insertional mutant is more sensitive to Zn and Cd. Thus, AtHMA3 is thought to play a role in the detoxification of Zn and Cd by sequestering the metals into the vacuole (Morel et al. 2009). In *N.caerulescens*, HMA3 is involved in the hyperaccumulation and the hypertolerance of Cd (Ueno et al. 2011). The other P_{1B}-ATPase, HMA4, is an efflux transporter in the plasma membrane and it is expressed mainly in roots, stems and flowers. Together with HMA2, it is partly responsible for the root to shoot Zn and Cd translocation (Verret et al. 2004; Eren and Argüello 2004; Mills et al. 2005; Wong and Cobbett 2009). A triplication of HMA4, in addition to cis-regulatory mutations, in *A. halleri* and a tandem quatriplication in *N. caerulescens* are related to the adquisition of their metal hyperaccumulation trait in the evolution (Hanikenne et al. 2008; Ó Lochlainn et al. 2011).

The distribution of the hyper-accumulating species in different, rather distant botanical families indicates that the trait of metal hyperaccumulation has evolved multiple times independently in the plant kingdom (Cappa and Pilon-Smits 2014). Metal hyperaccumulation strategies, such an increased expression of some metal transporters and chelators, may represent a high energetic cost for the plant and thus the question arises why this trait has been selected in evolution.

1.2 DISEASE IN PLANTS

1.2.1 *Concept and types of plant diseases*

Plant disease has been defined as "an impairment of the normal state of a plant that interrupts or modifies its vital functions" (http://www.britannica.com/science/plant-disease). This definition implies both abiotic and biotic stress conditions. Despite the fact that plant pathologists mainly focus research on biotic stress, it is well established that abiotic environmental factors have large influence on the interaction of plants with their enemies. This is classically

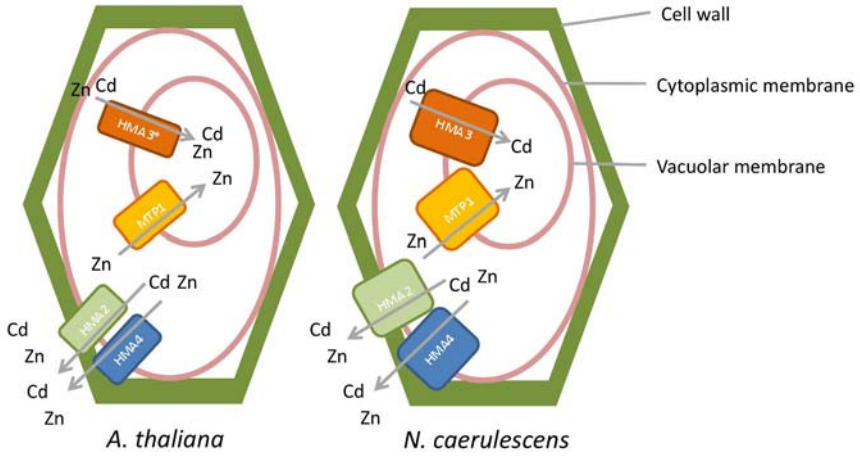


Figure 1.3: Plant cells with heavy metal transporters playing a role in Zn and Cd hyperaccumulation and hypertolerance in *N. caerulescens*. MTP1 and HMA3 are vacuolar influx transporters, while HMA2 and HMA4 are cytoplasmic efflux trasnporters. A greater size of the transporters in *N. caerulescens* compared to *A. thaliana*, indicates a higher gene expression. HMA3 in *N. caerulescens* has been only accounted for Cd transport. The gene encoding for HMA3* is only expressed in some *A. thaliana* ecotypes. (HMA2: Heavy metal ATPase 2; HMA3: Heavy metal ATPase 3; HMA4: Heavy metal ATPase 4; MTP1: Metal tolerance protein 1).

described by the so-called disease triangle highlighting the importance of interactions between plants-pathogens and the environment (Poschenrieder et al. 2006).

Plants, like animals, are affected by infectious diseases caused by pathogenic bacteria, fungi, viruses and viroids. In response to pathogen attack, plants have developed defence mechanisms capable of recognizing the pathogen and activating a series of responses at both the local and the systemic level. Pathogens, in turn, have evolved virulence mechanisms that allow them to overcome plant defence strategies. Coevolution between plants and their enemies, herbivores and pathogens, is an important driving factor for the large chemical diversity produced by plant secondary metabolism (Bednarek and Osbourn 2009; Zhu et al. 2014).

1.2.2 *Disease effect on plant growth and yield*

Pathogenic fungi cause severe crop yield losses. Many pathogenic fungi are characterized by high sporulation rates providing huge inoculums, short latent periods, and large-distance dispersion through water or wind. These fungi produce toxins and/or enzymes able to destroy cell compartmentation (necrotrophs) or they retrieve nutri-

ents from living cells (biotrophs) and alter plant growth and development by the synthesis or induction of phytohormones (Strange and Scott 2005).

After virus, pathogenic fungi are the second most important agents of emerging infectious diseases in plants (EIDs). These pose a threat on global food security, moreover taking into account that on a global scale human food supply mainly depends on four staple crops; namely rice, wheat, maize, and potato (Anderson et al. 2004). The dependence of world population on a reduced number of staple crops that are prone to catastrophic fungal diseases makes research into sustainable crop protection a priority in order to assure global food availability.

1.2.3 *Plant diseases control systems*

Within the frame of intensive farming systems, control of plant diseases is mainly based on chemicals. The initially widely used inorganic formulations containing sulphur, copper, arsenic or even mercury have been progressively substituted by synthetic broad-spectrum organic compounds. However, at present, efficient disease control demands an integrated approach combining more sustainable crop management, including crop rotation and measurements to improve biodiversity, with breeding efforts and biocontrol agents (Savary et al. 2012).

1.3 Pathogenesis of fungal diseases and mechanisms of plant resistance

Pathogenic fungi are classified within three groups depending on their modes of nutrition. According to this classification, fungi are necrotrophs, biotrophs or hemibiotrophs. While necrotrophs secrete high amount of toxins and induce host cell death to obtain nutrients, biotrophs feed on living plant tissue by means of a specialized structure that penetrates into de cells, the haustoria. Hemibiotrophs combine both strategies in a sequential manner, in two phases with variable duration: a first biotrophic phase is followed by a second necrotrophic phase. In all cases, penetration into the plant tissues is a first fundamental step to achieve infection. Besides the possibility to penetrate through natural openings (stomata hydatodes, lenticels) or wounds, most pathogenic fungi are able to directly penetrate through cuticles and cell walls by the release of degrading enzymes. This mode of penetration is a first step where signalling events between fungi and hosts can lead to recognition and activation of plant defence responses. In incompatible interactions, where an avirulent pathogen penetrates into a resistant plant, signals from the pathogen (elicitors) are recognized by plant receptors. A fast hypersensitive response in the form of localized cell death inhibits the infection. More-

over, the activation of secondary signals such as salicylic acid and the production of pathogenesis related proteins can lead to systemic acquired resistance, avoiding new infections throughout the plant. In compatible interactions, penetration is followed by invasion and colonization of the plant, which develops disease symptoms in the form of chlorosis, necrosis, wilting, and inhibited or abnormal growth (Agrios 2005). The topic of plant necrotrophic pathogens, with special focus on *A. thaliana* as a host, has been recently reviewed by Laluk and Mengiste (2010).

Classically plant defence mechanisms have been divided into constitutive or pre-existent and inducible defences. However, currently some authors consider that the discrimination between constitutive and induced defences is artificial since many defences fall in both classes and traits associated with defences can have roles in other primary and secondary physiological processes as well (Alba et al. 2011). Defences are, in principle, those plant traits that, when absent, make a plant more palatable for a plant consumer. Pre-existent defences are, for example, wax layers, trichomes, and thorns, but can also be secondary metabolites (Strauss et al. 2002) and protective surface proteins (Shepherd et al. 2005). They are considered as constitutive because they are present in the plant before suffering any pathogen attack. Contrastingly, the expression of inducible defences previously requires an interaction between the pathogen and the target plant.

1.3.1 *Mineral nutrition and infectious plant diseases*

Higher plants need at least 17 mineral elements in order to complete their life cycle. Besides the macronutrients, which are required in the range of mM concentrations (C, H, O, N, S, P, K, Ca, and Mg), micronutrients (Fe, Mn, Zn, Cu, B, Mo, Cl, and Ni) with a concentration in the µM range are necessary for optimum growth and development (Epstein and Bloom 2005). Moreover, Si is considered essential for grasses and Na for obligate halophytes (Epstein 2009; Subbarao et al. 2003).

Carbon and oxygen are mainly taken up in gaseous from the atmosphere, and hydrogen is provided by water. All other nutrient elements are usually taken up from the soil solution. Both deficient and excess supply can severely damage plants fitness by impairing vital functions such as cell wall and membrane stability, water relations, photosynthesis, respiration, alteration of secondary metabolism, and phytohormone balance, among others. Thus, adequate concentrations are not only required for general plant fitness, but also for specific defence mechanisms against biotic stress (Walters and Bingham 2007).

Microorganisms and plants share most of the essential nutrients and there is a hard competition amongst these organisms for achieving sufficient amounts. This is of special importance in plant pathogen

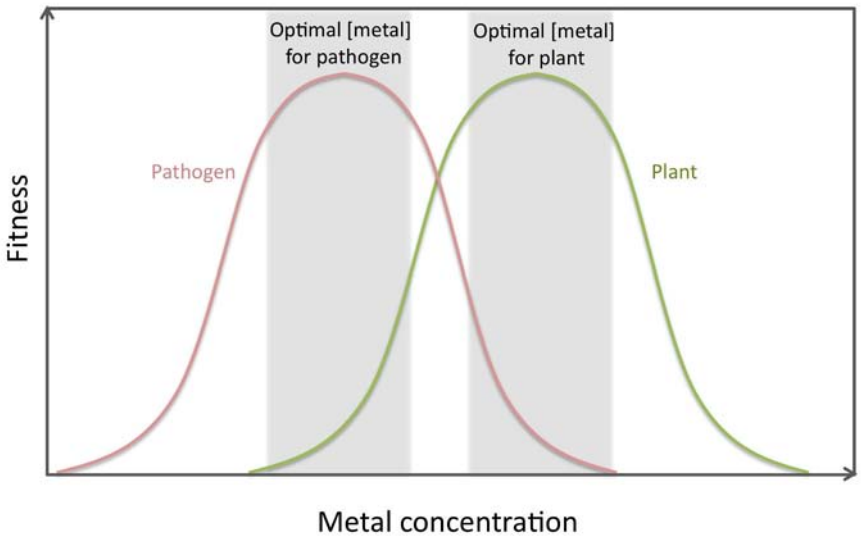


Figure 1.4: Model of a plant-pathogen interaction where optimal metal concentration for the plant fitness lays within the range of toxicity for the pathogen, thus leading to plant protection by elemental defence in case the optimal metal concentration for the plant is reached.

interactions were pathogenic fungi or bacteria obtain the essential mineral nutrients directly from the host plant. Response curves representing performance as a function of the concentrations of an essential nutrient can differ between host plants and the pathogen with optimal response in the plant occurring either at higher or at lower concentrations than in the pathogen. The first situation may lead to the so-called elemental defence (see below) (Figure 1.4). Moreover, plants not only take up essential elements from the soil. Non-essential, potentially toxic elements can inadvertently been taken up by transport mechanisms with low ion specificity. Under these conditions a higher susceptibility in the pathogen than in the host can also lead to protection against infection by elemental defence (Poschenrieder et al. 2006).

1.3.2 *Metal defence*

Several hypotheses have been forecast to explain the evolutionary advantages of metal hyperaccumulation. Among those, the hypothesis of the metal defence or, more generally, of the elemental defence is one of the most attractive and is receiving most support (Boyd and Martens 1998) . According to this, hyperaccumulation helps the plant to better defend itself against herbivores, pathogenic microorganisms, and even competing plant species. Although other scenarios are possible, such as amelioration of abiotic stress factors due to elemental

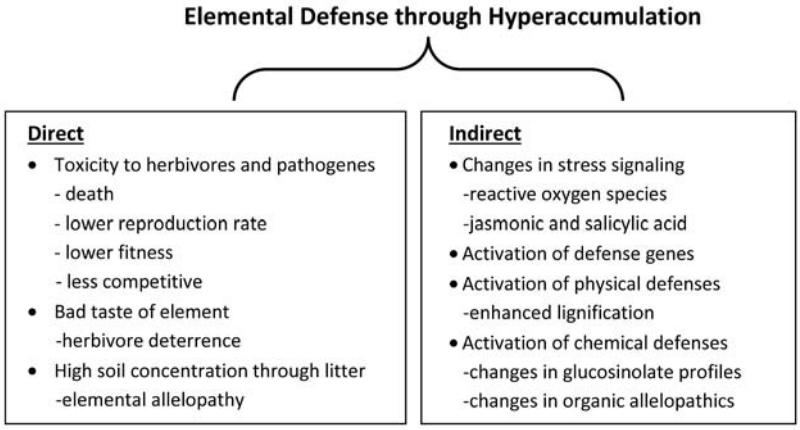


Figure 1.5: Direct and indirect mechanisms of elemental defence (Poschenrieder et al. 2014)

hyperaccumulation. Currently the mechanisms behind the protective effects against biotic stress are under intense investigation.

Different modes of action can lead to defence by elemental accumulation (Poschenrieder et al. 2006). Direct and indirect mechanisms can be distinguished (Figure 1.5, Poschenrieder et al. 2014). The accumulated elements can be directly toxic to the herbivore, pathogen, or competing neighbour plant. Indirect mechanisms include either or both elemental-induced activation of specific defence genes (i.e. the trace elements are acting as elicitors of defence signalling pathways) and alterations in the metabolism producing organic substances that can deter leaf-consuming herbivores, hamper tissue spreading of microorganisms, or act as allopathic substances delivered from decaying leaves (Morris et al. 2008).

The investigation of indirect mechanisms of the elemental defence has stimulated further research into the close interactions between mineral nutrition and plants' tolerance to biotic stress. In fact, both the observation that trace element levels below the hyper-accumulator level can get toxic to generalist herbivores (Coleman et al. 2005) and the relevance of essential trace elements such as Fe, Zn, Cu, or B in several defence reactions, including reactive oxygen species, (Fe and Cu), protein synthesis (Zn), and cell wall stability (B) has extended the elemental-defence research even to species with concentrations below the hyperaccumulation (Cheruiyot et al. 2013). The possibility of accumulating relatively low metal concentrations that are toxic for herbivores and pathogens has been regarded as a hypothetical model on how hyperaccumulation trait started being selected. This is the defensive enhancement hypothesis (Boyd 2012). Moreover, it is getting more an more clear that multiple defence mechanisms can cooperate to preserve plant fitness under biotic stress attack (Poschenrieder et al.

2011); this has been summarized by Boyd (2012) in the so-called joint effects hypothesis.

As an example, multiple factors are conditioning herbivorism by the common garden snail, *Helix aspersa* on *Noccaea* species. Snails do not seem to have a taste for Zn and glucosinolates largely determine leaf consumption in *N. caerulescens* under Zn-hyperaccumulation (Noret et al. 2005). Moreover, hyper-accumulation of either Zn or Cd alters the glucosinolate profile in *N. praecox* (Tolrà et al. 2006; Pongrac et al. 2007). Leaf Cd hyper-accumulation had a clear inhibitory effect on leaf consumption by snails in *N. praecox*, while consumption was stimulated by total leaf sugar concentration (Llugany et al., unpublished). Likewise, multiple factors determine the interactions between pathogenic fungi and metal hyper-accumulation. Under low metal supply hyper-accumulating species use to be highly sensitive to fungal infection. Cadmium protected *N. praecox* against infection by the biotrophic *Erysiphe* by direct toxicity and/or fungal induced jasmonate signalling (Llugany et al. 2013). In the case of foliar pathogens, as the biotrophic bacteria *Pseudomonas syringae*, it was demonstrated that Zn concentrations in the apoplast of Zn-treated *N. caerulescens* are sufficient to limit bacterial growth (Fones et al. 2010). Moreover, the deposition of callose and the expression of PR genes, but not SA synthesis, were unaffected upon pathogen infection and ROS was induced by Zn, but not by *Pseudomonas* (Fones and Preston 2013). This suggested that metal hyperaccumulation may have evolved by saving metabolic costs associated with defence response: this is a tradeoff between inorganic and organic-based defences. Nontheless, in the same study, the *Pseudomonas* type III secretion system (T3SS), typally activated to disable plant defences, conditioned the ability of the bacteria to grow in low Zn-treated plants, so some other plant defences may be playing a role.

Currently, there is a considerable number of research results supporting the elemental defence hypothesis through either of both direct and indirect mechanisms. Nonetheless, there are also negative results. Moreover, it has to be taken into account that metal-rich soils are strong selection factors for the evolution of metal tolerance not only in plants, but also for high metal tolerance in the pathogens and herbivores living there together with the host plant. The interaction of the above mentioned metabolic factors and their influence in the selection of the hyperaccumulation trait have been extensively reviewed by Hörger et al. (2013).

1.3.3 *Induction of plant defences*

Pathogens present conserved motifs termed pathogen or microbial-associated molecular patterns (PAMPs or MAMPs). These motifs can be recognized by high affinity receptors located in plant membrane

cells, the pattern recognition receptors (PRRs) (Boller and He 2009). The interaction between the PAMPs and the PRRs in the host triggers a first immune response at the site of infection, the PAMP-triggered immunity (PTI). As a consequence, the plant releases resistance proteins (RPs) to stop the infection. At the same time, the pathogen secretes effectors to suppress the PTI and thus it may give way to the activation of a second host immune response: the effector-triggered immunity (ETI). Still at the site of infection, this response bursts the formation of reactive oxygen species (ROS) and activates the hormonal signalling network, typically starting with salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Fujita et al. 2006; Torres et al. 2006; Koornneef and Pieterse 2008). This hormonal signalling network will be further discussed. As a consequence of ROS and SA formation a third response may take place: the hypersensitive response (HR) (Zaninotto et al. 2006). This response consists of the induction of host cell death and prevents the pathogen from spreading into the tissue. The SA also induces the synthesis of pathogen related proteins (PR proteins) in the plant and is an important player in the systemic acquired resistance (SAR) (Spoel and Dong 2012). A general scheme of plant defence response induction is shown in Figure 1.6.

1.3.4 *Key role of hormones in plant defence*

Phytohormones can be divided into typically developmental hormones such as auxins (IAA), gibberelins (GAs), cytokinins (CKs), brassinoesteroids (BRs) and abscisic acid (ABA) and typically defence response hormones like Et, JA and SA. Traditionally, the hormones SA, JA and Et have been linked to the response against biotic stress. Furthermore, the implication in plant defence of developmental hormones such as IAA, ABA, GAs, CKs, BRs, and peptide hormones has been reported (Figure 1.7), adding complexity to the network regulating the response to pathogens (Bari and Jones 2008).

Hormonal responses and their interactions in plants under biotic stress largely depend on the nature of the pathogen. It is not possible to assign to one hormone a general positive or negative influence on plant defence responses, as this varies depending on the pathogen. Recent review on the topic include the articles from Pieterse et al. (2009) and Verhage et al. (2010). A short outline of main response characteristics of specific hormones is given as follows:

1.3.4.1 *Salicylic acid*

SA is involved in defence responses against biotrophic and hemibiotrophic pathogens and in the development of SAR. SA plays an important role in both local and systemic defence responses and can induce the expression of pathogenesis related proteins (PRs) (Gao et al. 2015).

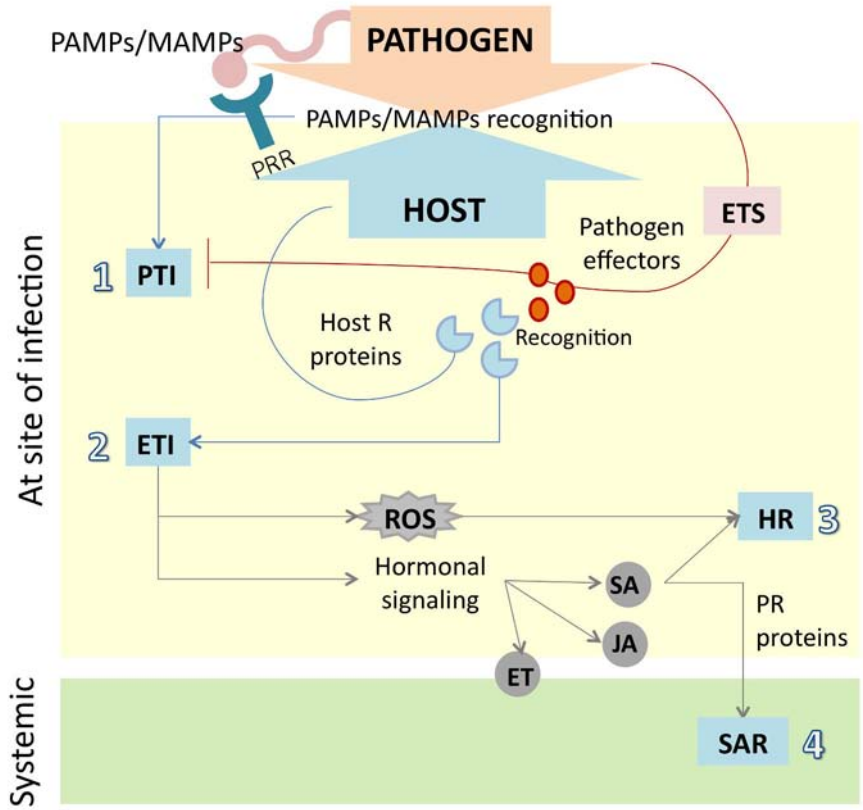


Figure 1.6: General scheme of plant immune response to pathogens showing pathogen recognition and signalling in an incompatible pathogen-host interaction. PAMPs: Pathogen-associated molecular patterns, PRR: Pattern recognition receptor; PTI: PAMP-triggered immunity, R proteins: Resistance proteins, ETS: Effector triggered susceptibility, ETI: Effector triggered immu¬nity, ROS: Reactive oxygen species, HR: Hypersensitive response, SA: Salicylic acid, JA: Jasmonic acid, ET: Ethylene, PR proteins: Pathogen related proteins, SAR: Systemic acquired response.

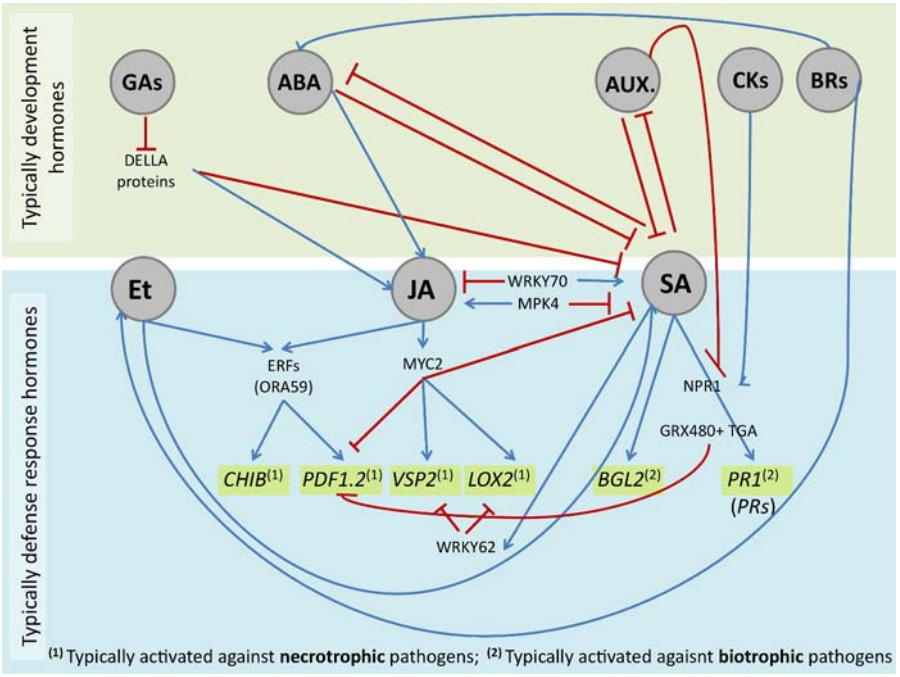


Figure 1.7: Hormone cross-talk in plant responses to biotic stress. GAs: gibberellins. ABA: Abscisic acid. AUX.:Auxins. CKs: citoquinins. BRs: brassinosteroids. Et: Ethylene. JA: Jasmonic acid. SA: Salicylic acid. ERFs: Ethylene response factors. ORA59: ethylene-responsive factor octadecanoid-responsive *Arabidopsis* AP2/ERF 59. *CHIB*: basic endochitinase (gene). *PDF*1.2: Plant defensin 1.2 (gene). *VSP*2: vacuolar storage protein 2 (gene). *LOX*2: lypoxigenase 2 (gene). NPR1: nonexpresser of PR genes 1.*PRs*: pathogen related (genes). *PR*1: pathogen related 1 (gene) 1. *BGL*2: β-1,3-glucanase 2 (gene). MYC2: transcription factor, jasmonate insensitive 1. WRKY70: WRKY DNA-binding protein 70. MPK4: mitogen-activated protein kinase 4. WRKY62: WRKY DNA-binding protein 62. GRX480: glutaredoxin-GRX480. TGA: Transcription factor.

1.3.4.2 *Jasmonic acid and ethylene*

JA/Et are known to be active against necrotrophic pathogens and herbivorous insects. It has been proposed that jasmonates could act as a mobile signal in *Arabidopsis* pathogen immunity (Truman et al. 2007). However, recently, it has been reported in *Arabidopsis* that JA is not the mobile signal in SAR, as the fatty acid desaturase-dependent SAR inducing activity was found not to be dependent on JA (Chaturvedi et al. 2008). JA and Et response integration has been reported to be controlled by ORA59, a transcription factor from the AP2/ERF transcription factors family (Pré et al. 2008).

SA, JA, and Et are mutually antagonistic, but some synergistic interactions have also been reported. Although, the signalling network is specific for every pathogen, plants need to be ready for different types of pathogens and thus the response network is composed of different pathways. The *Arabidopsis* WRKY70 has been found to regulate the antagonistic interaction between SA-and JA-mediated defences (Bari and Jones 2008). Overexpression of WRKY70 resulted in constitutive expression of SA-responsive *PR* genes and enhanced resistance to the biotrophic pathogen *Erysiphe cichoracearum*, but repressed the expression of JA responsive marker gene PDF1.2 and compromised resistance to the necrotrophic pathogen *Alternaria brassicicola* (Li et al. 2004, 2006). In contrast, suppression of WRKY70 expression caused an increase in *PDF*1.2 transcript levels and enhanced resistance to *A. brassicicola* (Li et al. 2006). These results suggest that WRKY70 acts as a positive regulator of SA-dependent defences and a negative regulator of JA-dependent defences and plays a pivotal role in determining the balance between these two pathways. WRKY62 has been reported to be synergistically induced by MeJA and SA. In addition, the analysis of loss and gain of function mutants in *Arabidopsis* plants revealed that WRKY62 downregulates JA-responsive *LOX*2 and *VSP*2 genes. These results suggest potential involvement of WRKY62 in the SA-mediated suppression of JA-responsive defence in *Arabidopsis* (Mao et al. 2011). Mitogen activated protein kinase 4 (MPK4) has been identified as another key component involved in the antagonism between SA-and JA-mediated signalling in *Arabidopsis*. The *Arabidopsis mpk*4 mutants show elevated SA levels, constitutive expression of SA responsive *PR* genes and increased resistance to Pst. In contrast, the expression of JA responsive genes and the resistance to *A. brassicicola* were found to be impaired in *mpk*4 mutants (Petersen et al. 2000; Brodersen et al. 2006). These results indicate that MPK4 acts as a negative regulator of SA signalling and positive regulator of JA signalling in *Arabidopsis*. Another important regulator that affects an¬tagonism between SA and JA-mediated signalling is a glutaredoxin, GRX480. Glutaredoxins are disulfide reductases, which catalyze thiol disulfide reductions and participate in the redox regulation of protein activities involved in a variety of cellular processes

(Meyer 2008). GRX480 has been shown to interact with TGA transcription factors involved in the regulation of SA responsive PR genes (Ndamukong et al. 2007). The expression of GRX480 is induced by SA and requires TGA transcription factors and NPR1. Furthermore, the expression of JA responsive *PDF*1.2 gene was inhibited by GRX480 (Ndamukong et al. 2007). These findings suggest that SA-induced NPR1 activates GRX480, which forms a complex with TGA factors and suppresses the expression of JA-responsive genes. Transcription factor WRKY53 represents an additional component involved in mediating the cross-talk between SA and JA signalling (Miao and Zentgraf 2007). WRKY53 has been shown to interact with the JA-inducible protein epithiospecifying senescence regulator (ESR). More importantly, the expression of these genes is antagonistically regulated in response to JA and SA suggesting that WRKY53 and ESR mediate negative cross-talk between pathogen resistance and senescence in *Arabidopsis* (Miao and Zentgraf 2007). The JA-responsive transcription factor JIN1/MYC2 acts as a negative regulator of SA signalling during Pst DC3000 infection in *Arabidopsis*. The *jin*1 mutant plants showed increased accumulation of SA, enhanced expression of *PR* genes and increased resistance to *Ps*t DC3000 compared to wild plants (Laurie-Berry et al. 2006). Moreover, the roles of SA, JA and Et are related to other phytohormones.

1.3.4.3 *Auxins*

IAAs are important players in these signalling networks. Many pathogens can produce IAAs or interfere with the host's IAA biosynthesis and so alter plant developmental processes. Plants, in turn, have evolved mechanisms to inhibit IAA signalling (Wang et al. 2007). Expansins promote the loosening of the cell wall favouring plant growth, but also growth of the pathogen. GH3-8, a gene encoding an indole-3-acetic acid–amido synthetase, that maintains IAA homeostasis by conjugating excess IAA to amino acids, suppresses expansin and mediates resistance to *Xanthomonas oryzae* pv. *oryzae* in rice by SA and JA independent pathways (Ding et al. 2008). Moreover, SA treatment did not change the level of free IAA, rather it caused a general repression of IAA–related genes, including the TIR1 receptor, and stabilized Aux/IAA repressor proteins; as a consequence IAA signalling was inhibited (Figure 1.8). This suppression of IAA signalling is considered to play an important role in the mechanisms of SA-mediated resistance to pathogens (Wang et al. 2007).

1.3.4.4 *Abscisic acid*

The ABA-deficient tomato mutant sitiens showed more resistance to *B. cinerea* (Audenaert et al. 2002), *Pseudomonas syringae* pv. tomato (Thaler et al. 2004), *Oidium neolycopersici* (Achuo et al. 2006) and

Erwinia chrysanthemi (Asselbergh et al. 2008) than wild plants. Similarly, the ABA-deficient *Arabidopsis* mutant *aba*2 − 1 showed more resistance to *Fusarium oxysporum* (Anderson et al. 2004) and the *aba*1 − 1 mutant showed less susceptibility to *Hyaloperonospora arabidopsidis* (Mohr and Cahill 2003) compared to wild plants. Exogenous application of ABA enhanced the susceptibility of various plant species to bacterial and fungal pathogens. For example, application of ABA increased the susceptibility of *Arabidopsis* to *Pst* (de Torres-Zabala et al. 2007), soybean plants to *Phytophthora sojae* (Mohr and Cahill 2003) and rice plants to *Magnaporthe grisea* (Koga et al. 2004). Recently, Yasuda et al. (2008) reported that ABA treatment suppressed SAR induction, indicating that there is an antagonistic interaction between SAR and ABA signalling in *Arabidopsis*. The role of ABA as a positive regulator of defence has also been reported (Mauch-Mani and Mauch 2005). ABA activates stomatal closure that acts as a barrier against bacterial infection (Melotto et al. 2006). As a result, ABA deficient mutants showed more susceptibility to *Pseudomonas syringae* ptv. tomato. In other studies it was documented that treatment with ABA protected plants against *A. brassicicola* and *P. cucumerina*, indicating that ABA acts as a positive signal for defence against some necrotrophs (Ton and Mauch-Mani 2004). In contrast, ABA deficient mutants are more sensitive to infection by the fungal pathogens *A. brassicicola*, *Pythium irregulare* (Adie et al. 2007) and *Leptosphaeria maculans* (Kaliff et al. 2007). Nonetheless, ABA is not a positive regulator of plant defence against all necrotrophs and its role depends on individual plant pathogen interactions. As a general effect, ABA has been shown to induce resistance partly through priming the deposition of callose (Flors et al. 2008). Hernández-Blanco et al. (2007) provided evidence for a direct involvement of ABA signalling in the control of *Arabidopsis* resistance to *R. solanacearum*. Consistently, *Arabidopsis* mutants in cellulose synthase genes required for secondary cell-wall formation showed increased induction of ABA-responsive defence-related genes. This suggests that ABA could exert its effect on plant defence by modulating cell wall metabolism in *Arabidopsis*. Recently, it has been shown that ABA induced the expression of a catalase (*CAT*1), a scavenger of H_2O_2, and at the same time activated H_2O_2 production (Xing et al. 2008). Taking together, accumulating evidence suggests that ABA regulates defence responses through its effects on callose deposition, production of reactive oxygen intermediates and regulation of defence gene expression.

1.3.4.5 *Gibberelins*

The *Arabidopsis* quadruple-della mutant that lacks four DELLA genes (*gai-t6*, *rga-t2*, *rgl1-1*, *rgl2-1*) is very susceptible to the fungal necrotrophic pathogens *A. brassicicola* and *B.cinerea*. In *Arabidopsis*, DELLA proteins promote resistance to necrotrophs by activating the

JA/ET-dependent defence responses, but susceptibility to biotrophs by repressing the SA-dependent defence responses (Navarro et al. 2008).

1.3.4.6 *Cytokinins*

Several plant growth-promoting bacteria have been found to produce CKs and the beneficial effects of these microbes have been attributed to CKs production and signalling. Nonetheless, results of CKs effects on pathogenic interactions are less clear. Transgenic plants overexpressing CK oxidase/dehydrogenase genes showed resistance against *P. brassicae* infection, suggesting that CKs act as key factors in the development of clubroot disease in *Arabidopsis* (Siemens et al. 2006). In this species, CKs have also been found to upregulate phytoalexin biosynthesis and to enhance resistance against *P. syringae* in a SA-independent pathway (Grosskinsky et al. 2011).

1.3.4.7 *Brassinosteroids*

It has been reported that BRs enhances resistance to TMV, *Pst* and *Oidium* sp. in tobacco. Similarly, BRs were shown to increase the resistance of rice plants against *Magnaporthe grisea* and *Xanthomonas oryzae* infection (Nakashita et al. 2003). The BRs-mediated resistance is independent of SA-mediated defence signalling pathways. BRs sprayed potato plants showed resistance to infection by *Phytophthora infestans* and this resistance was found to be associated with increases in the levels of ABA and Et (Krishna 2003). BAK1 is known to interact with the BR receptor, BRI1, and mediate BR signal transduction in plants (Li et al. 2002; Nam and Li 2002). BAK1 (also known as SERK3, somatic embryogenesis-related kinase 3) is up regulated in response to PAMPs (such as flg22 and elf18) and *Arabidopsis* mutant *bak*1 plants are compromised in PAMP responses, as evidenced by loss of ROS burst and growth inhibition in response to flg22 (Chinchilla et al. 2007; Heese et al. 2007). Interestingly, *bak*1 mutants developed spreading necrosis upon pathogen infection. Furthermore, *bak*1 mutants showed enhanced susceptibility to necrotrophic pathogens such as *A. brassicicola* and *B. cinerea*, whereas resistance to biotrophic pathogen *H. parasitica* was enhanced in the mutant compared to wild type plants (Kemmerling et al. 2007). BAK1 has been found to interact with the flagellin receptor, FLS2, in a ligand-dependent manner (Chinchilla et al. 2007; Heese et al. 2007). These data suggest a model where binding of flagellin to FLS2 promotes the formation of an active complex with BAK1, which results in the activation of downstream signalling components. The function of BAK1 in plant defence is BR-independent suggesting that BAK1 has dual role in the regulation of plant defence and development.

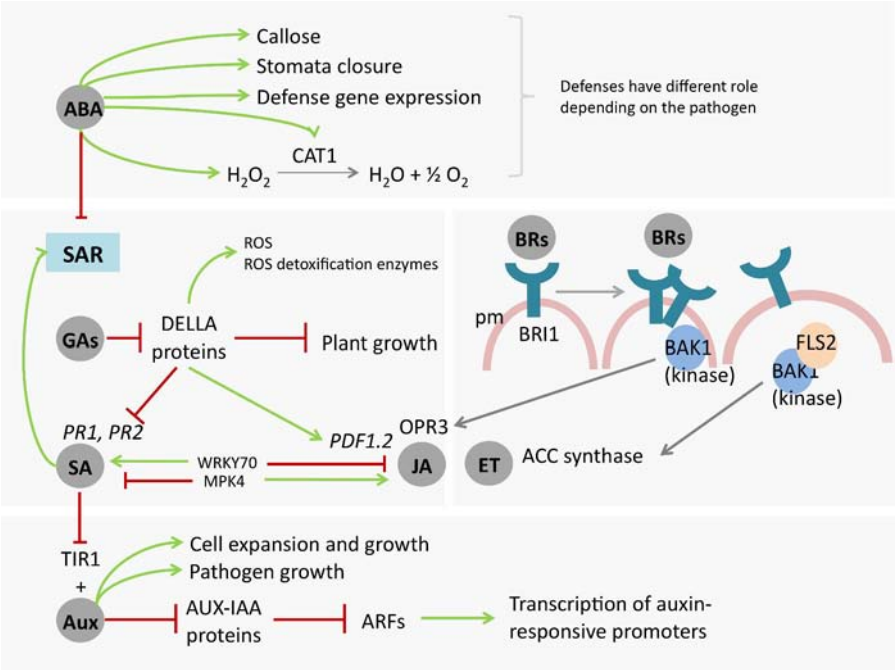


Figure 1.8: Some of the roles of phytohormones in biotic stress signalling in plants and some interaction amongst them. ABA: absciscic acid. CAT1: catalase. SAR: systemic acquired resistance. GAs: gibberelins. ROS: reactive oxygen species. *PR*1, *PR*2: pathogenesis releted genes. SA: salicylic acid. WRKY70: WEKY DNA-binding protein 70. MPK4: mythogen activated kinase 4. *PDF*1.2: pathogen defence factor gene. pm: plasmatic membrane. BRs: brassinolides. BRI1: brassinosteroid-insensitive 1 receptor. FLS2: flagellin-sensitive receptor. BAK1: BRI1-associated receptor kinase. OPR3: 12-oxophyto-dienoate reductase. ACC synthase: 1-aminopropane-1-carboxylic acid synthase. JA: jasmonic acid. Et:ethylene Aux: auxin. IAA: idol acetic acid. TIR1: Transport inhibitor response 1. ARFs: auxin responsive factors.

1.3.4.8 *Peptide hormones*

These peptides are from 18 to 23 amino acids in length, are processed from wound-and JA-inducible precursor proteins, and play roles in the activation of local and systemic responses against wounding and pest attack (Matsubayashi and Sakagami 2006).

A general scheme of main roles of phytohormones hormones in biotic stress signalling in plants is presented in Figure 1.8.

1.3.5 *Glucosinolates and plant defence*

Glucosinolates (GSs) are a group of more that 120 forms of beta-thioglucoside N-hydroxysulfates that are secondary metabolites in certain plant families, mainly in *Brassicaceae*, and that have been accounted for their role in plant defence against pests and herbivores.

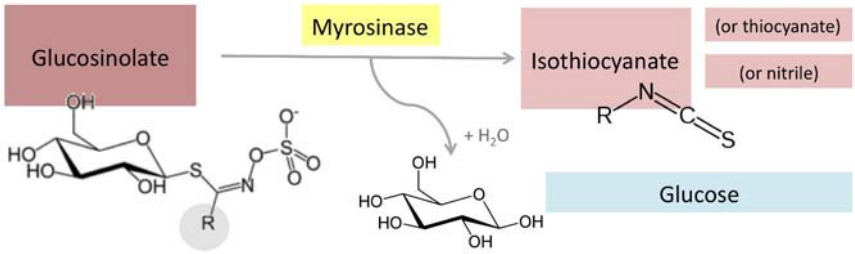


Figure 1.9: Glucosinolate degradation. Glucosinolates are degraded by the action of the enzime myrosinase into glucose and the active compound isothicyanate or thiocyanate or nitrile. The -R in the glucosinolate formulation is a radical that varies depending on the nature of the glucosinolate.

The active form of GSs, mainly isothiocyanate, is formed as a consequence of an enzymatic reaction driven by the enzyme Myriosinase, in which glucose is hydrolyzed and released (Figure 1.9). Myriosinase is stored in the cell separately from GSs to prevent isothiocyanate formation, although it can be released upon cell break caused, for example, by the attack of an herbivore or a necrotrophic fungus such as *A. brassicicola*. More detailed information about the glucosinolates biochemistry and functions is accessible in the reviews from Fahey et al. (2001), Halkier and Gershenzon (2006) and Sønderby et al. (2010).

1.4 CASE STUDIES

In the present study, *Arabidopsis thaliana* and *Noccaea caerulescens* were chosen as experimental plant species due its well established contrasting response to high Zn concentrations, while *Alternaria brassicicola* and *Botrytis cinerea* were selected as model necrotrophic fungi as they have a broad host spectrum in contrast to some other necrotrophs like *Alternaria alternate* or *Cochliobolus carbonum* that produce host- specific toxins (Lawrence et al. 2011; Mengiste 2012).

1.4.1 Arabidopsis thaliana *and* Noccaea caerulescens

Much progress in current knowledge on the role of hormones in biotic stress signalling in plants is derived from studies using the model plant *A. thaliana*. In this species, large array of hormone defective mutants are available. Some relevant examples are as follows:

1.4.1.1 npr1

One of the important regulatory components of SA signalling is the non-expressor of PR gene 1 (NPR1), which has been demonstrated to directly bind SA and so act as a receptor for this hormone. Moreover, NPR1 interacts with TGA transcription factors that are participate in

the activation of the SA-responsive *PR* genes, which are involved in plant defence, specially against biotrophic pathogens (Dong 2004; Wu et al. 2012).

1.4.1.2 pad1

The phytoalexin-deficient mutant has a defect in the metabolic pathway for camalexin synthesis. PAD1, together with PAD4, regulates the expression of PAD3, which is ultimaltely involved in the synthesis of camalexin (Zhou et al. 1999). Camalexin is the characteristic phytoalexin of *A. thaliana,* typically induced by a variety of pathogenic microorganisms and involved in plant immune response. In *A. thaliana* inoculated with *Alternaria brassicicola, pad1* mutants have been found to accumulate as much camalexin as WT plants and to be as susceptible as WT to infection, but showing an increased induction of the *PR1* gene, related to SA signalling, whereas *PDF1.2* expression, activated by the JA/Et pathways, being considerably reduced (Thomma et al. 1999; Wees et al. 2003; Glawischnig 2007; Ahuja et al. 2012). Glazebrook et al. (2003) also found a inability of *pad1* mutants treated with JA to induce the expression of the gene that encodes for the plant defensine *PDF1.2*, related to the JA pathway.

1.4.1.3 coi1

The coronatine insensitive 1 encodes a F-box protein involved in the SCF-mediated protein degradation by the 26S proteasome and is required for most JA-mediated responses (Xie et al. 1998). It has been reported that the COI1 or COI1-JAZ complex acts as a receptor for JA-Ile in *Arabidopsis* (Katsir et al. 2008).

1.4.1.4 etr1

The ethylene receptor 1 is involved in ethylene mediated responses to biotic stress. The *etr1* mutants lacking ethylene receptor are highly sensitive not only to necrotrophic fungi but even to non-pathogenic opportunistic soil fungi (van Loon et al. 2006).

Figure 1.10 summarizes the stress signalling pathways affected by mutations in NPR1, PAD1, COI1 and ETR1, as well as their resulting effects.

Amongst the more than 500 metal hyperaccumulating plants known to date, *Noccaea caerulescens*, a Zn and Cd hyperaccumulator is one of the most studied. A major reason is that as a Brassicaceae species, *Noccaea caerulescens* is closely related to *Arabidopsis thaliana* and many molecular tools developed for *A. thaliana* have been used directly or after few modifications for *N. caeruslescens* (Assunção et al. 2003; Rigola et al. 2006).

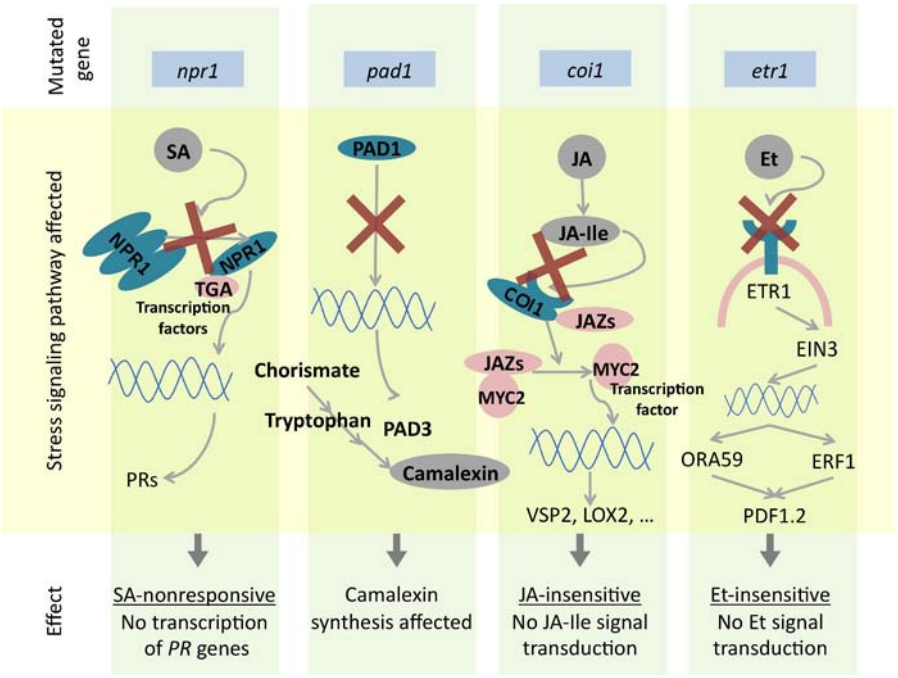


Figure 1.10: Alteration of signalling pathways and the corresponding effects in defence gene mutants. *npr*1/NPR1: non-expressor of *PR* genes. *pad*1/PAD1: phytoalexin-deficient 1. *coi*1/COI1: coronatine insensitive 1. *etr*1/ETR1: ethylene receptor 1: SA: salicylic acid. PRs: pathogenesis related (proteins). PAD3: phytpaexin-deficient 3 (protein). JA: jasmonic acid. JA-Ile: jasmonic acid conjugated with isoleucine. JAZs: jasmonate-zim domain (proteins). MYC2: Transcirption factor. VSP2: vacuolar storage protein 2. LOX2: lypoxigenase 2. Et: Ethylene. EIN3: Ethylene-insensitive 3. ORA59: ethylene-responsive factor octadecanoid-responsive *Arabidopsis* AP2/ERF 59. ERF1: ethylene response factor 1. PDF1.2: Plant defensin 1.2

1.4.2 Botrytis cinerea *and* Alternaria brassicicola

Currently, up to 30 species are recognized within the genus *Botrytis*. Some *Botrytis* species have a restricted host range, while others as *Botrytis cinerea* attack a wide variety of crop plants, causing grey mold in flowers, fruits, vegetables and bulbs, especially under cool humid environmental conditions. *Botrytis* also can cause leaf spot symptoms or dumping-off of seedlings (Agrios 2005). Its capacity to grow fast on different sources of nutrients and to attack many different types of plants makes *Botrytis* an ideal model species for studying plant-fungal interactions.

Botrytis cinerea infection is favoured by several virulence factors. The fungus penetrates into plant tissues by enzymatic breakdown of cuticles (cutinase, lipase) and cell walls (endopolygalacturonase). Host cells are killed by secretion of toxins. Several of these virulence factors are enhanced by the fungal production of oxalic acid. Reactive oxygen species also produced by the fungus further contribute to plant cell death (Rolke et al. 2004; Choquer et al. 2007). Besides the ROS of fungal origin, plants also can produce ROS in response to fungal attack contributing to the induction of resistance mechanisms.

Recently, it has been shown that in wild native and non-native plants *Botrytis* can present an endophytic lifestyle and that *Botrytis* – plant interactions can be more complex than previously thought, including the possibility of a switch between lifestyles from endophytic to necrotrophic under certain environmental conditions (van Kan et al. 2014). These findings open new perspectives for studying recognition and signalling events in pathosystems including *Botrytis* sp.

Many *Alternaria* species have saprophytic lifestyle. However, *A. brassicicola* can cause black spot disease in virtually all Brassicacean crops such as cabbage, canola, or mustard and *Alternaria* infection can severely affect economic yield. The virulence factors in *A. brassicicola* are still not clearly established. Inhibition of toxin production or of enzymes that degrade cell wall components such as pectins and cellulose has little influence on the fungal virulence. Nonetheless, the orchestrated action of cutinase, lipase, and cell wall degrading enzymes regulated by transcription factors seem to be important for pathogenesis of the fungus. The ability to degrade brassinin, a major resistant factor in the host plants makes a large contribution to the virulence of *A. brassicicola* (Srivastava et al. 2013).

Signalling of the plant defence responses differs not only between necrotrophic and biotrophic fungi, but also among the necrotrophs according to the specific or unspecific nature of their toxins. While cell death due to hypersensitive response is part of the plant immune response against biotrophic fungi, usually leading to non-infection and incompatible host-fungal interactions, cell death caused by necrotro-

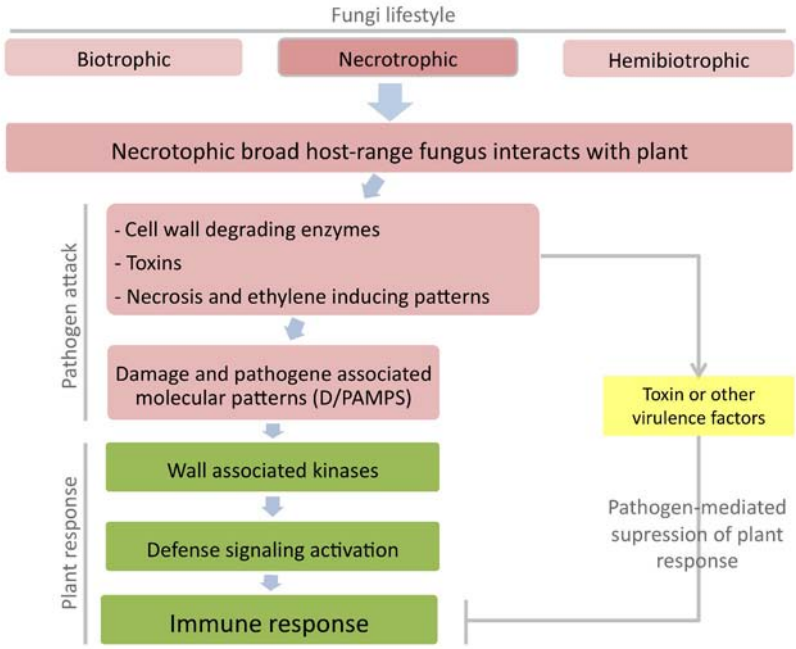


Figure 1.11: Major D/PAMS, virulence factors and immune responses in broad host- range necrotrophic fungi.

phic fungi is symptom of successful infection and fungal virulence in a sensitive host (Agrios 2005).

Figure 1.11 summarizes the main signalling events in necrotrophs producing unspecific toxins (modified from Mengiste 2012). Broad host-range necrotrophs produce cell wall degrading enzymes, toxins and necrosis and ethylene inducing proteins that in turn cause the production of damage and pathogen associated molecular patterns (PAMPS) that, through wall associated kinases, trigger defence signalling pathways and immune responses. However, toxins or other virulence factors can also lead to the inactivation of the plants defence mechanisms yielding high infection scores.

PAMPs motifs can be recognized by high affinity receptors located in plant membrane cells, the pattern recognition receptors (PRRs). The interaction between the PAMPs and the PRRs in the host triggers a first immune response at the site of infection: the PAMP-triggered immunity (PTI). As a consequence, the plant releases resistance proteins (RPs) to stop the infection. At the same time, the pathogen secretes effectors to suppress the PTI and thus it may give way to the activation of a second host immune response: the effector-triggered immunity (ETI). Still at the site of infection, this response burst the formation of ROS and activates the hormonal signalling network. In plants attacked by necrotrophic fungi the JA and Et signalling pathways are typically triggered, while in interactions with biotrophic and hemibiotrophic fungi the salicylic acid pathway is activated.

Finally, a general scheme considering all the above mentioned and how the plant, the pathogens and the Zn interact in relation with the metal defence and the related hypothesis is presented (Figure 1.12).

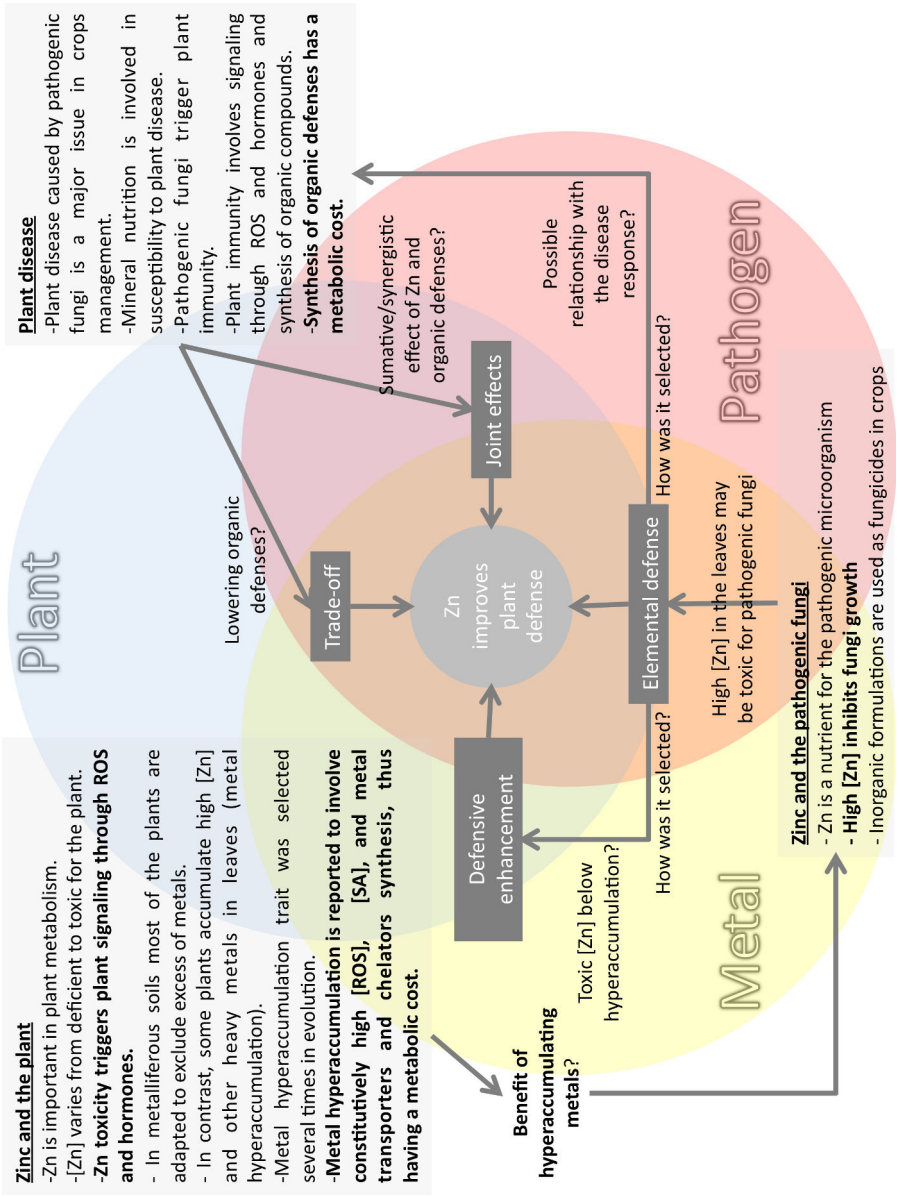


Figure 1.12: Key points in the global picture regarding the interaction of Zn in plant defence.

Part II

OBJECTIVES

2

OBJECTIVES

The main objectives of this thesis were to test the metal defence hypothesis in two model plants: the metal hyperaccumulating species *Noccaea caerulescens* and the non-hyperaccumnualting species *Arabidopsis thaliana*. Both direct defence through the accumulation of foliar concentrations beyond the toxicity threshold for the pathogen and indirect mechanisms by metal-induced enhancement of defence signalling pathways were considered. For this purpose three pathosystems with necrotropic foliar pathogens were established under laboratory condition. Moreover, natural occurring populations of the genus *Noccaea* were located in the Pyrenees, identified and their Zn and Cd accumulation ability characterized with a view to future field experiments. In this thesis, Zn was the linking factor that interconnected studies in plant disease and in metal (hyper)accumulation (Figure 2.1). More specifically, the three main objectives were:

1. Evaluation of the influence of Zn supplied to the metal hyperaccumulator *Noccaea caerulescens* growing in hydroponic solution over its response against the disease caused by the foliar necrotroph fungus *Alternaria brassicola.*

2. Analysis of the influence of Zn supplied to *Arabidopsis thaliana* growing in hydroponic solution over its response against the disease caused by the foliar necrotroph fungi *Alternaria brassicola* and *Botrytis cinerea.*

3. Localization, identification and characterization in relation with metal accumulation of plant populations from the *Noccaea* genus, a posteriori classified as *Noccaea brachypetala*, in the Eastern Iberian Peninsula.

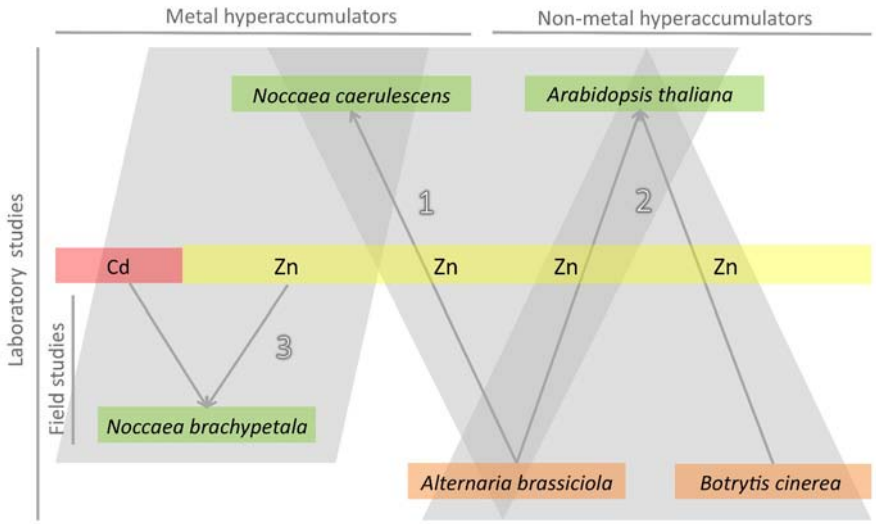


Figure 2.1: Interconnection amongst the plant species *N. caerulescens*, *A. thaliana* and *N. brachypetala* and the factors that affected their physiology in the studies comprised in this thesis. The scientific questions were organized in three general objectives, assessing: 1: Influence of Zn over the metal hyperaccumulating plant *N. caerulescens* response to *A. brassicicola* infection; 2: Influence of Zn over the non-metal hypearaccumulating plant *A. thaliana* and over its response to *A. brassicicola* or *B. cinerea* infection; 3: Localization, identification and characterization in relation with tolerance to Cd and Zn of plant populations classified *a posteriori* as *N. brachypetala* in the Eastern Iberian Peninsula.

Part III

EXPERIMENTAL

3

THE ROLE OF ZN IN THE NOCCAEA CAERULESCENS - ALTERNARIA BRASSICICOLA PATHOSYSTEM

This study focuses in the role of Zn in plant disease in the frame of the metal defence hypothesis in hyperaccumulator plants (Poschenrieder et al. 2006; Boyd 2012). For that purpose, the pathosystem *Noccaea caerulescens* – *Alternaria brassicicola* was chosen. *Noccaea caerulescens* has been largely studied since it was first described as a hyperaccumulator (Vázquez et al. 1992), and has been selected as a model plant for genetic studies in the Brassicaceae family (Peer et al. 2006). Since evidence for the metal defence hypothesis raised, it has been subjected to numerous studies regarding disease and herbivore resistance. *Alternaria brassicicola* is a necrotrophic pathogen that infects a large number of *Brassicaceae* and it has been largely used as a model organism for necrotrophic fungi. The species of study are more widely described in the introduction (1.4 on page 24).

3.1 MATERIALS AND METHODS

3.1.1 *Plant growth and experimental conditions*

Several experiments were conducted to examine the role of Zn in the response of *Noccaea* to *Alternaria* infection. From those, two first experiments were used to set up experimental conditions, such as Zn concentration in the solution and humidity during the infection stage. Amongst all the experiments performed, two with standardized experimental conditions were selected for testing the effect of the pathogen one week after the inoculation, and other two were performed to test the action of the pathogen only 24 h after the inoculation. Specific experimental conditions are detailed below. Seeds from *Noccaea caerulescens* (J. Presl & C. Presl) F.K. Mey. (Ganges ecotype) were germinated in soil mixed with vermiculite, with a previous 4-day stratification period in dark at 4°C. Average light intensity was 100 μmol m^{-2} s^{-1}, with 8 h day period and temperature set to 23-25°C. After two weeks, plants were transferred to a modified Hoagland solution at 25% ionic strength (pH 5.5) (Table 3.1). The solution was aerated and renewed at one week intervals. After 8 weeks, plants were individually transferred to 100 ml pots and pretreated during 4 weeks with $ZnSO_4$ added to the solution to reach concentrations of 2, 12 and 102 μM of Zn. Afterwards, half of the plants were leaf

Table 3.1: Modified Hoagland solution at 25%

Macronutrients	[ ] (mM)	Micronutrients [] (µM)	[ ] (μM)
KNO_3	1.5	KCl	50
$Ca(NO_3)_2$	1	H_3BO_3	25
$NH_4H_2PO_4$	0.5	$MnSO_4$	2
$MgSO_4$	0.25	$ZnSO_4$	2
		NH_4Mo	0.5
		$CuSO_4$	0.5
		Fe-EDDHA	20

inoculated with a spore suspension of *Alternaria brassicicola*. Plants were sampled one week after inoculation.

For the 24 h inoculation experiments, plants were germinated and grown in hydroponic solution with a previous 4-day stratification period in dark at 4°C. After 2 weeks, 3 plants were transferred to 100 ml pots and afterwards, only one plant per pot was left for 9 weeks.

3.1.2 *Fungal material and inoculation*

A. brassicicola (strain CBS 567.77; Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Nederlands) was grown in Petri dishes on potato dextrose agar (PDA; Sigma-Aldrich, Steinheim, Germany). Spore suspension for the inoculations was obtained from 2-week old *Alternaria* cultures. The mycelium was soaked with 5 ml of a potato dextrose broth (PDB; Sigma-Aldrich, Steinheim, Germany), scraped with a sterile blade and collected apart. After mycelia disaggregation by pipetting the suspension up and down, it was vacuum filtered through a sterile cloth using a Büchner funnel and a Kitasato flask. A spore suspension was obtained and adjusted to 20-30·10^4 spores·ml^{-1} in a Neubauer chamber. Then, all well-developed leaves from *N. caerulescens* plants were inoculated with one 10 µl droplet of the spore suspension in the central part of each leaf with no needle puncturing. A droplet of PDB without *Alternaria* spores was used as a negative control applied to one leaf of each of the inoculated plants. All plants were kept for one week inside a transparent plastic chamber and a humidifier was used to provide high humidity conditions (75-90% relative humidity) during the first three days in order to facilitate the start of the infection process (Figure 3.1).

3.1.3 *Plant growth measurements*

Plant growth was assessed by measuring rosette area before and after the treatment with Zn, while shoot and root dry weight was quan-

Figure 3.1: *N. caerulescens* plants growing inside the humidity chamber after inoculation with *A. brassicicola*.

tified at the end of the experiment. Rosette and root fresh weights were measured 7 days after the inoculation. Then, plant tissue was oven-dried at 60°C for 48 h and placed at room temperature at least one day before it was weighted again to obtain the dry weight.

3.1.4 *Ion concentration*

Dry leaf and root tissues were finely powdered using a mortar and pestle. About 0,1 g of tissue was predigested overnight in 2:5 ml of H_2O_2 30%: HNO_3 69% (v/v) and then digested during 4 h at 110 °C (Hot Bloc model 154-240, Environmental Express, Charleston, South Carolina, USA). The final volume was adjusted to 25 ml with Milli-Q water and ion concentration was analyzed by inductively coupled plasma mass spectrometry (Perkin-Elmer, Elan-6000). BCR 62 Olea europaea certified material was used as an internal control for the ion concentration.

3.1.5 *Disease symptoms: necrotic leaf area quantification*

The necrotic, chlorotic and healthy leaf areas were measured one week after inoculation. Scaled pictures of every leaf were taken. Then, different colors were assigned to each area (Photoshop C5S) and pixels were counted (Image-Pro Plus 6.0).

3.1.6 *Relative gene expression with quantitative reverse-transcription PCR*

The expressions of five genes related with the defence response and the stress signalling pathways of SA, JA and Et were quantified (Table 3.2 on the next page). Additionally, the expression of a constitutive gene of *Alternaria* was used to assess the degree of the disease in leaves of *N. caerulescens*. For that purpose, the entire rosette from each plant was frozen in liquid nitrogen. The tissue was collected in plastic tubes, finely pulverized in liquid nitrogen with a metallic spatula and

Table 3.2: *N. caerulescens* defence signalling marker genes quantified.

Hormonal pathway		Gene
SA	*PR*1	Pathogenesis-related 1
	*BGL*2	ß-1,3-Glucanase 2
JA/Et	*PDF*1.2	Plant defensin 1.2
	CHIB	Basic chitinase
JA	*LOX*2	Lipoxygenase 2

stored at -80°C. RNA was extracted following the TRIzol® Reagent (Invitrogen, Molecular Research Center Inc., OH, USA) according to manufacturer instructions and quantified at 260 nm with Nanodrop 2000 (Thermo Scientific, DE, USA). One microgram of RNA was used for reverse-transcription to cDNA using the cDNA Synthesis kit (Bio-Rad, CA, USA) according to the manufacturer instructions. cDNA was diluted 1:50 in Milli-Q sterile water. Quantitative PCR was performed on a CFX384 or a CFX 96 Real-Time System (Bio-Rad, CA, USA). Each reaction contained 5µl of iTaqTM Universal SYBR®Green Supermix (Bio-Rad, CA, USA), 2 µl of the sense and antisense primers at a final concentration of 2 µM and 3 µl of a dilution 1:10 or 1:50 of cDNA. The amplification program was performed by preincubating the cDNA for denaturation at 95°C for 10 min, followed by 40 cycles of a denaturation, annealing, and extension steps. After each cycle, fluorescence was measured at 72°C. A negative control without a cDNA template was run in each assay. Oligonucleotide primer sequences for the target genes are listed in Appendix A (v on page 125). *Tubulin* from *N. caerulescens* was used as reference gene. Standard curves from serial dilutions of sample cDNA were used to determine primer efficiency. Expression of the target gene relative to the expression of the reference gene was calculated using the Pfaffl method (Pfaffl 2001). Three technical replicates were used in all cases per sample, dilution and non-template control.

For the quantification of *A. brassicicola* in the leaves, primer sequences from the constitutive gene *AbActin* were used. The expression of *AbActin* was referred to that of the plant housekeeping gene, *NcTubulin* (see primer sequences in Appendix A, v on page 125). One sample from a non-inoculated plant was used as negative control.

The suitability of the *A. thaliana* primers for amplification of the defence genes sequences in the *N. caerulescens* transcriptome was checked. The products of qPCR performed for the five defence genes, the housekeeping from Noccaea, *Tubulin*, and their correspondent non-template controls were run in a 2% agarose gel (Standard Low –mr Agarose. Bio-Rad Laboratories, Inc. US) stained with SYBER® Safe DNA gel stain (Invitrogen. CA. US). The conditions for the qPCR are described in detail above. The amplified DNA sequences were

purified from the rest of the qPCR reaction components according to ExoSAP-IT® protocol (Affymetrix,US), suited for small DNA fragments. The purified amplicons were sequenced (GATC Biotech AG, Germany) and the nucleotide sequences from the single strands using the forward and reverse primers of each gene were matched using the Bioedit software (version 7.0.7.0) (Hall 1999) . The fully amplified sequences were then checked for homologies in the NCBI database through the Basic Local Alignment Search Tool (BLAST, blast.ncbi.nlm.nih.gov). The constitutive gene from *A. brassiciola*, *AbActin*, was also checked for correct amplification in the transcriptome of plant tissue colonized by the fungi. A PCR was carried out in a final volume of 20 µl (10 µL of Master Mix (Biotools, US) 3 µl RNAse free H_2O and 2 µM template). The sequence of the amplified DNA fragment from *Alternaria* was obtained following the same steps as for the plant defence genes. The results are presented in Appendix B (v on page 127).

3.1.7 *Hormone concentration*

Leaf concentrations of SA, JA, the ethylene precursor ACC, ABA, IAA, and GA were measured by LC–ESI(-)–MS/MS system according to Segarra et al. (2006). 200-250 mg of frozen tissue were grinded to powder in a mortar with liquid nitrogen and then homogenized at 4°C with 500 µl of the extraction solution MeOH–H_2O–HOAc (90:9:1 v/v/v). The homogenate was then centrifuged at 10 000 rpm for 1 min and the supernatant was stored at 4°C. The pellet was homogenized in 750 µl of the extraction solution and the extraction process repeated twice. Pooled supernatants were dried under vacuum, resuspended in 200 µl of 0.05 % HOAc in H_2O–MeCN (85:15 v/v), and filtered with a Millex-HV 0.45 µm filter from Millipore (Bedford, USA). Deuterated salicylic acid and 1-aminocyclopropane-1- carboxylic acid (both 98 atom% D -Sigma-Aldrich, Steinheim, Germany) at 500 ppb were used as internal standards in all the samples. Salicylic acid and JA quantification was done using a standard addition calibration as described in Llugany et al. (2013).

3.1.8 *Glucosinolate concentration*

Total leaf glucosinolate concentrations were measured following the enzymatic reaction of glucose release as described in Noret et al. (2005). Glucose is a component of all glucosinolates and can be used to quantify total glucosinolate concentration after enzymatic release. About 500 mg of pooled frozen tissue from the one week inoculation experiments were heated at 75°C in 10 ml of 70% methanol for 3 min and homogenized for 7 min using an Omni Mixer (OCI Instruments, Waterbury, CT, USA). Cooled mixtures were centrifuged for 3 min

at 3000 rpm, supernatants were collected into new tubes with 0.5 ml Pb–Ba acetate (0.5 M) and centrifuged again. The supernatants were kept at 4°C and 0.5 ml aliquots of each were used for glucosinolate separation by column chromatography (1 ml of DEAE Sephadex A-25 diluted in pyridine acetate 0.5 M). Columns were washed with 2 ml of distilled water, then myrosinase (250 µl; 2/3 EU ml^{-1}; Sigma T4528) was injected and incubated in the column for 15 h, time after which the column was washed again with 2 ml of water. Myrosinase hydrolyses the glucosinolates that were adsorbed in the column, allowing the release of the glucose and its collection in the last elution step. Finally, glucose concentration, which is stoichiometrically equivalent to the glucosinolate concentration, was quantified with a glucose kit (Diffchamp Group, Västra Frölunda, Sweden).

3.1.9 *Alternaria brassicicola tolerance to zinc in vitro*

The *A. brassicicola* spore suspension was obtained as described for the inoculum preparation (Subsection 3.1.2 on page 38), but at double concentration of PDB. Sterile water solutions of different $ZnSO_4$ concentrations ranging from 0 to 8 mM were mixed 1:1 with the spore suspension in 96-well plates. The growth of the spores was measured as the increment of optical density at 450 nm at the beginning of the experiment (t_0) and 48 h after (t_{48}). The same experiments were carried out using the chlorides $ZnCl_2$ and KCl, to dismiss a possible effect of the ion sulfate and chloride. Spore growth at different KCl concentrations was expected not to vary and so it was used as a control. A theoretical correspondence between Zn concentration in solution and in leaf was estimated. It was assumed that 1 g of leaf material is equivalent to 1 ml in volume and taken into account the water content in *Noccaea* aerial parts using the data from our experiments (Equation 3.1)

$$Leaf[Zn](\mu g g^{-1} DW) = -16,73 + 477,72 * Solution[Zn](mM) \quad (3.1)$$

3.1.10 *Statistical analysis*

Data analyses were carried out using Statistica 7 (StatSoft Inc., Tulsa, OK, USA). Factorial ANOVA was run when the assumptions for ANOVA were met and Multiple Comparison Fisher LSD test was used when ANOVA threw significant differences among the groups at a confidence interval of 95%. Otherwise, Kruskal-Wallis Test was used and, in case of finding any significance among the groups, Wilcoxon Rank Sum Test was performed. In the figures, means ± SE are represented and significant differences, when present, are marked with different letters.

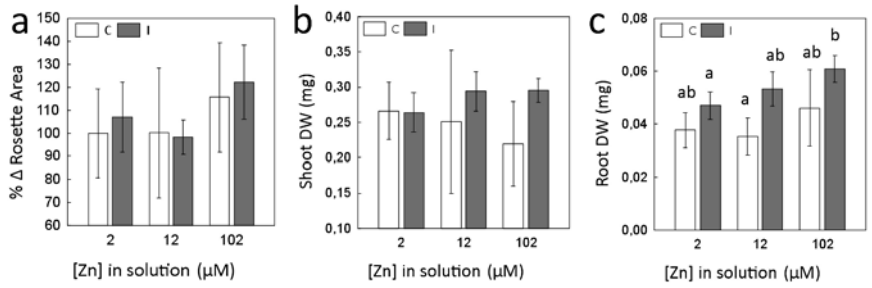


Figure 3.2: Rosette area and rosette and root DW of 3-month-old *N. caerulescens* treated with Zn (2, 12 or 102 µM) for 5 weeks. Four weeks after Zn addition, half of the plants in each treatment were inoculated with *A. brassicicola*. Factorial ANOVA was performed, followed by LSD Fisher test when $p < 0,05$. Error bars represent SE. Letters above the bars indicate statistically significant differences.

3.2 RESULTS AND DISCUSSION

3.2.1 *Plant Growth*

The Zn-treated plants exhibited no signs of stress such as chlorosis or reduced biomass. Moreover, no significant differences were found in rosette area, shoot or root DW amongst Zn treatments (Figure 3.2). These results suggest that all Zn treatments used were in the Zn-sufficient range, as important growth reduction under Zn-deficient conditions has been previously reported in *N. caerulescens* (Saison et al. 2004). Positive correlations between the Zn concentrations in the treatment and growth have been documented by Tolrà et al. (2006) at optimal Zn concentrations around 500 µM, where the increase on shoot dry weight was correlated with the shoot Fe concentration . In our study, the range of Zn concentration to which we submitted the plants was relatively low in comparison to other studies and so no remarkable differences in growth were spotted.

3.2.2 *Alternaria brassicicola*

3.2.2.1 *Alternaria growth in vivo*

Alternaria brassicicola has been extensively used as a model pathogen for the study of plant interaction with necrothrops in *A. thaliana* and other *Brassicaceae*. In metal hyperacululating plants it has been used before in the assessment of the protective role of metals in the Ni hyperaccumulator *Streotanthus polygaloides*, where *A. brasscicicola* growth was inhibited in the leaves of the plant treated with Ni (Boyd et al. 1994). Hanson et al. (2003) have also found an improved resistance to *Alternaria* in *Brassica juncea* treated with Se. As in the cited

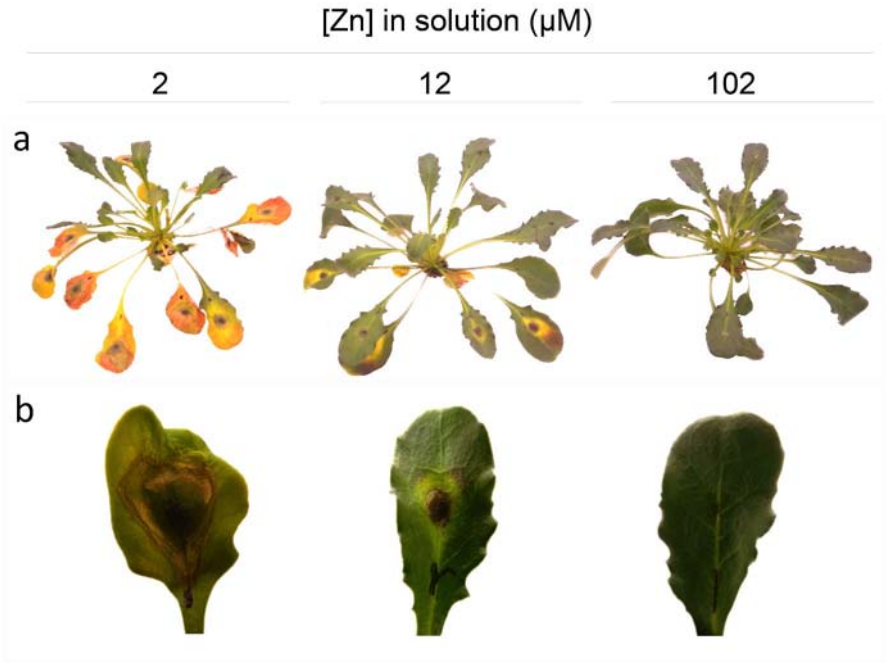


Figure 3.3: Full rosettes (a) and leaves (b) of 3-month-old solution- cultured *N. caerulescens* treated with Zn (2, 12 or 102 µM) for 5 weeks, one week after inoculation with *A. brassicicola*. Representative samples from one experiment are shown.

cases, a number of other studies have linked before a higher concentration of heavy metals in shoots of several hyperaccumulators to a greater resistance against different pathogens and herbivores, stating the so-called elemental defence hypothesis. The particular studies on this topic have been reviewed in the last years by Poschenrieder et al. (2006), Poschenrieder et al. (2011) and Hörger et al. (2013).

In our study, the overall degree of infection varied amongst the experiments, but the pattern remained constant: interestingly, the area of the leaf necrotic spot caused by *A. brassicicola* and the adjacent chlorotic area were visibly smaller in plants treated with high Zn (Figures 3.3 and 3.4). Infection of *A. brassicicola* in the leaves was also assessed at the molecular level by qRT-PCR, where the expression of *AbActin*, a housekeeping gene from the fungus, relative to a constitutive gene from *N. caerulescens*, *NcTubulin*, was quantified. Consistent with the visual symptoms observed, the relative expression of *AbActin* was lower in plants containing more Zn in the leaves (Figure 3.5), hence correlating a higher Zn concentration with a greater resistance against *A. brassicicola*.

3.2.2.2 *Alternaria growth in vitro*

Given the importance of *A. brasscicicola* in Brassicaceae family crops, *in vivo* and *in vitro* toxicity assays have been performed for different *A. brasscicicola* strains since long (Miller 1950; Channon 1970;

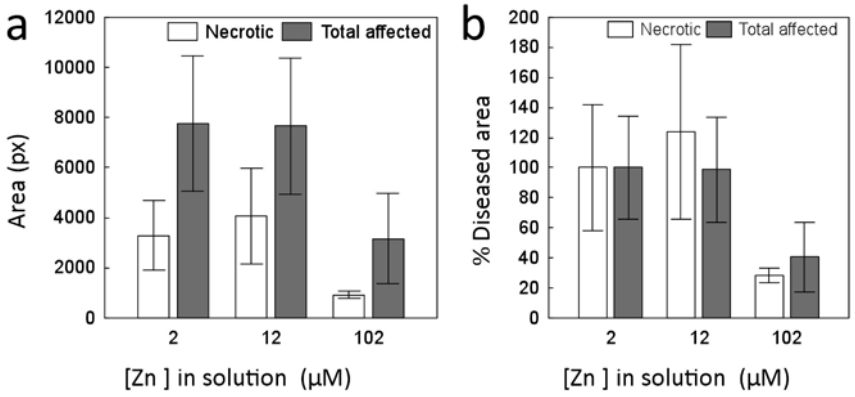


Figure 3.4: Leaf affected area in infected leaves of 3-month-old solution- cultured *N. caerulescens* treated with Zn (2, 12 or 102 µM) for 5 weeks, one week after inoculation with *A. brassicicola*. Magnitudes are expressed as absolute values (a) or referred to the lowest Zn treatment (b). Non parametric Kruskal-Wallis test was carried out. Error bars represent SE.

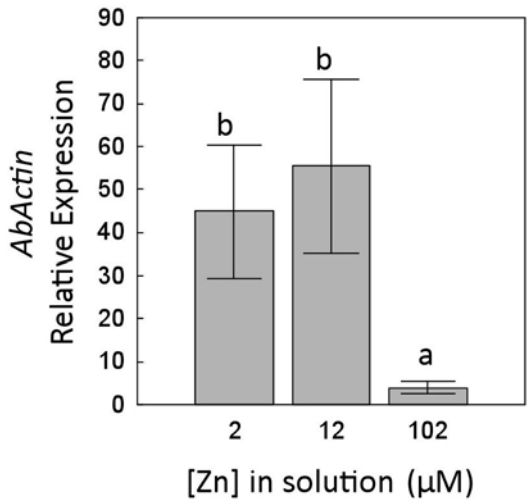


Figure 3.5: Relative gene expression of the *AbActin* gene from *A. brassicicola* in leaves of 3-month-old solution- cultured *N. caerulescens* treated with Zn (2, 12 or 102 µM) for 5 weeks. 4 weeks after Zn addition, half of the plants in each treatment were inoculated with *A. brassicicola*. *Tubulin* from *N. caerulescens* was used as a reference gene and the group of plants under 102 µM Zn treatment was set as a calibrator (Relative gene expression = 1). Representative data from one out of two independent experiments are presented. Factorial ANOVA and LSD Fisher test were performed. Error bars represent SE. Letters above the bars indicate statistically significant differences.

Pandey and Narang 2004). Here, the tolerance of our specific strain of *A. brassicicola* to high Zn concentrations was assessed *in vitro*. Spore growth in PDB with different $ZnSO_4$ concentrations was measured as increment of optical density at 450nm with an incubation period of 48 h (Figure 3.6). *A. brassicicola* growth decreased exponentially with increasing $ZnSO_4$ concentration, so this tendency was adjusted to the exponential model (Equation 3.2).

$$O.D._{450nm} = 0,76e^{-2,2[Zn]} \tag{3.2}$$

Interpolation in the exponential curve showed that Zn concentrations around 440 µM caused a 50% growth inhibition of *A. brassicicola* (EC_{50} = 440 µM) compared to the control without Zn and that *A. brassicicola* growth was totally inhibited at Zn concentrations equal or higher than 2 mM.

A. brassicicola exposure to $ZnCl_2$ and KCl was also assessed. Growth at increasing $ZnCl_2$ concentrations followed the same pattern as the described for $ZnSO_4$ and KCl did not affect spore growth at any of the concentrations tested. Thus, within the concentration range considered, sulfate and chloride ions had no effect over the spore development and the inhibition detected was totally due to Zn.

The role of Zn as an inorganic defence is determined by the toxicity and deficiency thresholds for the plant and the pathogen together. A theoretical correspondence between Zn concentrations in the solution and in the dry leaf was established in order to evaluate the potencial toxic effects of Zn accumulated in the plant (see materials and methods, Subsection 1.4.1.2 on page 25). The results showed that the average Zn concentration found in *N. caerulescens* leaves after 5 weeks under 102 µM Zn treatment, 5000 µg/g, was equivalent to 10 mM of Zn in solution, a concentration 20 times higher than the EC_{50} for *A. brassicicola* i*n vitro*. Experimental determination of the Zn concentration to which the pathogen is exposed inside the leaf is still a challenge, due to the necrotrophic lifestyle of *A. brassicicola* and to the metal chelation in the plant. Although metal chelation cannot be overlooked, given the relation between *Alternaria* susceptibility to Zn and *Noccaea* leaf Zn concentration, there is a strong possibility that Zn plays a role as inorganic defence in this model.

Proves for a direct toxic effect of Zn against a pathogen have been obtained in hyperaccumulators. Studies from Fones et al. (2010) and Fones and Preston (2013) have found that the Zn present in the apoplast of *N. caerulescens* had a toxic effect on the biotroph bacteria *Pseudomonas syringae*, without significant increment of the oxidative burst, callose deposition and activation of genes related with SA, typically associated with the defence against biotrophs, hence attributing to Zn a main role of inorganic defence in their model. However, the view of a trade-off between Zn and the mechanisms

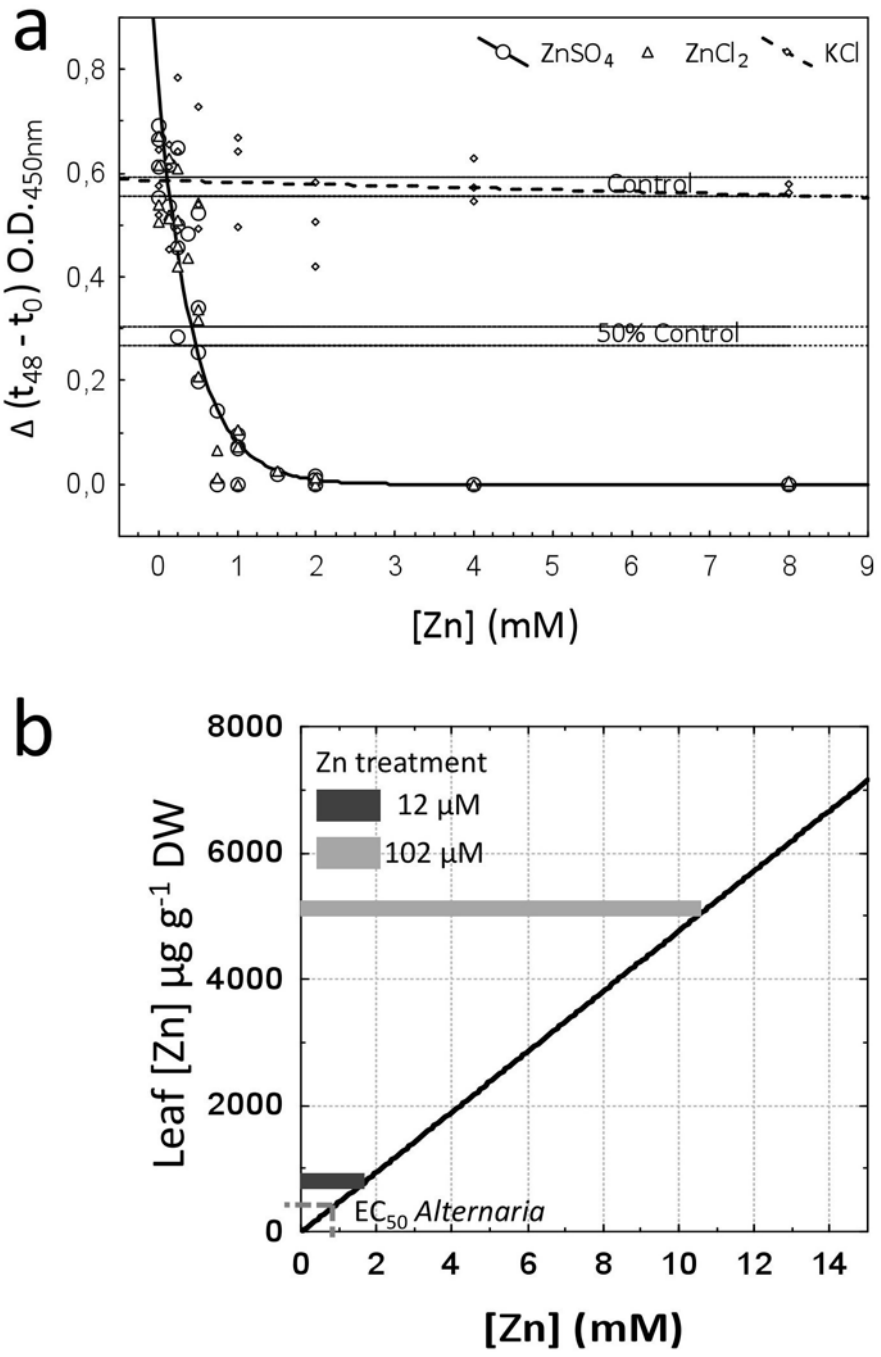


Figure 3.6: *Alternaria brassicicola* tolerance to Zn in vitro (a) and theoretical correspondence between [Zn] in dry leaf and [Zn] in solution (b), where the dashed line indicates the [Zn] at the EC_{50} for Zn and *Alternaria* and the bars represent the average [Zn] in *Noccaea* plants treated with 12 or 102 μM of Zn in our experiments.

typically activated upon pathogen attack has not been supported by other studies. In the Zn and Cd hyperaccumulator *Noccaea praecox*, Cd was correlated with a greater resistance to the biotrophic fungus *Erysiphe cruciferarum* (Llugany et al. 2013), lowering the SA concentration previously activated by Cd, but increasing the accumulation of JA induced by both fungus and Cd in inoculated plants. At lower Cd concentrations, a similar behavior has been described for the non-hyperaccumulator *A. thaliana* inoculated with the necrotrophic fungus *Botrytis cinerea* (Cabot et al. 2013). Hereafter, the possible mechanisms underlying the correlation between a higher Zn concentration and a greater resistance to *Alternaria* will be discussed.

3.2.3 *Mineral Nutrients*

3.2.3.1 *Tissue Ion Concentrations*

As expected, large increases in shoot and root Zn concentrations were found in Zn-treated plants with respect to the control group (Figure 3.7). In 102 µM Zn-treated plants, Zn shoot concentrations were found to be above the Zn hyperaccumulation threshold (Ent et al. 2012) with values up to 5000 µg/g DW, while plants grown at the lowest Zn treatment showed shoot Zn concentrations at least 10 times lower. In fact, *N. caerulescens* is a hyperaccumulator species able to accumulate up to 40 000 mg Kg^{-1} of Zn (Saison et al. 2004).

Many studies have addressed the influence of mycorrhyzal fungi in metal uptake from the soil in metal hyperaccumlating and no-metal hyperaccumulating plants. However, there is a lack of bibliography about leaf pathogenic fungi controlling plant metal uptake or translocation from the roots to the shoots. Our results show that the shoot Zn concentration was influenced by inoculation with *Alternaria*, as well as by the interaction of the Zn treatment and the inoculation factors. Inoculated plants grown at the highest Zn treatment presented approximately half of the Zn concentration found in control. Therefore, *Alternaria* did not trigger neither Zn uptake nor its translocation to the inoculation site in the leaves. On the contraty, Zn translocation from root to shoot was lower in inoculated plants grown at 102 µM Zn (One way ANOVA, p=0,023). The shoot to root Zn concentration ratio tended to decrease over the Zn treatment ranging from 2-1 at the 2 µM treatment and from 1-0.5 at the 102 µM treatment, respectively. This decrease could be related to the large amounts of Zn remaining in the roots at the highest treatment (Figure 3.7). Despite this, no differences in the expression of the *HMA*4 transporter, mainly involved in the root to shoot translocation, were found amongst the treatments The expression of *HMA*3, encoding for an influx vacuolar transporter, was also not affected (Figure 3.10).

Previous results have shown an interaction between Zn and other essential elements such as Ca, Mg, P, Fe and S in *N. caerulescens* (Sai-

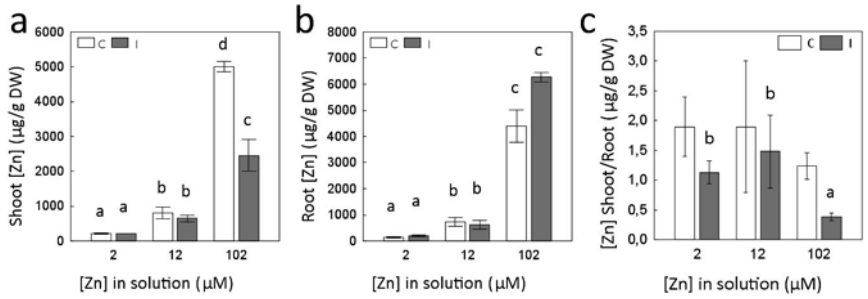


Figure 3.7: Zn concentrations in 3-month-old solution- cultured *N. caerulescens* treated with Zn (2, 12 or 102 µM) for 5 weeks. In the 4th week, half of the plants were inoculated with *A. brassicicola* and samples taken after one week. Control plants are represented by "C" and inocualted plants by "I". Error bars represent SE. Letters above the bars indicate statistically significant differences.

son et al. 2004). In the present study, significant differences amongst treatments were found for Ca, Mg S, Cu and Mo in shoots and K, Mg, S, B, Mn and Mo in roots (Figures 3.8 and 3.9).

Plant K uptake decreased with Zn supply, nonetheless, K translocation from roots to rosettes was similar, and no differences in rosette K were observed (Figure 3.8). Therefore, decreased K concentrations did not show a relationship with the differences in *Alternaria* growth amongst Zn treatments. Previous studies have shown a positive correlation between Ca and Zn concentrations in shoots and the co-localization of both elements in epidermal vacuoles (Vázquez et al. 1992). Although contradictory results have been found by Küpper et al. (1999) and Saison et al. (2004), where concentrations of Ca, Mg, P and K in the epidermal sap of *N. caerulescens* were reported to decrease with increasing Zn supplementations. Recently, Dinh et al. (2015) also revealed a negative correlation between Zn and Ca concentration in shoots of *N. caerulescens* submitted to Zn treatments above 200 µM and confirmed the co-localization of both elements by SEM microscopy. In the present study, no significant differences in root Ca were observed amongst treatments. However, rosette Ca concentration slightly increased with Zn supply in control plants, while a negative relationship between shoot Ca and Zn supply was found in inoculated plants (Figure 3.8). Root Mg, with the exception of the highest Zn-treated control plants, showed a tendency to increase with Zn supply, independently of *Alternaria* inoculation. In rosettes, Mg decreased with increasing Zn supply in inoculated plants, while control plants did not show a clear tendency (Figure 3.8). The sulphur metabolism is closely related to glucosinolates and metal chelators such as metallothioneins, phytochelatins, gluthation, nicotianamine, methionine and cysteine (see Figures in Introduction 1.2 on page 6 and 1.9 on page 24). In the metal hyperaccumulating ecotype of *Sedum alfredii*,

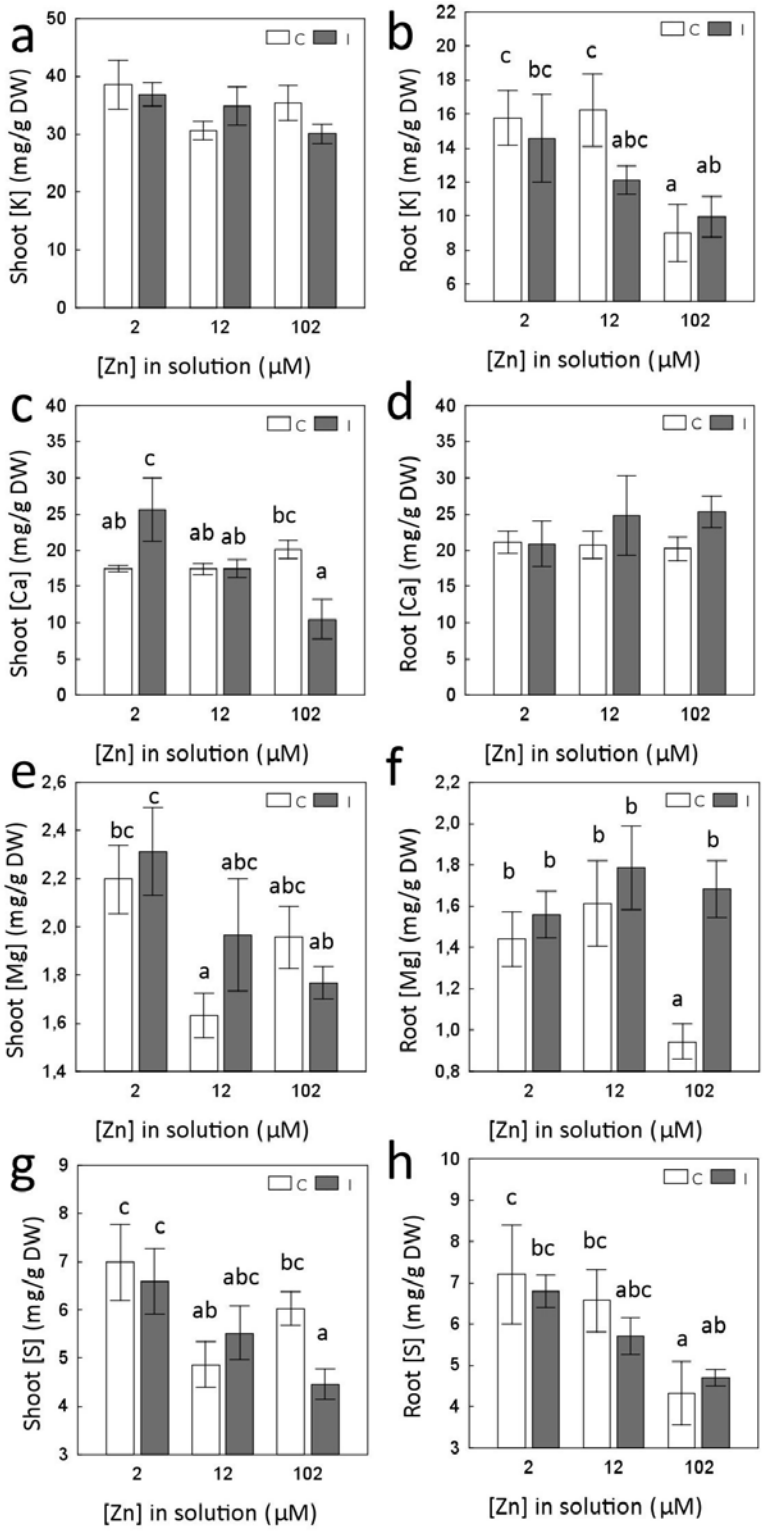


Figure 3.8: K (a, b), Ca (c, d), Mg (e, f) and S (g, h) concentrations in shoots and roots of 3-month-old solution- cultured *N. caerulescens* treated with Zn (2, 12 or 102 µM) for 5 weeks. In the 4th week, half of the plants were inoculated with *A. brassicicola* and samples taken after one week. Control plants are represented by "C" and inocualted plants by "I". Error bars represent SE. Letters above the bars indicate statistically significant differences.

Cd accumulation was positively correlated with nicotianamine synthase in the roots (Liang et al. 2014). Our results are not in line with a Zn-induced enhancement of S acquisition and increased S-based Zn detoxification, as in shoots and roots, a negative relationship was found between Zn and S concentrations (Figure 3.8). Sulphur levels decreased, especially in roots, with increased Zn supply. In shoots, the lower S values could be determined by a lower absorption of this element in the root. This effect was especially visible in inoculated plants. However, the shoot S concentrations in plants with high Zn supply remained higher than 0,4%, a level that is considered as adequate for other Brassicaceae species (Bergmann 1993). Taken all together, our results are in agreement with the previous studies cited above that showed decreased concentrations of Ca, Mg, P and K with increasing Zn concentration.

The increase in Zn supply and the inoculation with *Alternaria* also affected micronutrient uptake and distribution in *N. caerulescens*. Amongst the micronutrient analyzed, B, Mn, Cu and Mo showed significant differences amongst Zn and/or inoculation treatments (Figure 3.9). Rosette B concentration showed a tendency to increase with Zn supply, especially in non-inoculated plants. In addition, a significant increase in root B concentration was shown in non-inoculated plants supplied with the highest Zn. Manganese levels were influenced by the Zn supply, more significantly in roots. Mn concentration decreased with increasing Zn supply in the roots of both inoculated and non- inoculated plants. Contrastingly, in the aerial part, Mn concentrations appeared to rise with increased Zn treatments in control plants, while the opposite was observed in inoculated plants. A significant interaction between Zn treatment and inoculation with *Alternaria* was found in rosette Cu, but not in root Cu. The Cu concentration appeared to increase along with Zn treatment in control plants, but decreased in the inoculated 102 µM Zn treatment (Figure 3.9). In shoots and roots, a negative relationship was found between Zn and Mo concentrations. Molybdenum values decreased especially in roots with higher Zn supply. In shoots, the lower Mo concentrations could have been determined by a lower absorption of this element in the roots. This effect was especially notorious in inoculated plants.

Summarizing, Zn treatment in the solution affected Zn, Ca, S, Mg and Mo concentration in shoot and K, S, Mn, Zn, B and Mo in roots. The factor inoculation considered alone only affected Zn concentration in shoots and Mg concentration in roots, while the interaction between the two factors Zn concentration and inoculation was significant for Zn, Ca and Cu concentration in shoots (Table 3.3). Appart from Zn concentration, the most marked differences were found in K, S, Mn and Mo concentration in the roots, where they were negatively correlated with Zn concentration.

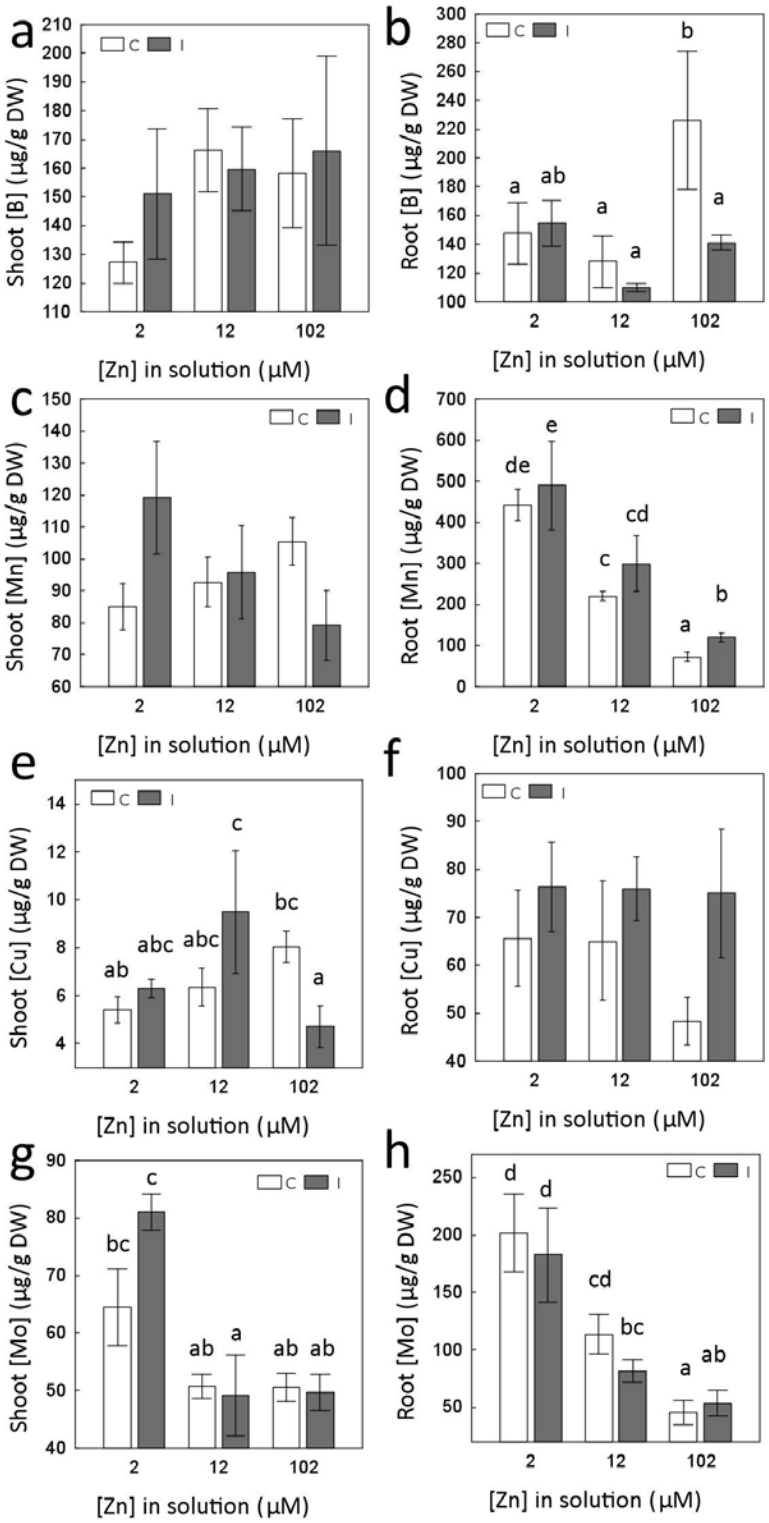


Figure 3.9: B (a, b), Mn (c, d), Cu (e, f) and Mo (g, h) concentrations in 3-month-old solution- cultured *N. caerulescens* treated with Zn (2, 12 or 102 µM) for 5 weeks. In the 4th week, half of the plants were inoculated with *A. brassicicola* and samples taken after one week. Control plants are represented by "C" and inocualted plants by "I". Error bars represent SE. Letters above the bars indicate statistically significant differences.

Table 3.3: Factors (Zn in the solution, inoculation -with *Alternaria*- or the interaction of both factors) that affected significantly (factorial ANOVA, p<0,05) the concentration of ions in shoot, root or both in *N. caerulescens*.

Plant part	**Factor**		
	Zn in the solution	**Inoculation**	**Zn*Inoculation**
Only shoot	Ca, Mg	Zn	Zn, Ca, Cu
Only root	K, Mn, B	Mg	
Shoot and root	Zn, S, Mo		

3.2.3.2 *Zn Transporters Gene Expression*

Plant Zn homeostasis depends on the expression and activity of different membrane transporters. Additionally, in *Arabidopsis thaliana,* a new role has been assigned to the the iron trasporter IRT1, also involved in Zn homeostasis, as it has been recently accounted for being at the cross-talk between heavy metals homeostasis and immunity. The *irt1 A. thaliana* mutants showed a reduced defence gene expression and callose deposition, as well as an increased susceptibility to the bacteria *Dickeya dadantii,* while treatement with the ferric chelator siderophore deferrioxamine protected the plant agaisnt *Pseudonomas syringae* (Vert et al. 2002; Henriques et al. 2002; Aznar et al. 2014). In search of possible new links between heavy metals and immunity, the role of the P-type ATPases *HMA3* and *HMA4,* members of the type 1B heavy metal-transporting subfamily of the P-type ATPases, was considered, given that both have been related to metal hyperaccumulation in *N. caerulescens* (see Introduction, 1.1.3 on page 6). In roots, *HMA*4 expression has been related to Zn transfer from the root to the shoot (Hanikenne et al. 2008) and in leaves, *HMA*4 has been found to be 2.7-fold more abundant in microsomal fraction of epidermal than in mesophyll tissue. In contrast, the vacuolar transporter *HMA*3 is significantly enriched in the mesophyll tissue (Schneider et al. 2013). Here, relative gene expressions of *HMA*3 and *HMA*4, were quantified in two replicate independent experiments by means of q-RT-PCR, showing no differences in expression upon pathogen attack for none of the genes (Figure 3.10). To our knowledge, the influence of the infection over the expression of these metal transporter genes had not been assessed before. In addition, no relationship was found between these two Zn transporter expression and shoot Zn concentrations (Figure 3.7).

3.2.4 *Stress Hormones*

The influence of Zn and infection caused by *A. brassicicola* on the stress hormone pathways was measured in leaves of *N. caerulescens*.

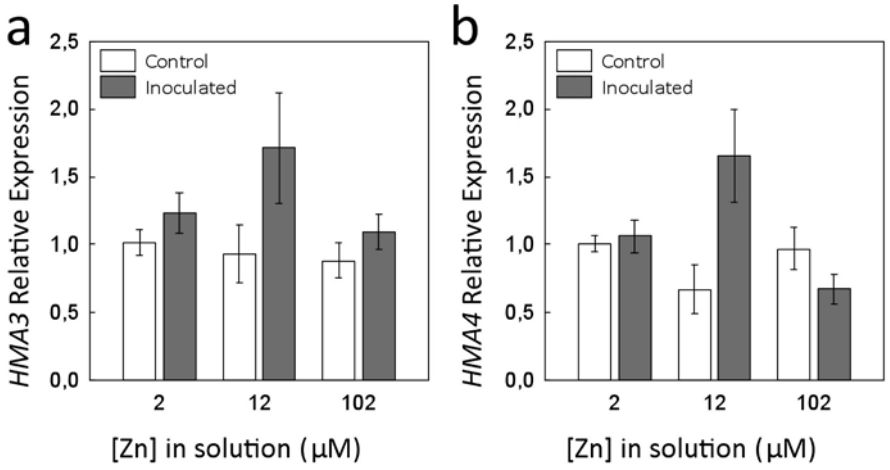


Figure 3.10: Relative gene expression of the heavy metal transporters *HAM*3 (a) and *HMA*4 (b) in leaves of 3-month-old solution- cultured *N. caerulescens* treated with Zn (2, 12 or 102 µM) for 5 weeks. 4 weeks after Zn addition, half of the plants in each treatment were inoculated with *A. brassicicola*. The group of control plants under the 2 µM Zn treatment was set as a calibrator (Relative gene expression = 1). Factorial ANOVA was performed. Error bars represent SE.

Concentrations of JA, SA, ABA, as well as the ET precursor ACC were quantified. Among these routes, JA/Et, JA and SA were chosen for a closer study in gene expression, given their key role in the defence response. Relative expression of the following marker genes was assessed: *PDF*1.2 and *CHIB* for JA/Et pathway, *LOX*2 for JA and *PR*1 and *BGL*2 for SA (see introduction, 1.3.4 on page 16). Both hormone concentration and relative gene expression were measured 24 h and one week after the inoculation with *Alternaria*. Results of gene expression are presented together with their reference hormone, either JA or SA. Prior to their use, the suitability of *A. thaliana* primers for amplification of the gene sequences in the *Noccaea caerulescens* transcriptome was positively verified (see Appendix B v on page 127)

3.2.4.1 *Ethylene*

The concentrations of the ethylene precursor, ACC, did not significantly change amongst Zn or *Alternaria* treatments at the two periods of time considered (Figure 3.11).

3.2.4.2 *Jasmonic Acid*

Jasmonic acid concentrations did not vary in response to Zn (Figure 3.12). This result is in agreement with previously reported data by Llugany et al. (2013), that did not find any increase in jasmonic acid

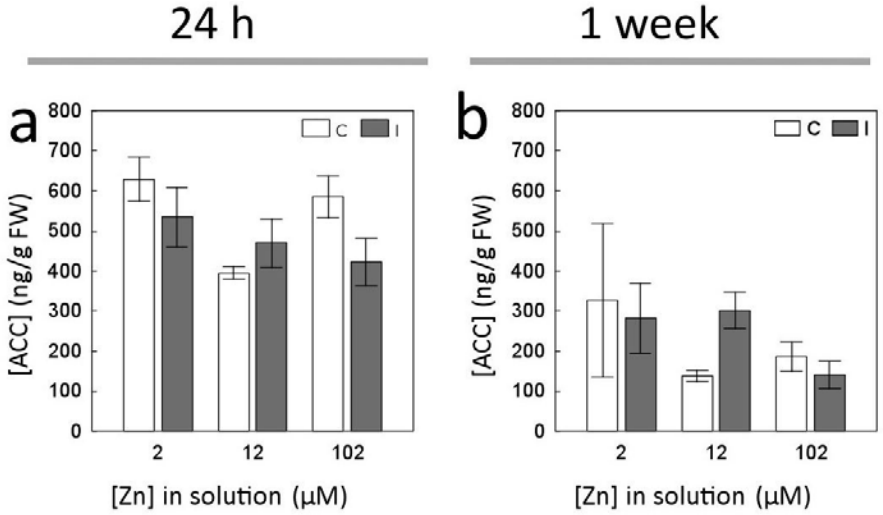


Figure 3.11: ACC concentration in leaves of *N. caerulescens* growing in nutritive solution with Zn (2, 12 or 102 µM) under two different experimental conditions. Half of the plants were inoculated with *A. brassicicola* and samples taken 24 h (a) or 1 week after inoculation (b). Not inoculated control plants are represented by "C" and inoculated plants by "I". Data from one representative experiment out of two independent experiments for each of the experimental conditions are presented. Error bars represent SE.

in response to Cd in the closely related hyperaccumulator *Noccaea praecox*.

JA synthesis was strongly induced in inoculated plants already 24 h after the inoculation with *A. brassicicola*. In the leaves of the inoculated plants, JA concentration reached values of 50-70 ng/g as an average, while in the controls the presence of JA was almost not detected. One week after inoculation, the JA concentration in controls remained close to zero and in the inoculated plants it increased on an infection-dependent manner. Plants under the 2 µM Zn treatment accumulated nearly 1500 ng/g of JA in fresh leaves in the advanced stages of infection. Contrastingly, plants that had been able to cope better with the disease, especially plants under 102 µM Zn treatment, presented less JA in the leaves (250 ng/g as average) than those with severe infection symptoms. The JA/Et and JA signalling pathway marker genes *PDF*1.2 (JA/ET), *CHIB* (JA/Et) and *LOX*2 (JA) were upregulated in inoculated plants 24 h after contact with *Alternaria* conidia. While *PDF*1.2 was expressed only around two times more than in the control, *CHIB* and *LOX*2 were greatly enhanced 24 h after inoculation. *CHIB* was overexpressed from 10 to 20 times more in inoculated plants and *LOX*2 from 15 to 25. After one week of infection, induction of *PDF*1.2 remained in the inoculated plants under 2 and 12 µM Zn treatment, but not in plants treated with 102 µM of Zn. No differences among the treatment were found anymore for

*LOX*2 expression. Although no significant, there was a tendency to lesser JA concentrations and lower expression of *PDF*1.2, *CHIB* and *LOX*2 in plants under the higher Zn treatment already 24 h after inoculation. Therefore, there is a negative relationship between leaf Zn concentration and *Alternaria* growth and JA leaf concentration and JA-dependent gene expression markers that supports the view of the trade-off hypothesis.

3.2.4.3 *Salicylic Acid*

In non-metal hyperaccumulating plants, SA is involved in the hypersensitive response (HR) displayed upon pathogen attack, linked to an explosion of the oxidative burst and to cell dead at the site of infection (Alvarez 2000). However, salycilic acid has been found to be constituvely high in a number of metal hyperaccumulators, such as *N. caerulescens* and *N. praecox*, compared to the non-hyperaccumulators *A. thaliana* and *N. arvense* (Freeman et al. 2005). Given the constitutively high levels of SA and ROS in hyperaccumulators and based on experimental experience (Fones et al. 2010; Fones and Preston 2013), Fones et al. (2010) proposed an alternative defence signalling pathway in *Noccaea caerulescens*, independent of the oxygen reactive species signalling pathway. Nonetheless, in contrast, Llugany et al. (2013) reported an increase in salycilic acid in response to Cd in *Noccaea praecox*. In our study, salycilic acid concentrations did not change in response to Zn, but SA, as well as the relative expression of the SA-induced genes *PR*1 and *BGL*2, were regulated in response to *Alternaria* infection (Figure 3.13). SA synthesis was upregulated 24 h and one week after plants were submitted to *Alternaria* inoculation. In the early response, while basal SA concentration in control plants was 100 ng/g as an average, inoculated plants experienced an increase of SA of 100 to 250 ng/g in fresh leaves. One week after the inoculation with the fungus, SA concentration dramatically rose up in infected plants. In the most heavily infected individuals, those without Zn supplementation, SA values reached more than 2500 ng/g. Following the same tendency of ABA and JA concentration, SA accumulation showed to be dependent on the degree of infection. Among the inoculated plants, those under the high Zn treatment did not concentrate in their tissues more than 700 ng/g. This result does not support the view of Fones and Preston (2013), as SA is accumulated upon pathogen attack in our pathosystem. As well as SA synthesis, expression of SA related genes *PR*1 and *BGL*2 was upregulated in inoculated plants 24 h and more strongly one week after inoculation with *Alternaria*. At both sampling times, enhancement of *PR*1 in inoculated plants was approximately two times greater than that of *BGL*2. While *PR*1 relative expression ranged from 5 to 7 times more in inoculated plants under all Zn treatments, *BGL*2 relative expression varied from 2 to 5 folds. Inoculated plants under the 102 µM Zn treatment

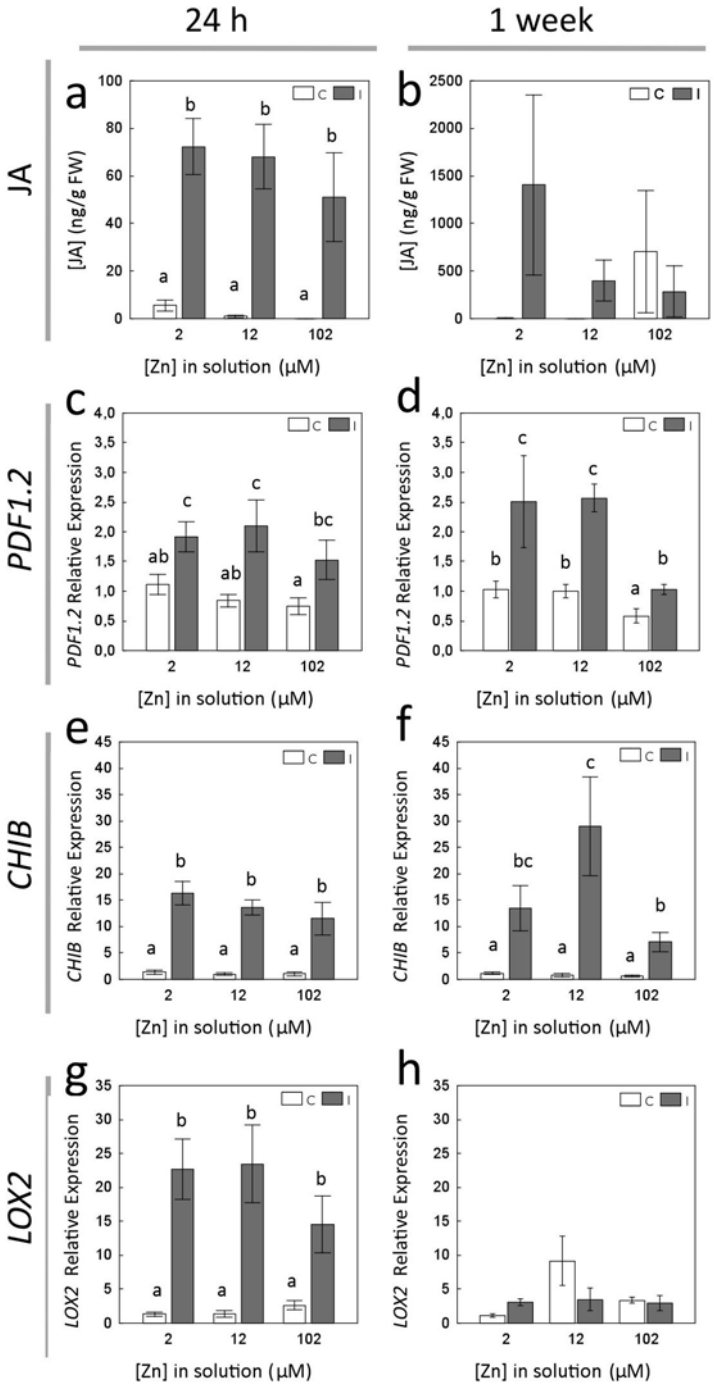


Figure 3.12: JA concentration and JA related marker genes *PDF*1.2, *CHIB* and *LOX*2 relative expression (R.E.) in leaves of *N. caerulescens* growing in nutritive solution with Zn (2, 12 or 102 µM) under two different experimental conditions. Half of the plants were inoculated with *A. brassicicola* and samples taken 24 h (a, c, e, g) or 1 week after inoculation (b, d, f, h). For the marker genes (c, d, e, f, g, h), the group of control plants under the 2 µM Zn treatment was set as a calibrator (Relative gene expression = 1). Not inoculated control plants are represented by "C" and inoculated plants by "I". Data from one representative experiment out of two independent experiments for each of the experimental conditions are presented. Error bars represent SE. Letters above the bars indicate statistically significant differences.

presented less expression of both genes one week but not 24 h after inoculation, probably related to a lower infection in plants with higher Zn content. Although SA pathway was activated by *Alternaria* evenly in all Zn treatments 24 h after the inoculation, one week later it was downregulated in the plants with the higher Zn concentration. As it was observed before for JA, this negative relationship between leaf Zn and leaf SA concentrations suggests the existence of a trade-off mechanisms between Zn and the plant defence pathways triggered by salycilic acid in *Alternaria* infected plants.

SA-mediated HR has been associated in a number of different pathosystems to a greater resistance to biotrophs (Glazebrook 2005). However, in metal hyperaccumulating plants, at least two cases have been documented where a combination of high metal accumulation plus infection by a biotroph have underregulated SA synthesis. This pattern has been described by Fones et al. (2010) and Fones and Preston (2013) in *N. caerulesces* treated with Zn and infected with *P. syringae* and by Llugany et al. (2013) in *N. praecox* exposed to Cd and infected with *E. cruciferarum*. Additionally, in the second case, JA was accumulated while not SA. In our case, both SA and JA pathways were activated upon pathogen attack and both downregulated one week later, suggesting that defence responses in hyperaccumulators may depend on the particular plant-metal-pathogen interaction. Thus, more pathosystems should be tested in order to draw a general picture of the defence in metal hyperaccumulators.

3.2.4.4 *Abscisic Acid*

Abscisic acid concentrations did not increase in response to high Zn (Figure 3.14). Nonetheless, ABA was synthesized in response to *A. brassicicola* at 24 h and in greater extent one week after inoculation. While in plants submitted to infection only for 24 h ABA concentrations remained under 2 ng/g of fresh leaf fresh weight, after one week this value rose up to nearly 600 ng/g as an average in the plants that were more affected by *Alternaria*. Although the fungus elicited ABA response equally in all Zn treatments in the early stage of infection, 7 days later ABA concentration was dependent on the degree of infection. In graph b, there is a decrease of ABA with greater Zn concentrations in inoculated plants, which may be a reflect of the lesser leaf damage and lower presence of *Alternaria* in plants under 12 and specially 102 µM Zn treatment.

The ABA response to Zn and *Alternaria* followed the same pattern shown by ACC, JA, and SA, as no differences in these defence regulators were found 12 h after the inoculation amongst Zn treatments. Nonetheless, in contrast, one week after the inoculation, the negative relationship found between leaf Zn and leaf JA, SA and ABA concentrations supports the existence of trade-off mechanisms between

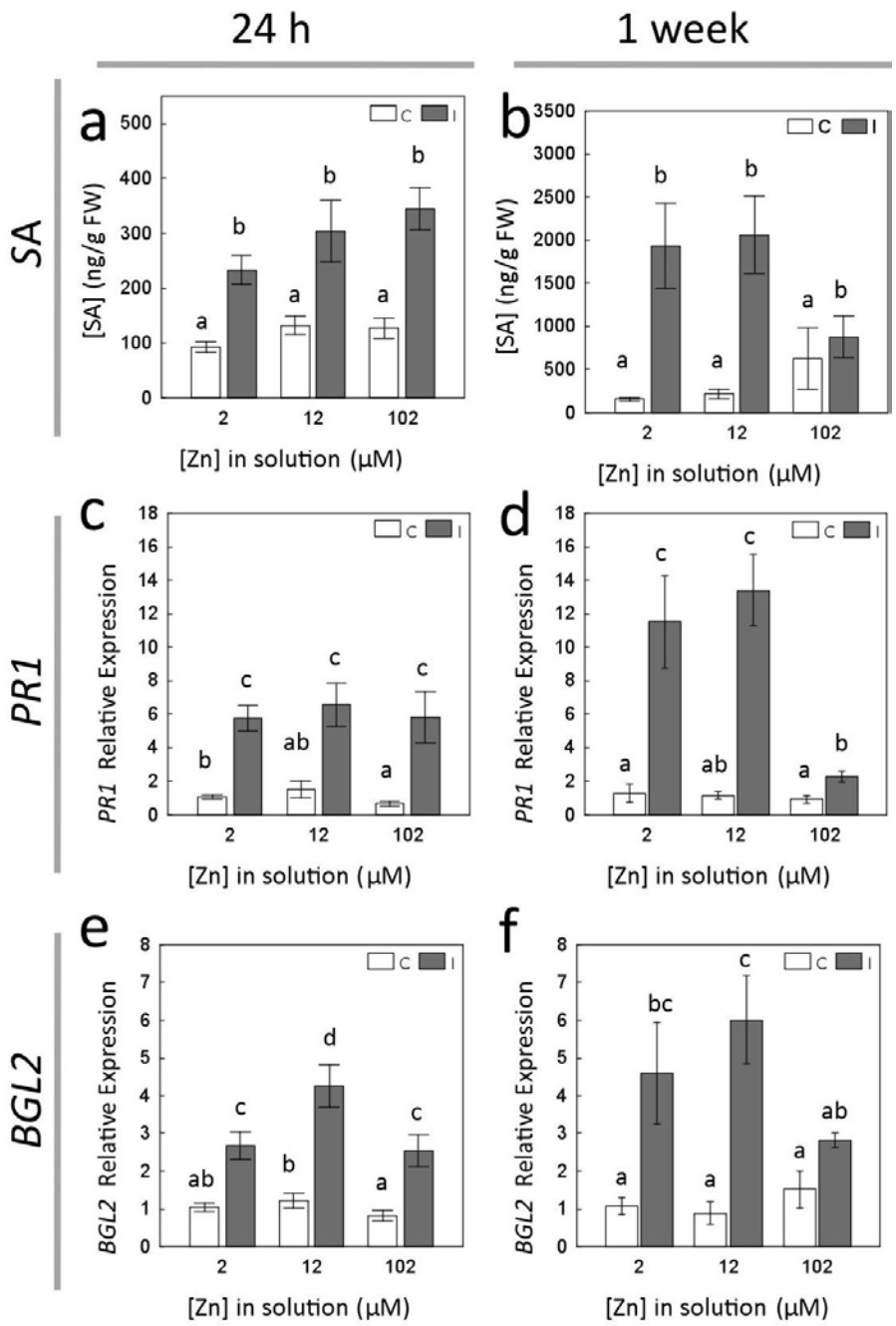


Figure 3.13: SA concentration and SA related marker genes *PR*1 and *BGL*2 relative expressions (R.E.) in leaves of *N. caerulescens* growing in nutritive solution with Zn (2, 12 or 102 µM) under two different experimental conditions. Half of the plants were inoculated with *A. brassicicola* and samples taken 24 h (a, c, e) or 1 week after inoculation (b, d, f). For the marker genes (c, d, e, f), the group of control plants under the 2 µM Zn treatment was set as a calibrator (Relative gene expression = 1). Not inoculated control plants are represented by "C" and inoculated plants by "I". Data from one representative experiment out of two independent experiments for each of the experimental conditions are presented. Error bars represent SE. Letters above the bars indicate statistically significant differences.

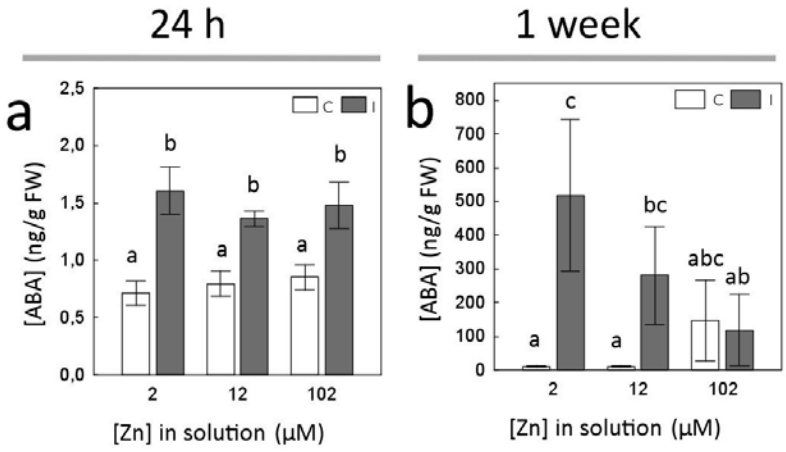


Figure 3.14: ABA concentration in leaves of *N. caerulescens* growing in nutritive solution with Zn (2, 12 or 102 µM) under two different experimental conditions. Half of the plants were inoculated with *A. brassicicola* and samples taken 24 h (a) or 1 week after inoculation (b). Not inoculated control plants are represented by "C" and inoculated plants by "I". Data from one representative experiment out of two independent experiments for each of the experimental conditions are presented. Error bars represent SE. Letters above the bars indicate statistically significant differences.

the Zn hyperaccumulation trait and the defence mechanisms in *N. caerulescens* (Figure 3.15).

3.2.5 *Glucosinolates*

Glucosinolates are glucose and sulphur-based compounds of the secondary metabolism of some plant families that play a role in defence against pathogens and herbivores (see introduction, 1.3.5 on page 23). Glucosinolate concentrations in shoots of *N. caerulescens* growing in increasing Zn concentrations have been measured before in the study from Tolra et al. (2001), where total GSs, as well as Sinalbin, the most abundant GS in *N. caerulescens*, decreased in shoots with the higher Zn concentrations, while increased in the roots. Zn has also been found to be negatively correlated with shoot GS concentrations in studies by Noret et al. (2007) in different populations of *N. caerulescens* and, more recently, by Asad et al. (2015). In agreement with them, a field study with *A. halleri* populations found a slight indication of a trade-off between glucosinolate-based organic and inorganic defences, but only in one chemotype and not clearly with all the metals tested (Kazemi-Dinan et al. 2015). However, another recent study from Foroughi et al. (2014) has shown the opposite, as total glucosinolates concentrations measured by liquid chromatography-mass spectrometry (LC-MS) were positively correlated to increasing Zn

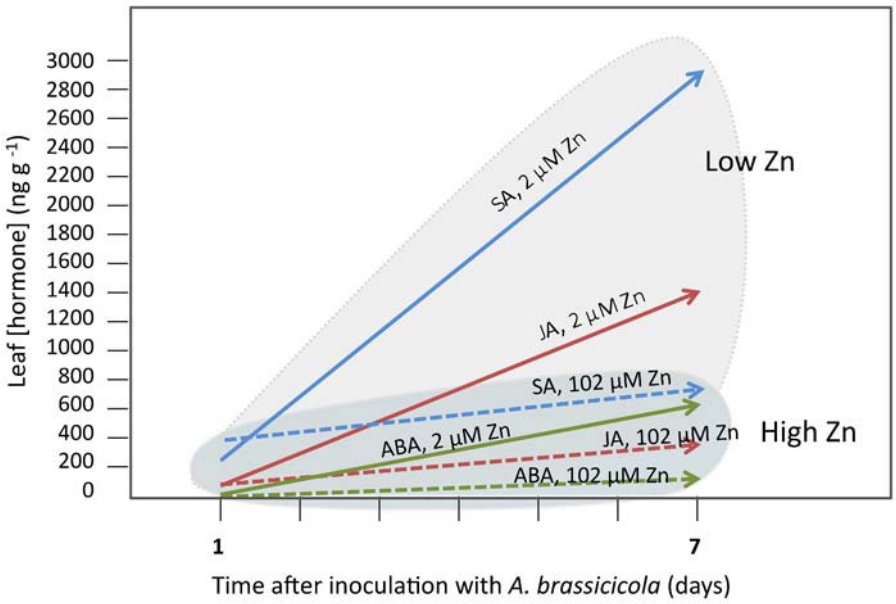


Figure 3.15: Leaf SA, JA and ABA concentration 24 h and one week after the inoculation with *A. brassicicola*. A trade-off between Zn and the hormones concentrations was only reported one week after the inoculation, where the high Zn-treated plants (High Zn) presented less hormone concentration than the low Zn-treated (Low Zn).

shoot concentration in the hyperaccumulator. In our study, the trade-off between Zn and GSs concentration in shoots of *Noccaea caerulescens* described by Tolra et al. (2001) and reported in other models, was also observed (Figure 3.16 on page 63). Here, total glucosinolate concentration in shoots of non–inoculated *N. caerulescens* decreased with increasing Zn concentration in the solution. Plants under 2 µM Zn treatment concentrated above 70 µg/g in fresh leaf, while in plants treated with 12 µM, GSs concentration did not reach 50 µg/g and those submitted to the highest Zn treatment, 102 µM, presented GSs in a concentration close to zero.

Although the relation between Zn and other metals and GSs has been assessed before in different studies, as well as their effect on herbivores feeding on the plants (Noret et al. 2004; Asad et al. 2015), the influence of Zn over GSs has not been quantified before in a hyperaccumulator inoculated with a foliar pathogen. In our study, contrary to the GS profile in control plants, GS in inoculated plants was positively correlated with Zn concentration (Figure 3.16). Inoculated plants from the 2 and 12 µM Zn treatment presented low GS concentration, less than 25 µg/g as an average. Contrastingly, inoculated plants submitted to 102 µM of Zn in solution, accumulated levels of GS close to 90 µg/g. Thus, inoculated plants with high Zn concentrated had approximately as much GS as healthy plants with low Zn.

According to previous data (Figures 3.3 on page 44 and 3.5 on page 45), lower Zn concentration in shoots determined a greater susceptibility to *A. brassicicola*. These low Zn-treated plants presented a decrease in total GS concentration one week after inoculation, possibly as a consequence of GSs pool being depleted by transformation into the GS active product isothyocianate. Opposed to that, plants growing at 102 µM Zn, which coped better with infection, presented low GS values when healthy and high values one week after the inoculation. This suggests that in inoculated, high Zn-treated plants *Alternaria* may have previously induced GSs synthesis, but as infection did not progress, GSs remained accumulated into the inactive form. Consistently, according to Foroughi et al. (2014), the induction of glucosinolate synthesis has been found to be mediated by jasmonate signalling, that was activated 24 h after the inoculation in our model. This view would not support the trade-off hypothesis in early stages of the disease. However, with the present data we are unable to determine how both synthesis and degradation of GSs contribute to generate the described differences. Quantifying the expression of genes involved in GSs synthesis as well as measuring the myriosinase activiy would help to clarify the role of Zn and GS during infection in *N. caerulescens*. In any case, Zn did not mediate a pre-activation of organic defences through GSs.

3.3 CONCLUDING REMARKS

To sum up, in the pathosystem *Noccaea caerulescens - Alternaria brassicicola*, *Alternaria* growth declined with increased leaf Zn concentrations. Nonetheless, neither Zn uptake by the roots nor its translocation to the shoots was favored by *Alternaria* infection. The leaf Zn concentration reached at the highest Zn supply was not related to an increase in the expression of Zn-transporters genes *HMA3* or *HMA4*, and showed a negative relationship with leaf Ca, Mg, S and Mo in the inoculated plants. One week after the inoculation, a trade-off response mechanism was found between leaf Zn and the concentration of the plant defence regulators JA, SA and ABA, but not the Et precursor, ACC. Leaf glucosinolates also showed a negative relationship with leaf Zn, but, in contrast, one week after the inoculation, glucosinolates concentration increased in the leaves of high Zn-treated plants. Further work is required to understand the relative role of Zn and glucosinolates in the control of *Alternaria* infection.

A summary of the evidences found in this study, supporting the different hypothesis to explain metal defence is presented in Table 3.4 on page 64.

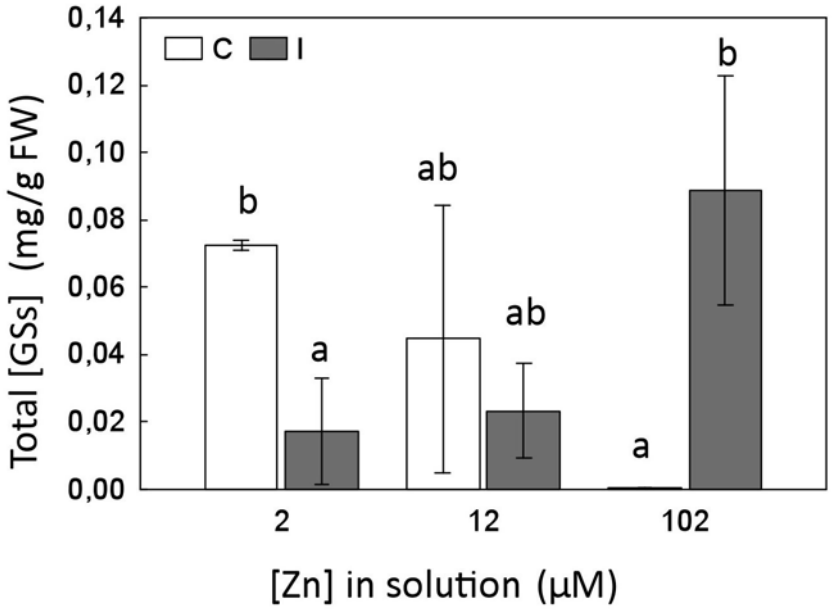


Figure 3.16: Total glucosinolates (GSs) concentration in leaves of *N. caerulescens* treated with Zn (2, 12 or 102 µM) for 5 weeks. Half of the plants were inoculated with *A. brassicicola* and samples taken one week after inoculation. Two independent experiments were performed. Each replicate for the GSs analyses combine plant material from two independent samples. The statistical analyses consider data from both experiments together. Factorial ANOVA and Fisher LSD test were performed. Error bars represent SE. Letters above the bars indicate statistically significant differences.

Table 3.4: Support for the current hypothesis in metal defence in our model, regarding the role and interaction of inorganic and organic defences and taking as a premise that Zn protects *N. caerulescens* from *A. brassicicola*.

Hypothesis	For	Against
Inorganic defences	Leaf [Zn] potentially toxic for *Alternaria*	
Trade-off	↓SA, JA and ABA in ↑[Zn] plants (1 week)	↑SA, JA and ABA in all infected plants (24 h)
	↓[GS] in ↑[Zn] healthy plants (1 week)	↑[GS] in ↑[Zn] infected plants (1 week)
Joint Effects	↑GSs in ↑[Zn] infected plants (1 week)	↑SA, JA and ABA in all infected plants (24 h)
		↓GSs in ↑[Zn] healthy plants (1 week)

4

THE ROLE OF ZN IN THE ARABIDOPSIS THALIANA - ALTERNARIA BRASSICICOLA / BOTRYTIS CINEREA PATHOSYSTEMS

The metal defence hypothesis, initially developed for metal hyperaccumulator plants with high metal concentrations, has recently been extended further including metal concentrations below the hyperaccumulator level (Coleman et al. 2005; Cheruiyot et al. 2013). In the case of Zn, concentration below the toxicity threshold for non-hyperaccumulating species are less likely to directly harm pathogens, as these threshold concentrations are relatively low in comparison to those found in hyperaccumulators. However, it has been found that close to toxicity threshold concentrations of some metals can elicit defence mechanisms in non-hyperaccumulating plants (Cabot et al. 2013). Enhanced Zn concentrations can elicit the plant hormonal pathways and there probably is considerable cross-talk between ionic stress and biotic stress signalling in the plants (Poschenrieder et al. 2006). In this scenario it is likely that plants exposed to slightly enhanced concentrations of the metal will undergo a pre-activation of the defence response signalling and, in consequence, will more efficiently cope with the pathogen attack.

To explore this hypothesis, in our studies, *A. thaliana* was used as a model for non-hyperaccumulator plants. Two pathosystems were stablished by infecting *A. thaliana* with *Alternaria brassicicola* or *Botrytis cinerea*.

Three sets of results are presented:

1. Influence of Zn on *Arabidopsis thaliana* (Section 4.1).
2. Interaction of Zn in the defence response in *Arabidopsis thaliana* and *Alternaria brassicicola* pathosystem (Section 4.2 on page 70).
3. Interaction of Zn in the defence response in *Arabidopsis thaliana* and *Botrytis cinerea* pathosystem (Section 4.3 on page 83).

4.1 INFLUENCE OF ZN ON ARABIDOPSIS THALIANA

4.1.1 *Materials and Methods*

4.1.1.1 *Plant growth and experimental conditions*

For plant growth measurements see Chapter 3, Subsection 3.1.3 on page 38 and for experimental conditions see in this Chapter 4.2.1.1 on page 70 and 4.3.1.1 on page 83.

Table 4.1: Experiments performed and parameters measured to assess Zn influence over *A. thaliana*.

Experiment	Plant growth	[Ion]	[Proteins]	[Pigment]
I	x	x	x	
II	x	x	x	x
III	x	x	x	x

4.1.1.2 *Ion concentration*

Inorganic nutrition was quantified as described in Chapter 3, subsection 3.1.4 on page 39.

4.1.1.3 *Proteins and pigments*

Photosynthetic pigments and soluble proteins were extracted from fresh leaves using bicine buffer (pH 8) (Lawlor et al. 1989). Photosynthetic pigments were extracted in 100% ethanol (1:10 v/v). The mix was kept at dark and cold for 10 min and then centrifuged at 12000 g during 2 min. The supernatant was collected and the absorbance was measured at 665, 649 and 470 nm for determination of chlorophyll a, chlorophyll b and the sum of carotenes and xanthophyll according to Lichtenthaler and Wellburn (1983). The rest of the leaf and bicine homogenate was centrifuged during 2 min at 12000 g. The concentrations of soluble proteins were determined following the method described by Bradford (1976).

4.1.2 *Results*

4.1.2.1 *Plant growth*

The growth of *A. thaliana* was affected by Zn supplementation in the solution (Figure 4.1). Root dry weight decreased in plants under the 27 µM Zn treatment, which may be a consequence of metal being over the toxicity threshold for *A. thaliana*. Therefore, in the following tests, the 27 µM Zn group was excluded from the experimental design.

4.1.2.2 *Mineral nutrition*

Zn concentration in shoots of *A. thaliana* varied from 100 to 250 µg/g of dry weight in plants submitted to 2 µM of Zn in the nutritive solution, in all the experiments performed. The Zn concentrated by plants under the 12 µM and the 27 µM Zn treatment remained around 500 and 900 µg/g in the aerial tissues, respectively. Zn was greatly accumulated in roots, from 3 to 4 times more than in shoot, reaching up to 3000 µg/g in plants treated with 27 µM of Zn (Figure 4.2).

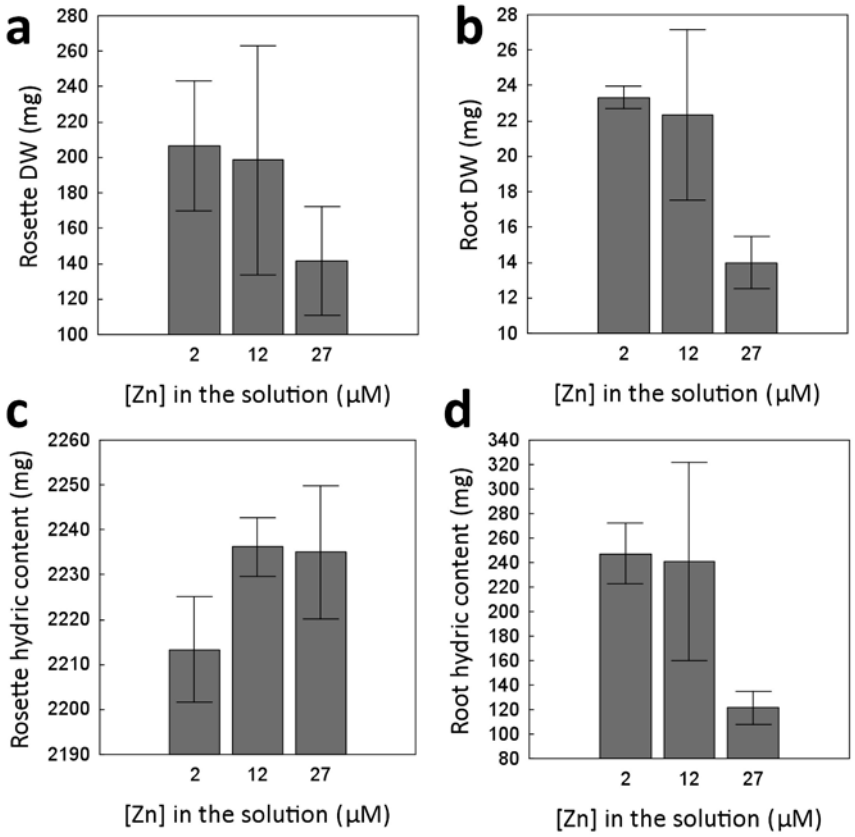


Figure 4.1: Growth of shoots (a) and roots (b) and hydric content of shoots (c) and roots (d) of 3-month-old *A. thaliana* treated with 2, 12 or 27 µM Zn for 5 weeks. Data from one representative experiment among three were selected. Error bars represent SE.

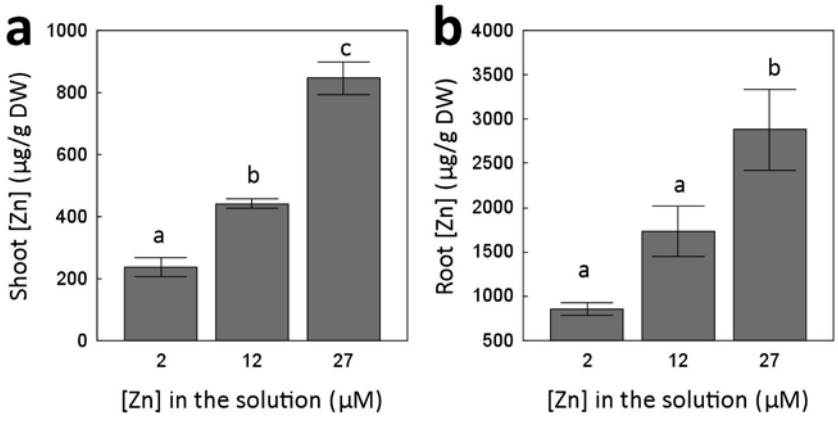


Figure 4.2: Zn concentrations in shoots (a) and roots (b) in 3-month-old *A. thaliana* treated with 2, 12 or 27 µM Zn for 5 weeks. Data from one representative experiment were selected. One-way ANOVA was performed, followed by LSD Fisher test. Error bars represent SE. Letters above the bars indicate statistically significant differences.

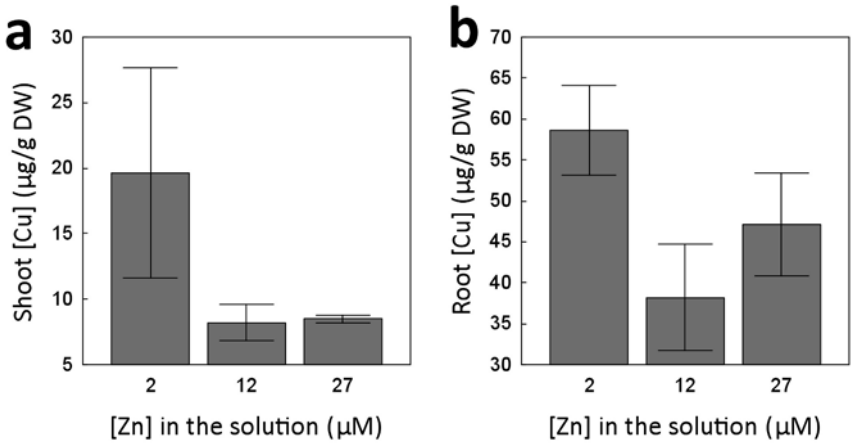


Figure 4.3: Cu concentrations in shoots (a) and roots (b) of 3-month-old *A. thaliana* treated with 2, 12 or 27 µM Zn for 5 weeks. Data from one representative experiment were selected. Error bars represent SE.

In both shoots and roots, Cu concentration tended to decrease already with 12 µM of Zn in the solution (Figure 4.3). Although there were no statistical differences among the treatments, the tendency to decrease was consistent along the experiments performed and may have influenced the plant growth.

The concentration of Mo was lower in roots of 12 and 27 µM Zn-treated plants in the two experiments performed. In shoots, it also tended to decrease only with 12 µM of Zn, but not with 27 µM (Figure 4.4 on the facing page).

4.1.2.3 *Proteins and pigments*

The concentrations of the total soluble protein and of the pigments chlorophyll a, b and both xantophylls and carotenes were quantified in the aerial tissues. While Zn did not have influence over the concentration of any of the pigments, it determined a higher concentration of proteins in plants treated with 27 µM of Zn (p= 0,037). This pattern was observed in both of the experiments performed (Figure 4.5 on the next page).

4.1.3 *Concluding remarks*

As expected, increasing Zn concentration in the solution significantly conditioned a higher Zn concentrtion in *A. thaliana* tissues. Nonetheless, the higher Zn concentration to which plants were submitted, 27 μM, tended to influence negatively *A. thaliana* growth.

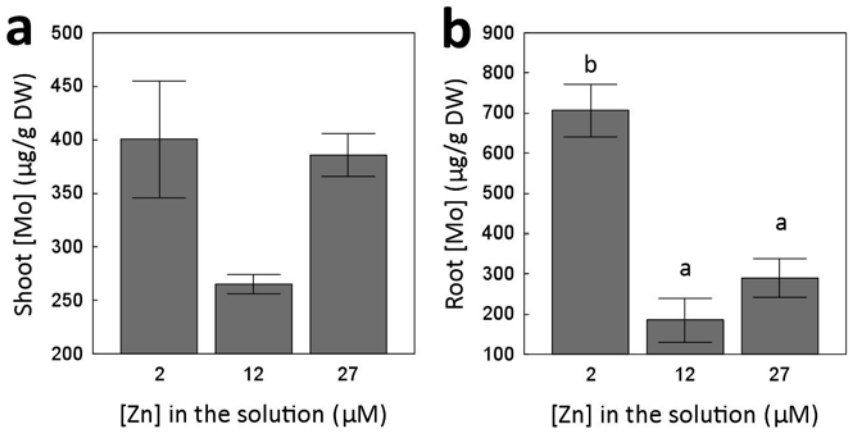


Figure 4.4: Mo concentrations in shoots (a) and roots (b) of 3-month-old *A. thaliana* treated with 2, 12 or 27 µM Zn for 5 weeks. Data from one representative experiment were selected. One-way ANOVA, followed by LSD Fisher test when p<0,05, were performed. Error bars represent SE. Letters above the bars indicate statistically significant differences.

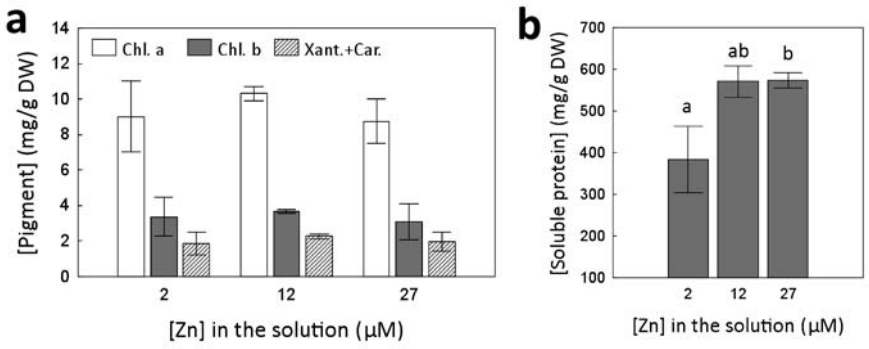


Figure 4.5: Pigments chlorophyll a (Chl. a), chlorophyll b (Chl. a) and xanthophyll and carotene (Xant. + Car.) (a) and total soluble protein (b) concentration in shoots 3-month-old *A. thaliana* treated with 2, 12 or 27 µM Zn. Data from two independent experiments are presented. One-way ANOVA was performed for each of the pigment concentration and the non-parametric Mann-Whitney U test for the protein concentration. Error bars represent SE. Letters above the bars indicate statistically significant differences.

Table 4.2: Experiments performed in the *A. thaliana* – *A. brassicicola* pathosystem

	Experiment	
	WT (Col-0)	WT (Col-0) and defence response mutants
Plant growth	x	
Disease symptoms quantification	x	
Alternaria quatification		x
[Ion]		x
Stress marker genes expression	x	
Hormone concentration		x

4.2 ARABIDOPSIS THALIANA - ALTERNARIA BRASSICICOLA PATHOSYSTEM

4.2.1 *Materials and methods*

4.2.1.1 *Plant growth and experimental conditions*

Two experiments were conducted using the *A. thaliana - A. brassicicola* pathosystem (Table 4.2). In both of them, plants were submitted to treatments of 2 and 12 µM of $ZnSO_4$ supplied to the hydroponic solution during 5 weeks (see solution in Chapter 3, Table 3.1 on page 38).

In the first experiment, seeds from *A. thaliana* WT (Col-0) were germinated and grown in vermiculite during 9 weeks at 24.5 °C with a photoperiod of 7 h light, and a light intensity of 156 PAR. Adult plants were transferred to 100 ml pots and were grown in aerated hydroponic solution, with the correspondent treatments during 5 weeks. At week four, two leaves from half of the plants were inoculated with *A. brassiciola* as described in Chapter 3, Subsection 3.1.2 on page 38. High relative humidity was maintained around the plant to favor pathogen infection by placing inoculated plants over a tray with water and covering them with perforated plastic bags. After one week, plants were harvested.

Seeds from *A. thaliana* WT and the mutant lines presented in the introducion (Section 1.4 on page 24) and in Table 4.3, were germinated in Petri dishes with agar. The criteria to select the mutants was to cover the three main hormonal signalling pathways involved in the immune response (SA, JA and Et) and the pathway of the camalexin synthesis, one of the major phytoalexins affecting *A. brassicicola* pathogenesis (Pedras and Minic 2014). Homozigotic *coi*1 mutants were selected in 50 µM MeJA MS agar, as did not suffer from

Table 4.3: *A. thaliana* mutant lines used in the experiments and the effect of the mutation over some of the hormonal signalling marker genes. ( ) indicates an indirect involvement.

Mutant	Pathway disrupted	Hormonal pathway involved	Gene affected down-stream
npr1 SA-nonresponsive	NPR1 interaction with TGA transcription factors for synthesis of *PR* genes	SA	*PR1*
pad1 Camalexin deficient	PAD1 induction of synthesis of PAD3 for synthesis of camalexin	(JA/Et)	(*PDF1.2*)
coi1 JA-insensitive	Interaction of COI1 with JA-Ile for activation of transcription factor MYC2 and synthesis of *VSP*2 and *LOX*2	JA	*VSP2*, *LOX2*, *PDF1.2*
etr1 Et-insensitive	Interaction with Et receptor	Et	*PDF1.2*

root growth inhibition and anthocyan production one week after germination Ramírez et al. (2010). After one week, all seedlings were transferred to nutritive solution with 2 µM of Zn and were grown hydroponically for 8 weeks under the conditions described above. Treatments with 2 and 12 µM Zn were administrated in 100 ml pots for 5 weeks. In the beginning of the week four, half of the plants were inoculated with *A. brassicicola*, as described in Chapter 3, Subsection 3.1.2 on page 38, and all the plants were kept under a plastic greenhouse-like cover with vaporized water to ensure pathogen penetration.

4.2.1.2 *Fungal material and inoculation*

A. brassicicola strain, culture and spore suspension preparation is described in Chapter 3, Subsection 3.1.2 on page 38.

4.2.1.3 *Plant growth measurements*

Method for plant growth quantification is described in Chapter 3, Subsection 3.1.1 on page 37.

Table 4.4: *A. thaliana* defence signalling marker genes quantified and the hormonal pathway they are related to.

Hormonal pathway		Gene
SA	*PR*1	Pathogenesis-related 1
	*BGL*2	ß-1,3-Glucanase 2
JA/Et	*PDF*1.2	Plant defensin 1.2
	CHIB	Basic chitinase
JA	*LOX*2	Lipoxygenase 2
	*VSP*2	Vegetative storage protein 2

4.2.1.4 *Ion concentration*

The procedure for mineral nutrition quantification is detailed in Chapter 3, subsection 3.1.4 on page 39.

4.2.1.5 *Disease symptoms*

The degree of the disease caused by *Alternaria* in the leaves was assessed by measuring the affected area (see Chapter 3, Subsection 3.1.5 on page 39).

4.2.1.6 *Hormone concentration*

The concentration of SA, JA, ABA and the precursor of Et, ACC, were quantified in the leaves of *A. thaliana* WT and the four mutants used in our experiment (Table 4.3 on page 71). The protocol for hormone extraction and quantification is described in Chapter 3, Subsection 3.1.7 on page 41.

4.2.1.7 *Relative gene expression*

The expressions of six genes related to the plant defence response (See Introduction, Subsection 1.3.4 on page 16 and Table 4.4) were quantified by means of qPCR. The gene of the Vegetative Storage Protein 2, *VSP*2, a marker gene for the JA pathway, was included in the set of genes that had been already quantified in *Noccaea* (primer sequences detailed in the Appendix A, v on page 125). For specifications in the protocol see Chapter 3, Subsection 3.1.6 on page 39.

4.2.2 *Results and discussion*

4.2.2.1 *Plant growth*

The growth of *A. thaliana* plants, measured through shoot and root weight and number of leaves, appeared to decrease in inoculated plants as a consequence of the infection with *A. brassicicola* (Figure

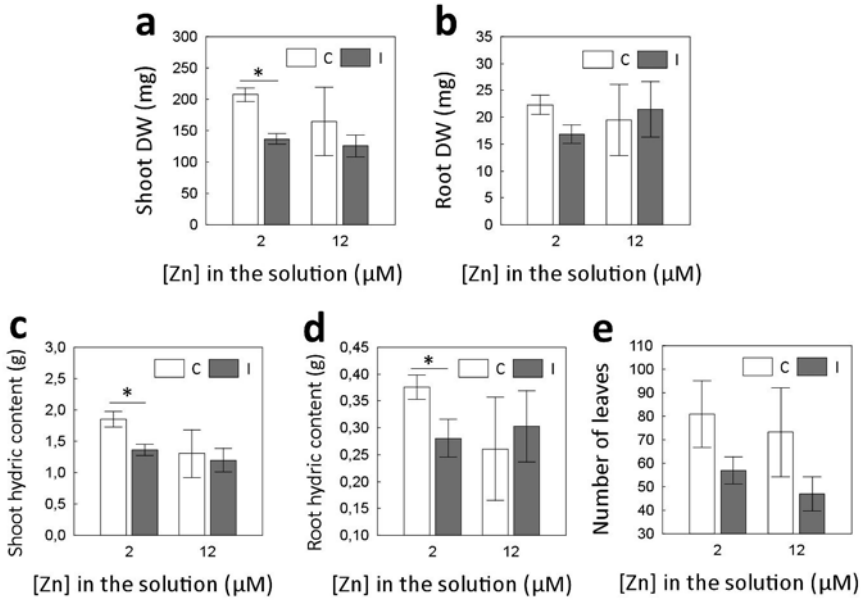


Figure 4.6: Shoot and root dry weight and leaf number of 3-month-old *A. thaliana* treated with 2 or 12 µM of Zn for 5 weeks. 4 weeks after Zn addition, half of the plants in each treatment were inoculated with *A. brassicicola* and measures taken one week after the inoculation. Factorial ANOVA was performed to check for interaction between factor [Zn] and inoculation and T-test was run to check for differences between two groups. Inoculated plants are represented by "I" and control, non-inoculated plants, by "C". Error bars represent SE. * Indicates statistically significant differences.

4.6). Plant growth was as well slightly reduced by Zn at a concentration of 12 µM, although no significant differences were found with the plants growing at 2 µM of Zn. However, in the plants submitted to the high Zn treatment, *Alternaria* did not cause significant growth reduction compared to the non-inoculated plants. In this case, Zn may have played in favor of the plant defences and thus, the costs of the metal accumulation reduced the additional costs of the response against the pathogen.

4.2.2.2 *Ion concentrations*

The effect of the inoculation with *A. brassicicola* over the ionic content in tissues of *A. thaliana* Col-0 and the four stress pathways mutants was measured. Zn uptake in the mutants was independent from the altered defence pathways, as none of them showed a different behavior regarding Zn accumulation in leaves compared to Col-0. Consequently, the absence of cross-talk between Zn accumulation and the immune response related to the disrupted pathways in the mutants, would allow us to differenciate a possible role of the accumulated

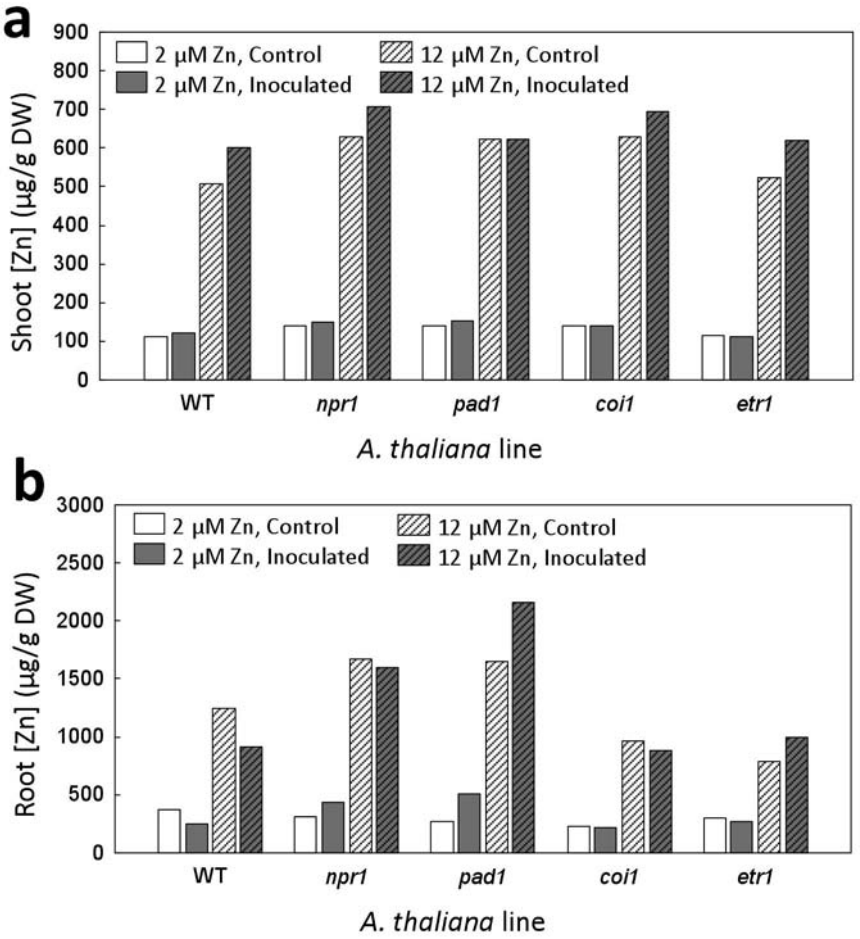


Figure 4.7: Zn concentrations in shoots (a) and roots (b) of 3-month-old *A. thaliana* WT and four defence defective mutants (*npr*1, *pad*1, *coi*1 and *etr*1) treated with 2 or 12 µM Zn for 5 weeks and inoculated with *A. brassicicola* in the 4th week. Data from pooled samples from one experiment are shown.

Zn as an inorganic defence and so it renders the mutants suitable for further analysis (Figure 4.7).

4.2.2.3 *Alternaria growth*

ZN TOXICITY AND ALTERNARIA GROWTH IN VITRO A theoretical correspondence between Zn in leaves of *A. thaliana* and Zn in solution was established. Data on *A. brassicicola* tolerance to Zn *in vitro* showed that the pathogen growth was reduced to the half at Zn concentrations close to 0.5 mM (EC_{50} = 480 µM) and Zn above 2 mM did not allow any growth (see Chapter 3, Subsection 1.4.1.2 on page 25 and Figure 3.6 on page 47). According to the average Zn concentration in *A. thaliana* rosette under 2 and 12 µM Zn treatments and the value of the EC_{50} for Zn and *Alternaria* (Figure 4.8), only plants

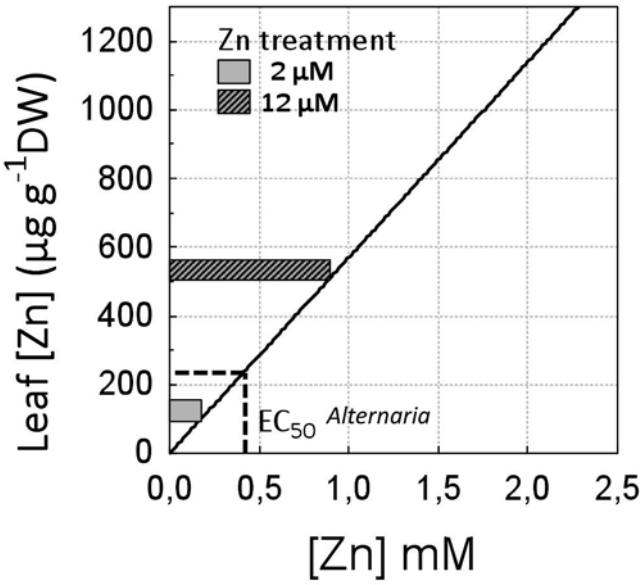


Figure 4.8: Theoretical correspondence between [Zn] in dry leaf of *A. thaliana* and [Zn] in solution. [Zn] in the leaves of plants submitted to the different Zn treatments (horizontal bars) and the tolerance of *B. cinerea* to Zn given as EC_{50} (dashed line) are represented.

treated with 12 µM of Zn would bear enough Zn to be potentially toxic for the fungus.

ALTERNARIA GROWTH IN VIVO In line with the hypothesis of a potential protection driven by Zn in *A. thaliana* 12 µM Zn-treated plants, the percentage of the necrotic and chlorotic area in the inoculated leaves tended to be smaller in this group. This tendency was not clearly perceptible to the eye and the high variability within the samples did not allow to spot statistical differences between the treatments (Figure 4.9). However, a close-up analysis at the molecular level provided a more sensitive approach to evaluate the spread of the disease. The expressions of both, plant and fungi, housekeeping *AtTubulin* and *AbActin*, were quantified by means of qPCR (Figure 4.10). Interestingly, data on relative expression of the genes showed that in WT non-treated plants, the pathogen grew twice as much as in the 12 µM Zn-treated leaves. Thus, Zn provided protection against *Alternaria* in WT *A. thaliana* plants. Surprisingly, none of the mutants was sensibly more susceptible to *Alternaria* than the lower-Zn WT plants. This results contrast with the findings from Thomma et al. (1998), Thomma et al. (1999) and Wees et al. (2003), where specially the JA-insensitive, *coi1*, mutant was less resistant to the disease caused by *Alternaria.*

Even if Zn concentration in the plant tissues did not vary in the different WT and mutant lines (Figure 4.7 on the preceding page), the

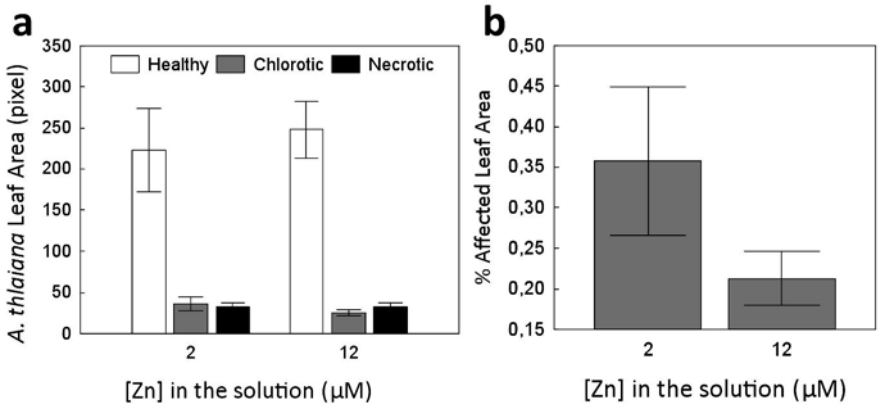


Figure 4.9: Leaf affected area in infected leaves of 3-month-old *A. thaliana* treated with 2 or 12 µM of Zn for 5 weeks and one week after inoculation with *A. brassicicola*. Quantification of affected area was absolute, taking into account the healthy, chlorotic and necrotic areas in the leaf (a), or relative to the total leaf area (b), where affected area is the sum of the necrotic and the chlorotic areas. Error bars represent SE.

protective action of Zn reported in the WT was not observed in the mutants. In all the four mutants, the pathogen growth was not significantly different in Zn-treated and non-treated plants and it was never lower than the quantified in WT 12 µM Zn-treated plants (Figure 4.10). In any case, if Zn alone would be toxic for the pathogen at the average concentration in the above-ground tissues of treated plants (550 µg/g of DW), the metal would also offer some protection in the impaired-defence pathways mutants, as well as in the WT. Not surprisingly, according to Coleman et al. (2005), metal concentrations in tissues from non-hyperaccumulator plants are considerably lower to that in hyperaccumulators and protection by direct effect of the metal in the first ones is possible but less probable than in the hyperaccumulators. In our experiments, as Zn did not substitute the defect in the organic defences, it is suggested that Zn as an inorganic defence is not a key factor in the protection of *A. thaliana* against *A. brassicicola* at the concentrations tested.

4.2.2.4 *Hormone concentration*

As the hypothesis that Zn alone can act as an inorganic defence against *Altenaria* infection in the non-hyperacccumulator *A. thaliana* was not supported by our results, further attention was focused on the possible interaction of Zn with the organic defences. The concentrations of the three hormones JA, SA, ABA and the precursor of Et, ACC, were quantified in leaves of WT and the mutant lines of *A. thaliana* (Figure 4.11 on page 79). First results show that, as a general pattern, in WT plants no enhancement of hormone synthesis was reg-

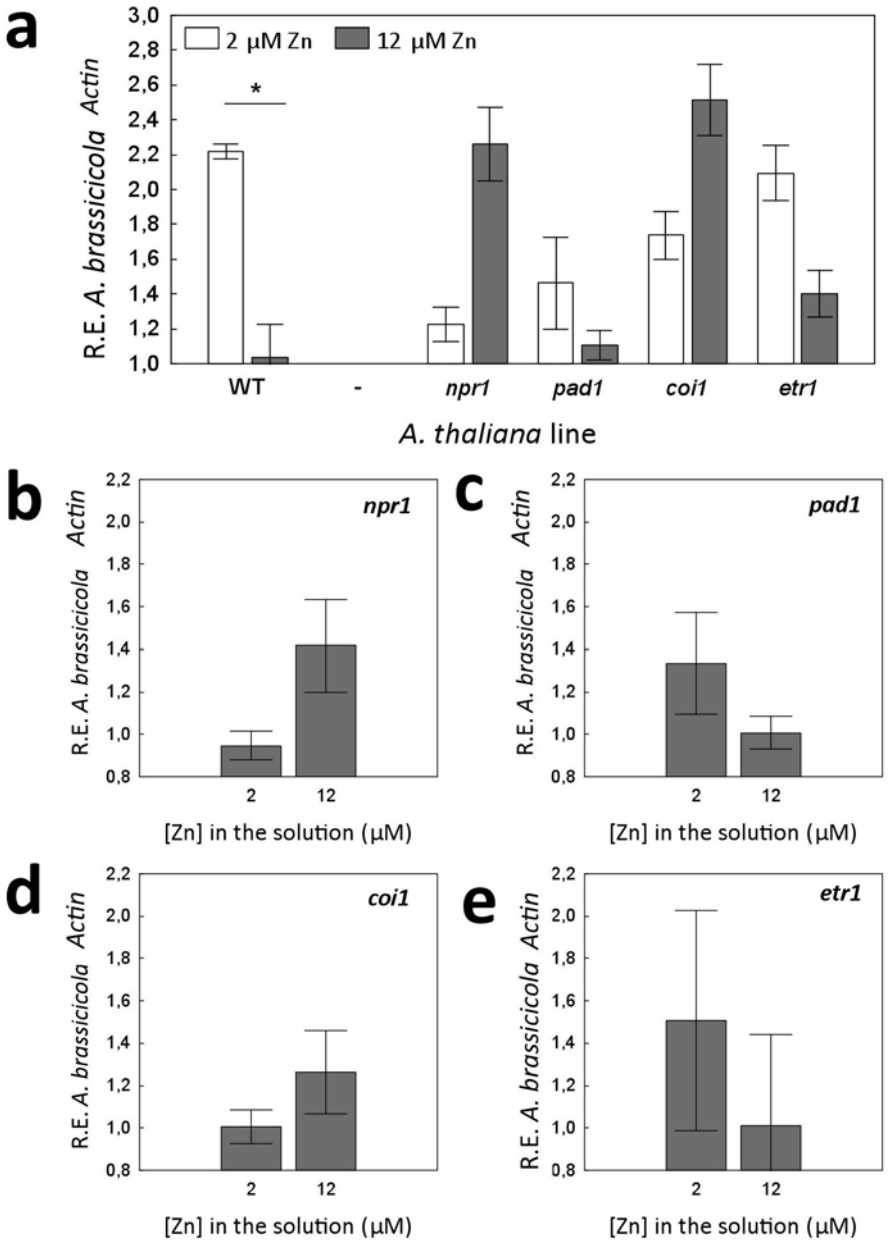


Figure 4.10: Relative gene expression (R.E.) of the *Actin* constitutive gene from *A. brassicicola* in leaves of 3-month-old *A. thaliana* WT and four defence response defective mutants (*npr*1, *pad*1, *coi*1 and *etr*1) treated with 2 or 12 µM of Zn for 5 weeks. 4 weeks after Zn addition, half of the plants in each treatment were inoculated with *A. brassicicola*. *Tubulin* gene from *A. thaliana* was used as a reference gene. The calibrator group (R.E.=1) was the group with lower absolute gene expression in each case: a) WT plants under 12 µM Zn treatment, b) *npr*1 plants under 2 µM Zn treatment, c) *pad*1 plants under 12 µM Zn treatment, d) *coi*1 plants under 2 µM Zn treatment and e) *etr*1 plants under 12 µM Zn treatment. Error bars represent SE. * Indicates statistically significant differences.

istered, while in the mutants, more response to Zn treatment than to *Alternaria* infection was found.

In *npr*1 plants, where the SA-induced synthesis of PR proteins is disrupted (see Introduction, Subsection 1.4.1 on page 24, Figure 1.10 on page 26), the most marked effect observed was on JA and ACC synthesis. Data from our experiment showed that Zn mediated an increase of both the concentration of JA and the precursor of Et, as plants under the 12 µM Zn treatment synthesized higher levels of JA and ACC. When the SA pathway was truncated downstream, JA and Et seem to compensate the defect for maintaining the Zn homeostasis. A markedly higher concentration of JA in Zn-treated and infected plants unable to activate the SA pathway, where there has been a elicitation of stress response (Figure 4.11: b, *npr1*), possibly highlights the antagonism between SA and JA (see Introduction, Subsection 1.3.4 on page 16). No effect was observed regarding SA synthesis, probably due to the existance of an alternative NPR1 non-dependent signalling pathway (Shah 2003).

The *etr*1 plants, lacking the Et receptor and having affected the downstream expression of genes such as *PDF*1.2 (see Introduction, Subsection 1.4.1 on page 24, Figure 1.10 on page 26), also responded to Zn treatment, but not to infection with *Alternaria*. The strongest response was observed in SA concentrations and, to less extent, in JA in plants treated with 12 µM Zn. This suggests that the Et pathway is important in Zn homeostasis and if it is defective, SA and JA are enhanced to cope with excess of Zn.

In the camalexin defective mutant, *pad*1 (see Introduction, Subsection 1.4.1 on page 24, Figure 1.10 on page 26), ACC synthesis was affected by Zn, as it seemed to be downregulated in the lower Zn-treated plants and enhanced in plants growing at 12 µM of Zn. Moreover, SA synthesis appeared to be progresively enhanced in inoculated, Zn-treated and inoculated plus Zn-treated plants. Coherently, *pad1* mutants inoculated with *A. brassicicola,* have been described to present an enhanced expression of *PR1*, typically induced by SA (Glazebrook et al. 2003). In this case, additionally, a possible enhancement of SA synthesis driven by Zn in the inoculated plants was observed (Figure 4.11: a, *pad1*).

In the *coi*1 mutants, where there is a defect in the JA pathway that affects the synthesis of genes such as *LOX*2 and *VSP*2 (see Introduction, Subsection 1.4.1 on page 24, Figure 1.10 on page 26), there was a clear interaction of Zn and SA, as SA concentration was increased in the Zn-treated plants (Figure 4.11: a, *coi1*).

A summary of the most remarkable tendencies found is presented in Figure 4.12. Nevertheless, these results should be analyzed cautiously, as more experimental replicates would help to elucidate the influence of Zn in the different stress signalling pathways.

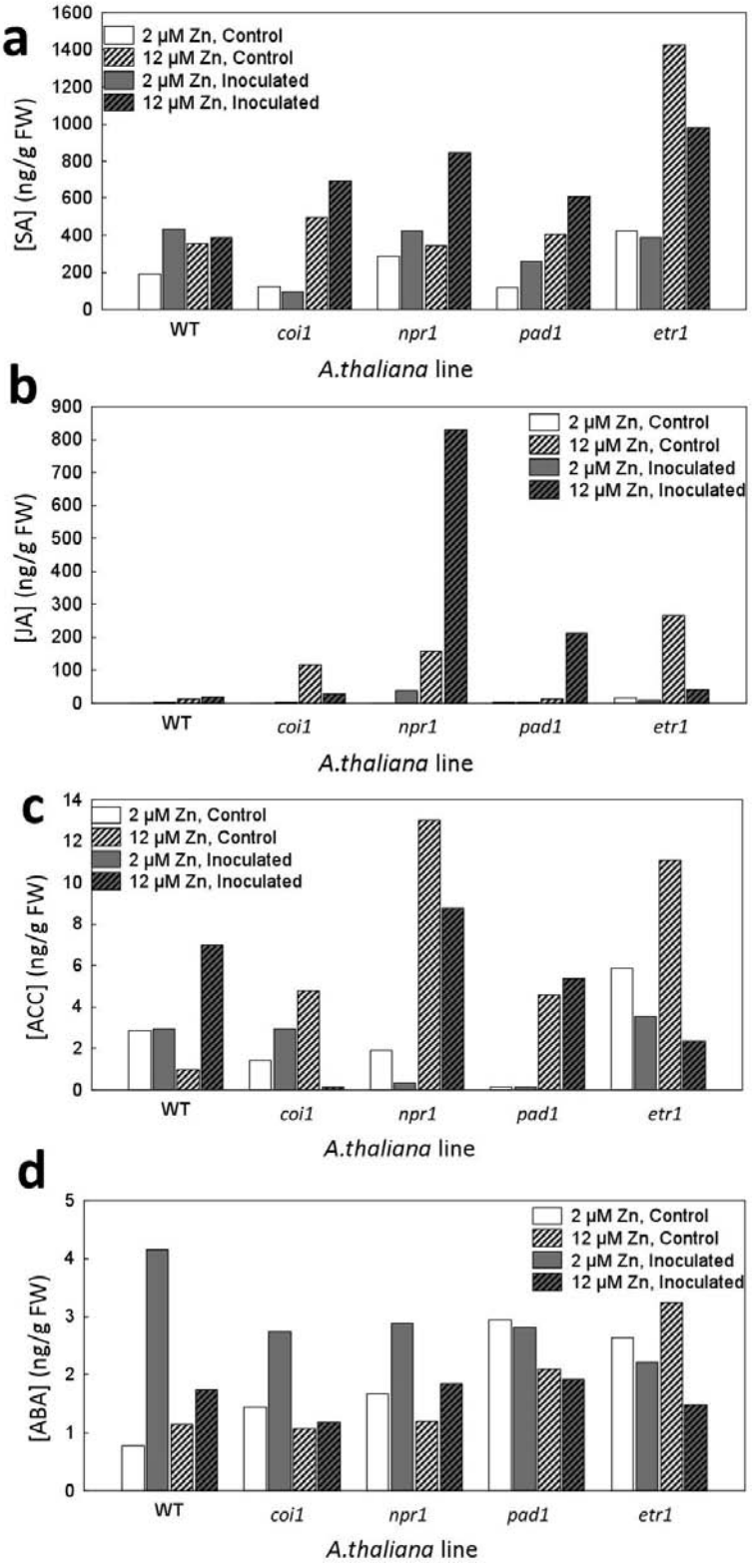


Figure 4.11: Hormone concentration of SA (a), JA (b), the Et precursor, ACC, and ABA (d) in leaves of 3-month-old *A. thaliana* WT and four defence response defective mutants (*coi*1, *npr*1, *pad*1 and *etr*1) treated with 2 or 12 µM of Zn for 5 weeks. 4 weeks after Zn addition, half of the plants in each treatment were inoculated with *A. brassicicola*. Data from pooled samples from one experiment are presented.

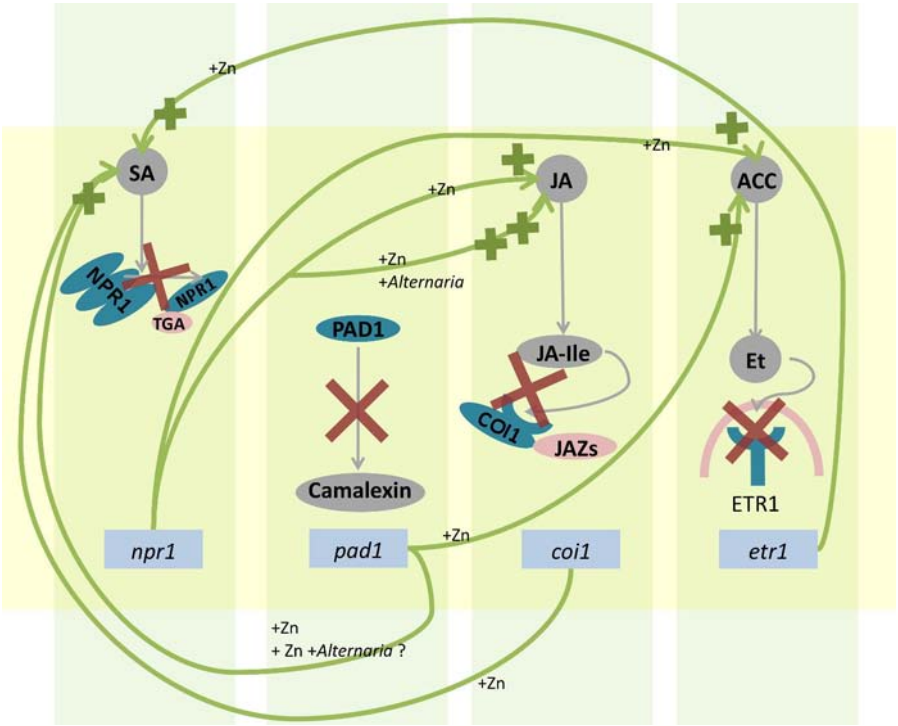


Figure 4.12: Putative effect of the stress response impairment over the SA, JA and ACC concentrations in four *A. thaliana* mutants submitted to Zn treatments of 2 or 12 µM of Zn for 5 weeks and inoculated in the last week with *A. brassicicola*. SA: salicylic acid. *npr*1/NPR1: non-expressor of *PR* genes. TGA: Transcription factor. *pad*1/PAD1: phytoalexin-deficient 1. JA: jasmonic acid. JA-Ile: jasmonic acid conjugated with isoleucine. JAZs: jasmonate-zim domain (proteins). *coi*1/COI1: coronatine insensitive 1. ACC: 1-Aminocyclopropane-1-carboxylic acid. *etr*1/ETR1: ethylene receptor 1. (+)/(++) indicates an enhacement / remarkable enhancement of the hormone (or ACC in the case of Et) synthesis. +Zn: induced in 12 μM Zn-treated plants. +*Alternaria*: induced in *A. brassicicola* inoculated plants.

4.2.2.5 *Relative gene expression*

As tentative results on hormones concentrations did not provide us with enough information to allow a better understanding on how the plant stress pathways interact with Zn and *Alternaria*, the relative expressions of 6 genes related to the SA (*BGL*2 and *PR*1), JA/Et (*PDF*1.2 and *CHIB*) and JA (*VSP*2 and *LOX*2) stress signalling pathways were quantified in *A. thaliana* WT plants (see Table 4.4 on page 72 and Figure 1.7 on page 18).

Interestingly, the expression pattern of the genes from the JA/Et and the SA pathway was similar, responding to inoculation with *Alternaria,* all except *PR1,* and, what is more remarkable, amplifying their expression with a combination of the Zn treatment and the inoculation (Figure 4.13: a, b, d). Amongst them, *PDF1.2*, a gene that had been linked before in a number of studies to the response to *A. brassicicola* in *A. thaliana* (Wees et al. 2003; Brown et al. 2003; Schenk et al. 2003), was the most expressed.

While the expressions of *PR1*, *BGL2* and *CHIB* varied in a similar range (up to 18 folds more expressión than the calibrator group), the relative expression of the JA/Et marker *PDF1.2* was considerably higher. In lower-Zn, inoculated plants, *PDF1.2* gene copy numbers were around 50 times higher than in the not inoculated and in the 12 µM Zn-treated plants the number increased up to 200-250 times more as an average (Figure 4.13: b). Similar results from Cabot et al. (2013) have been reported in *A. thaliana* treated with Cd and Si and inoculated with another necrotroph, *Botrytis cinerea*, where a major synergistic effect between Cd and the fungus was found. More recently, Cheruiyot et al. (2015) have tested the toxicity of a combination of different metals and metals with organic compounds over the beet armyworm, *Spodoptera exigua*, finding synergistic effects in most of the cases. Not surprisingly, high levels of Zn and the non-nutritional Cd in plant cause ROS production, which may elicit stress hormonal pathways (Lin and Aarts 2012).

Although the expression of the genes from the JA/Et pathway was enhanced by *Alternaria* and specially by a combination of Zn and the pathogen, the expressions of the genes from the JA signalling pathway, *VSP*2 and *LOX*2, were not affected by the treatments (Figure 4.13: c).

In short, both SA and JA/Et pathways reacted to the infection of *Alternaria* in control and Zn-treated plants. The expression of all the four genes was boosted by Zn in the infected plants and amongst the four, *PDF*1.2 was the one with more relevance in the defence response in this pathosystem. Our results suggest that in the range of heavy metals concentrations below the hyperaccumulation, which may be still low to harm the pathogens, plant improved resistance may be obtained by the cross-talk between heavy metal and biotic stress, supporting the joint effect hypothesis from Boyd 2012 and being a pos-

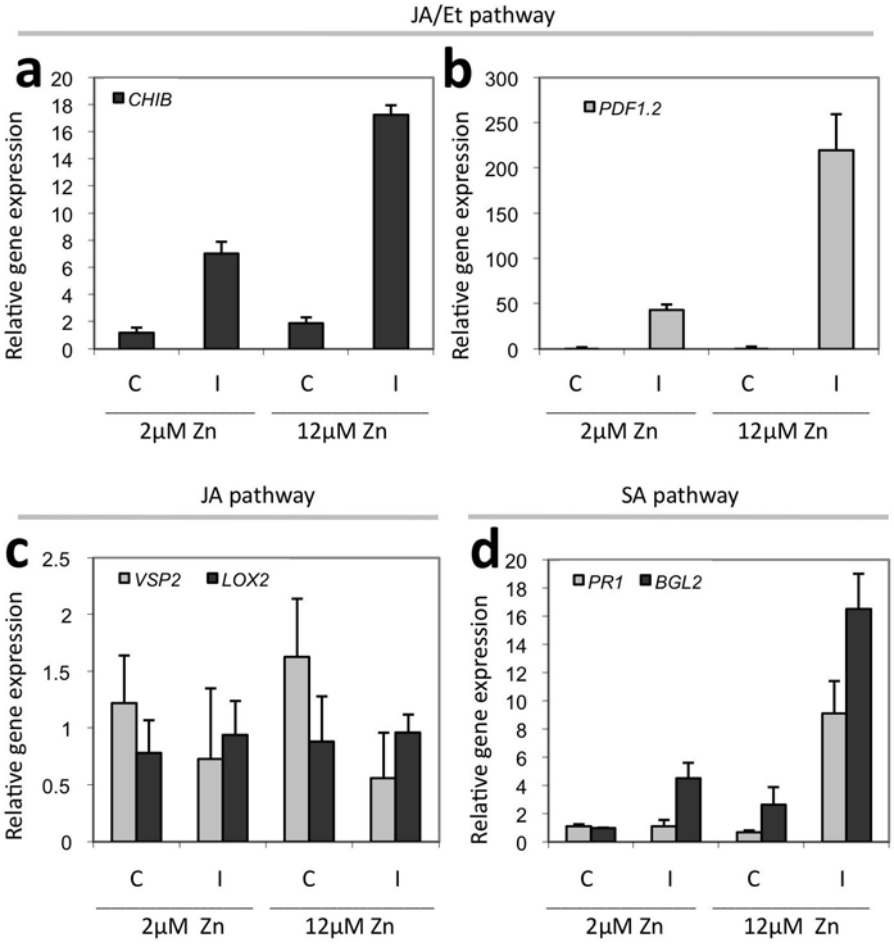


Figure 4.13: Hormonal signalling marker genes relative expression in leaves of 3-month-old *A. thaliana* plants treated with 2 or 12 µM of Zn during 5 weeks. In the 4th week, half of the plants were inoculated with *A. brassicicola* and samples taken 1 week after inoculation. JA/Et related genes: *CHIB* (a) and *PDF1.2* (b), JA related genes: *VSP2* and *LOX2* (c) and SA related genes: *PR1* and *BGL2* (d). The group of not inoculated control plants under the 2 µM Zn treatment was set as a calibrator (Relative gene expression = 1). Not inoculated control plants are represented by "C" and inoculated plants by "I". Error bars represent SE.

sible explanation for the acquisition of the hyperaccumulation trait. However, a further analysis of the stress marker genes expression in the mutant lines would contribute to draw a global picture of the complexity of the hormonal networks and how they interact in response to biotic and ionic stress.

4.2.3 *Concluding remarks*

A. thaliana plants growing in solution supplemented with 12 µM of Zn were able to concentrate in their aerial tissues an average of 500-600 µg/g of Zn, while the control group growing in solution with 2 µM of Zn only reached concentrations around 100 µg/g of the metal. Similarly, mutant *A. thaliana* lines *npr*1, *pad*1, *coi*1 and *etr*1, defective in different pathways involving the defence response against *Alternaria*, concentrated as much Zn in their tissues as the WT plants. This Zn concentration in the tissues of Zn-treated WT and mutants *A. thaliana* had the potential to be toxic for *Alternaria*. However, only *A. thaliana* WT plants treated with 12 µM of Zn were more resistant to *A. brassicicola* attack and not the mutant lines. As Zn did not compensate the metabolic defect in the mutants, it is suggested that the plant organic defences activated by the enhanced Zn levels play a more important role than Zn as a direct inorganic defence agent.

Firsts analysis on hormones, quantifying concentration of SA, JA, ABA and the precursor of Et, ACC, did not help us to explain the differences on the resistance to *Alternaria* in the Zn-treated WT plants. Noteworthy, in *A. thaliana* WT plants, Zn amplified the expression of genes related to the SA and JA/Et signalling pathway, such as *PR*1 and *BGL*2 for SA and *CHIB* and *PDF*1.2 for JA/Et. It was *PDF*1.2 the gene that more strongly responded to *Alternaria* attack and its expression was greatly amplified by Zn during the infection.

Taken all together, our results support the hypothesis of the joint effects between inorganic and organic defences under the metal hyperaccumulation threshold. However, in order to obtain a broader view, there is a need to validate the hypothesis in different pathosystems.

4.3 ARABIDOPSIS THALIANA - BOTRYTIS CINEREA PATHOSYSTEM

4.3.1 *Materials and methods*

4.3.1.1 *Plant growth and experimental conditions*

Several experiments were performed in the *A. thaliana* - *Botrytis cinerea* pathosystem (Table 4.5 on the following page) in WT plants and in the mutants from Table 4.3 on page 71 (for more information see Introduction, 1.4.1 on page 24). In all of them, plants were ex-

Table 4.5: Experiments performed in the *A.thaliana* – *B.cinerea* pathosystem.

	WT	Mutants	[Zn] (µM)	Growth	[Ion]	Botrytis quantification	Hormones
I	x		2, 12, 27	x			
II	x		2, 12, 27	x	x	x	
III	x		2,12		x	x	
IV	x		2, 12			x	x
V	x	x	2, 12		x	x	x

posed to treatments of $ZnSO_4$ supplied to the hydroponic solution (see Table 3.1 on page 38) during 5 weeks and were inoculated with *B. cinerea* in the 4th week. In the first two experiments, concentrations of 2, 12 and 27 µM of Zn were applied, while in the rest of the experiments the treatment with the higher concentration was discarded.

For methods on plant growth measurements see Chapter 3, Subsection 3.1.1 on page 37.

4.3.1.2 *Fungal material and inoculation*

Botrytis cinerea (strain MUCL43837; Mycothèque Université Catholique de Louvain, Louvain-la-Neuve, Belgium) was grown on potato dextrose agar (PDA; Sigma-Aldrich, Steinheim, Germany) in dark, at 20 °C. The spore suspension for the inoculations was obtained from 2-week old *Botrytis* cultures. Conidia were collected in potato dextrose broth (PDB; Sigma- Aldrich), mixing it with the mycelia to release the spores and filtrated with four layers of gaze. Spore density was adjusted to 10^6 spores/ml with PDB. One 10 µl droplet of the conidia suspension was placed in the center of each fully-developed leaf. A droplet of PDB without *Botrytis* spores was applied to one leaf of each of the inoculated plants as a negative control. All plants were kept for one week inside a transparent plastic chamber and a humidifier was used to provide high humidity conditions (75-90% relative humidity) in order to facilitate the start of the infection process.

4.3.1.3 *Ion concentration*

Tissue mineral concentrations were measured as described in Chapter 3, Subsection 3.1.4 on page 39.

4.3.1.4 *Disease assessment*

The spread of *B. cinerea* biomass in the leaves of *A. thaliana* was assessed by means of qPCR. The expression of the constitutive gene from *Botrytis*, *ActinA*, was quantified in relation with that of *A. thaliana*,

Tubulin (primer sequences in Appendix A, v on page 125). For more details in the qPCR protocol see Chapter 3, Subsection 3.1.6 on page 39.

4.3.1.5 *Hormone concentration*

The concentrations of the hormones JA, SA, ABA and the precursor of Et, ACC, were measured in *A. thaliana* leaves, where samples had been pooled by treatment. Two independent experiments were performed, one of them including the four stress response defective mutants, *npr*1, *pad*1, *coi*1 and *etr*1, presented in the Introduction, Subsection 1.4.1 on page 24, and in Table 4.3 on page 71. The protocol of extraction and quantification of the hormones is widely described in Chapter 3, Subsection 3.1.7 on page 41.

4.3.2 *Results and discussion*

4.3.2.1 *Plant growth*

The infection caused by *B. cinerea* in the *A. thaliana* plants did not have a significant impact on the biomass production. Only the fresh weight of shoots and roots of plants treated with 2 µM of Zn and the hydric content in shoots of all the inoculated plants tended to decrease (Figure 4.14).

4.3.2.2 *Tissue ion concentrations*

Tissue ion concentrations were measured in shoots and roots of *A. thaliana* WT and the stress response defective mutants. Similar results to those in the *A. thaliana - A. brassicicola* pathosystem were found, as Zn treatments boosted the Zn concentration in WT and mutant plants, with the particularity that in treated, inoculated plants highest Zn concentrations were reported (Figure 4.15).

4.3.2.3 *Botrytis growth*

ZN TOXICITY AND BOTRYTIS GROWTH IN VITRO The growth of *Botrytis cinerea* exposed to different $ZnSO_4$ concentrations was quantified. The effect of $ZnCl_2$ and KCl was also measured to dismiss possible interactions of the ion sulfate. As the growth decline with $ZnCl_2$ described the same curve as with $ZnSO_4$ and the ion chloride combined with K did not affect the growth at the concentrations measured, it was assumed that all the toxicity was due to Zn.

The data of *Botrytis* growth with $ZnSO_4$ was adjusted to the logarithmic model (Equation 4.1).

$$\%\Delta(t_{48} - t_0)O.D._{450nm} = 52,199 - 84,945 * log_{10}([ZnSo_4]) \quad (4.1)$$

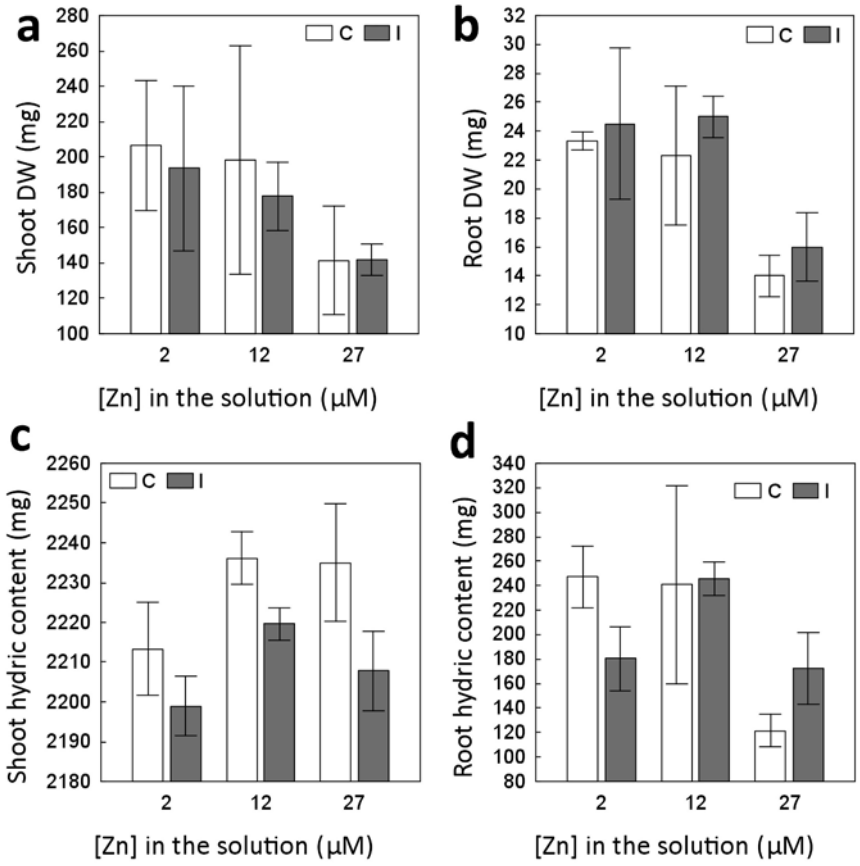


Figure 4.14: Shoot (a) and root (b) fresh and dry weight and hydric content in shoots (c) and roots (d) of 3-month-old *A. thaliana* treated with 2, 12 or 27 µM of Zn for 5 weeks. 4 weeks after Zn addition, half of the plants in each treatment were inoculated with *B. cinerea* and measures taken one week after the inoculation. Not inoculated control plants are represented by "C" and inoculated plants by "I". Error bars represent SE.

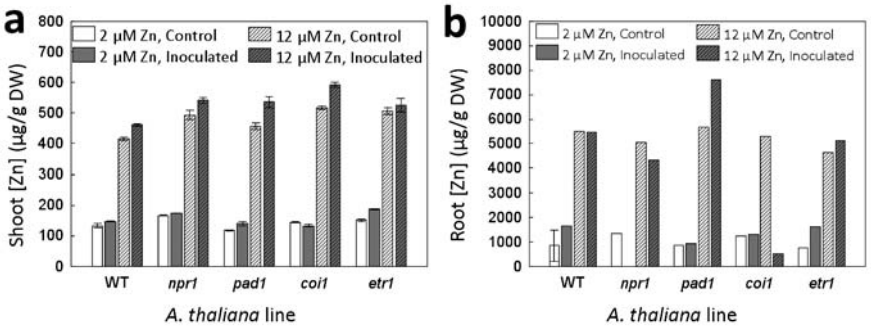


Figure 4.15: Zn concentrations in shoots (a) and roots (b) of 3-month-old *A. thaliana* treated with 2 or 12 µM Zn for 5 weeks and inoculated with *B. cinerea* in the 4th week. Error bars represent SE.

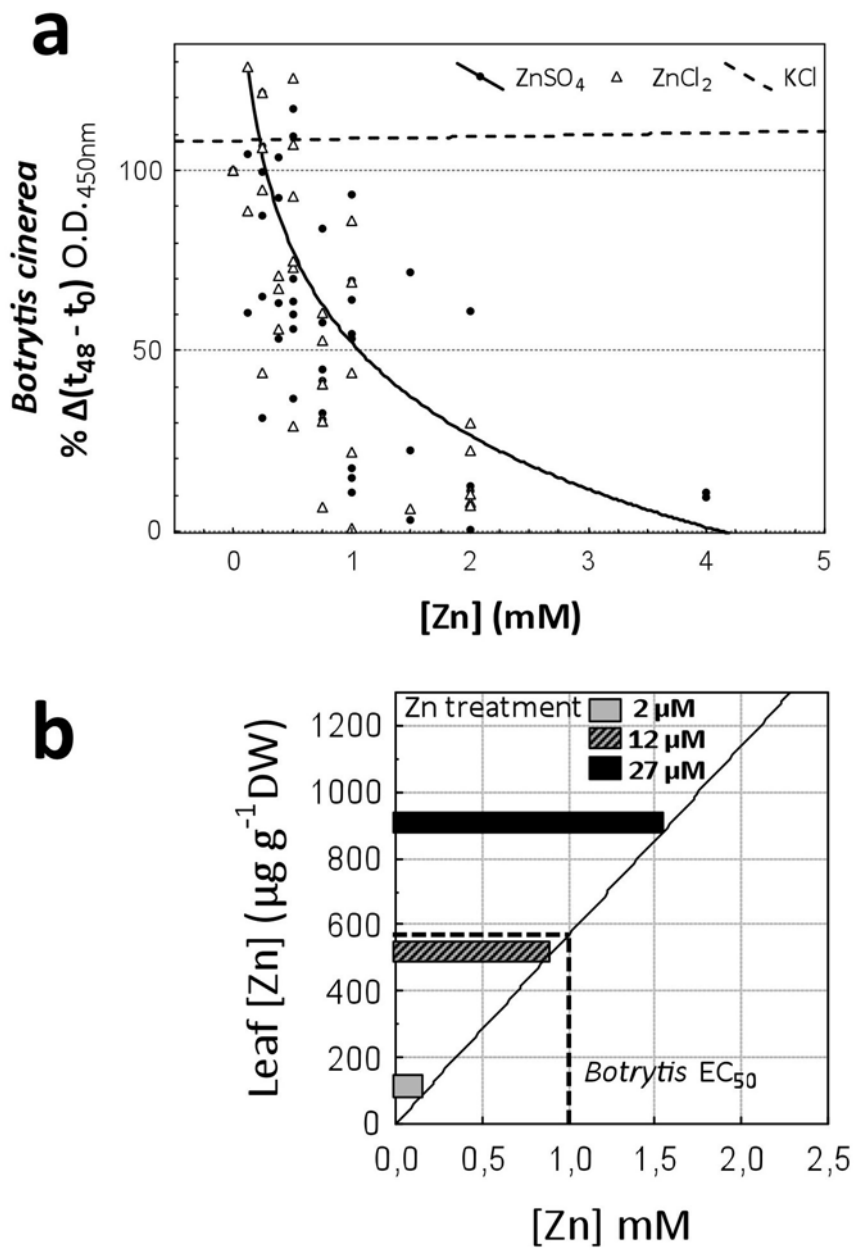


Figure 4.16: *Botrytis cinerea* tolerance to Zn in vitro (a) and theoretical correspondence between [Zn] in dry leaf of *A. thaliana* and [Zn] in solution (b). In b), [Zn] in the leaves of plants submitted to the different Zn treatments (horizontal bars) and the tolerance of *B. cinerea* to Zn given as EC_{50} (dashed line) are represented.

Figure 4.17: *A. thaliana* plants treated with Zn: 2 µM (a), 12 µM (b) and 27 µM (c) one week after being inoculated with *B. cinerea*.

From the model (Figure 4.16:a) it can be extracted that Zn concentrations around 1mM reduced *Botrytis* growth to the half (EC_{50}) and an increase to 4 mM barely allowed any growth. Taking into account data from Figure 3.6 on page 47, *Botrytis* tolerance threshold to Zn was around two times higher than that of *Alternaria*. If, again, this Zn concentration in solution is compared to that in the leaves (Figure 4.16: b), this time only the treatment of 27 µM Zn would provide enough Zn in the above-ground tissues to be potentially toxic to *Botrytis*. As most of the pathogenicity experiments were carried out in plants treated with 2 and 12 µM of Zn, it is expected that the metal did not act as an inorganic defence in these tests.

BOTRYTIS GROWTH IN VIVO In order to assess the effect of the inoculation with *Botrytis cinerea*, as no macroscopic differences on the spread of the disease were found (Figure 4.17), the expressions of both plant and fungi housekeeping genes were quantified by means of qPCR.

In two of the four experiments performed in WT (Figure 4.18), *Botrytis* grew 2 and 4 times more in the Zn-treated plants, while in other of the experiments it was in the low Zn group where it spread 6 times more than in the 12 µM Zn-treated. In a fourth test, no differences in *Botrytis* growth were found. As expected, stress response defective mutants *npr*1, *pad*1 and *etr*1 were more sensitive to the pathogen attack than WT plants, but, surprisingly, not *coi*1. Amongst the mutants, *etr1* was the most susceptible, which is coherent with previous studies that correlate the expression of *ETR1* with resistance to *B. cinerea* (Wang et al. 2013). Moreover, in this experiment, Zn did not protect from the disease in none of the mutants, and even it leaded to a greater *Botrytis* growth in *npr*1, *pad*1 and *coi*1. However, the inconsistency of the results along the trials in WT did not allow us to conclude if Zn plays a protective role against *Botrytis* or the metal rather interferes negatively with the defence response or if it is indifferent at the concentrations tested.

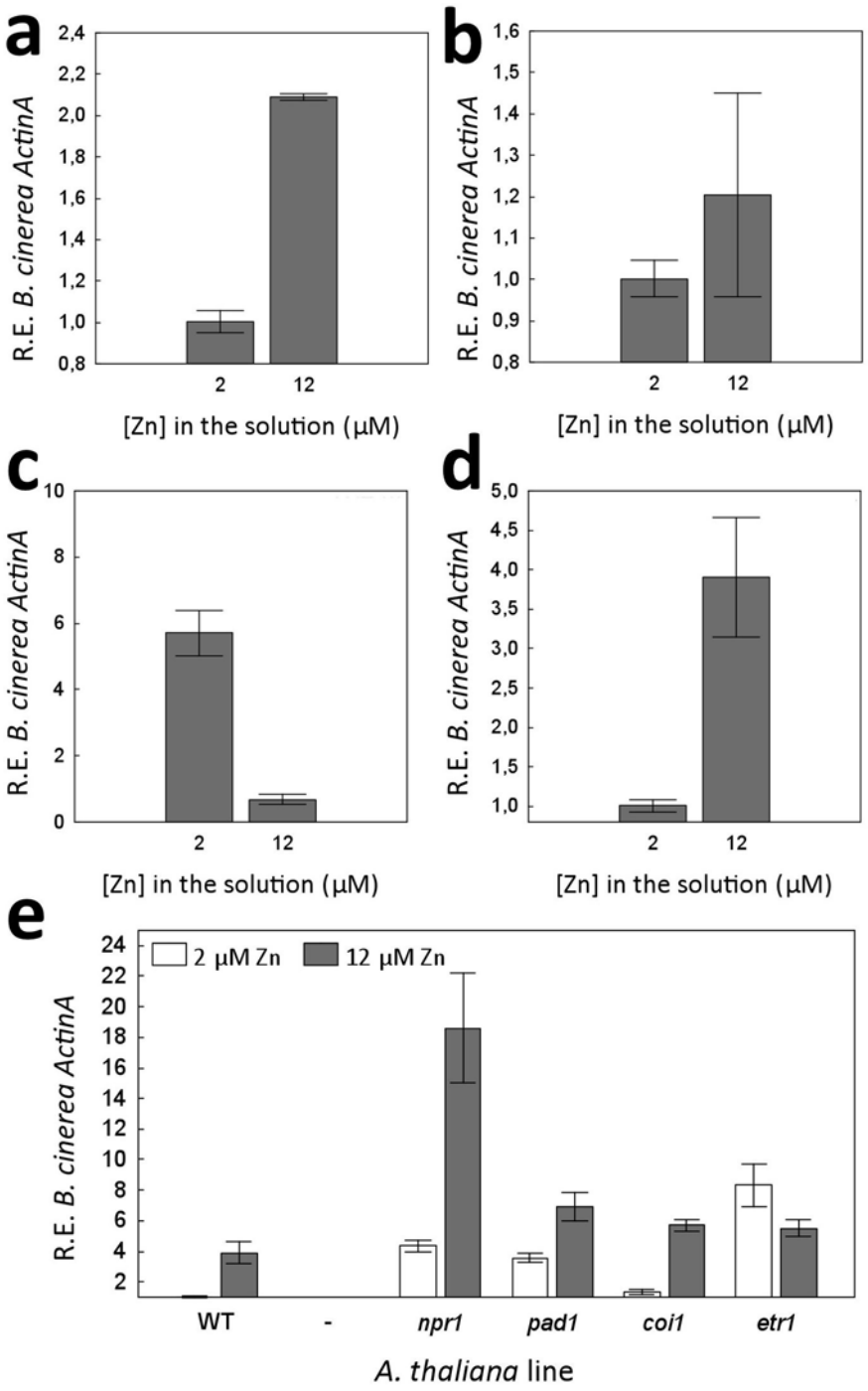


Figure 4.18: Relative expression (R.E.) of the *ActinA* gene from *B. cinerea* in leaves of 3-month-old *A. thaliana* treated with 2 or 12 µM of Zn for 5 weeks. 4 weeks after Zn addition, half of the plants in each treatment were inoculated with *B. cinerea*. *AtTubulin* was used as a reference gene. Data from four independent experiments in *A. thaliana* WT are presented (a, b, c, d). One of them (d) was part of an experiment including the stress response deficient mutants *npr*1, *pad*1, *coi*1 and *etr*1 (e). The calibrator group (R.E.=1) was the one with lower absolute gene expression in each case. Error bars represent SE.

4.3.2.4 *Hormones*

The concentrations of the hormones JA, SA, ABA and the precursor of Et, ACC, were measured in two independent experiments, one of them including the stress response mutants (Figures 4.19 and 4.20). The reaction towards the pathogen was variable along the experiments, as well as it was the growth of *Botrytis*, previously presented in graphs c, d and e from Figure 4.18 on the previous page. In the first experiment (corresponding to Figure 4.18, graph c), Zn may have driven a protective mechanism against *B. cinerea*, whereas Zn rather favored the spread of the fungus in the second experiment, including the mutants (Figure 4.18, graphs d and e).

JA synthesis was apparently induced by *Botrytis* in both experiments, as inoculated plants present higher concentrations of the hormone (Figure 4.19: a, b). In the case of SA (Figure 4.19: c, d), its average concentration reached around 250 ng/g in leaves of 2 µM Zn-treated plants from the first experiment and only 130-160 ng/g in plants from the 12 µM Zn treatment, where *Botrytis* grew less. On the contrary, in the second experiment, SA reached concentrations of around 90 ng/g in the 12 µM treated, infected plants, that were more susceptible to the disease than the low-Zn, inoculated plants, only with less than 70 ng/g of SA. Noteworthy, ACC was synthesized in response to the disease in the first experiment, but not in the second (Figure 4.19: e, f). In the first, moreover, ACC concentration was three times higher in the 12 µM Zn-treated plants than in the 2 µM, suggesting a possible enhancement of ACC by Zn during infection. In the case of ABA, its synthesis seemed to be affected mainly by Zn in both experiments, as its concentration was higher in plants submitted to the 12 µM Zn treatment (Figure 4.19: g, h). The most remarkable general pattern observed, suggest that SA is related to a greater susceptibility (Figure 4.19: c, d), while ACC, the Et precursor, to a greater resistance (Figure 4.19: e, f) to *B. cinerea.* Coherently, Et has been linked before to the resistance to *Botrytis* in *A. thaliana* and also in tomato (Diaz et al. 2002; Zhao et al. 2012), playing a major role.

When taking into account the stress response defective mutants (Figures 4.20: a, c, e, g), first results showed that *npr*1 and *etr*1 plants appeared to accumulate more SA and JA than WT plants. Not surprisingly, SA accumulation has been reported to be higher in pathogen-inoculated *npr1* mutants than in wildtype plants that are inoculated with pathogen (Shah 2003), and that SA signalling antagonizes with JA (see Introduction 1.3.4 on page 16). *etr*1 also seemed to have increased synthesis of ACC and, in a lesser extent, of JA, SA and ABA. *pad*1 and *coi*1 only accumulated slightly more ABA than WT. These results are summarized in Figure 4.21 on page 93. However, taking a look to the global response under all the treatments, the most remark-

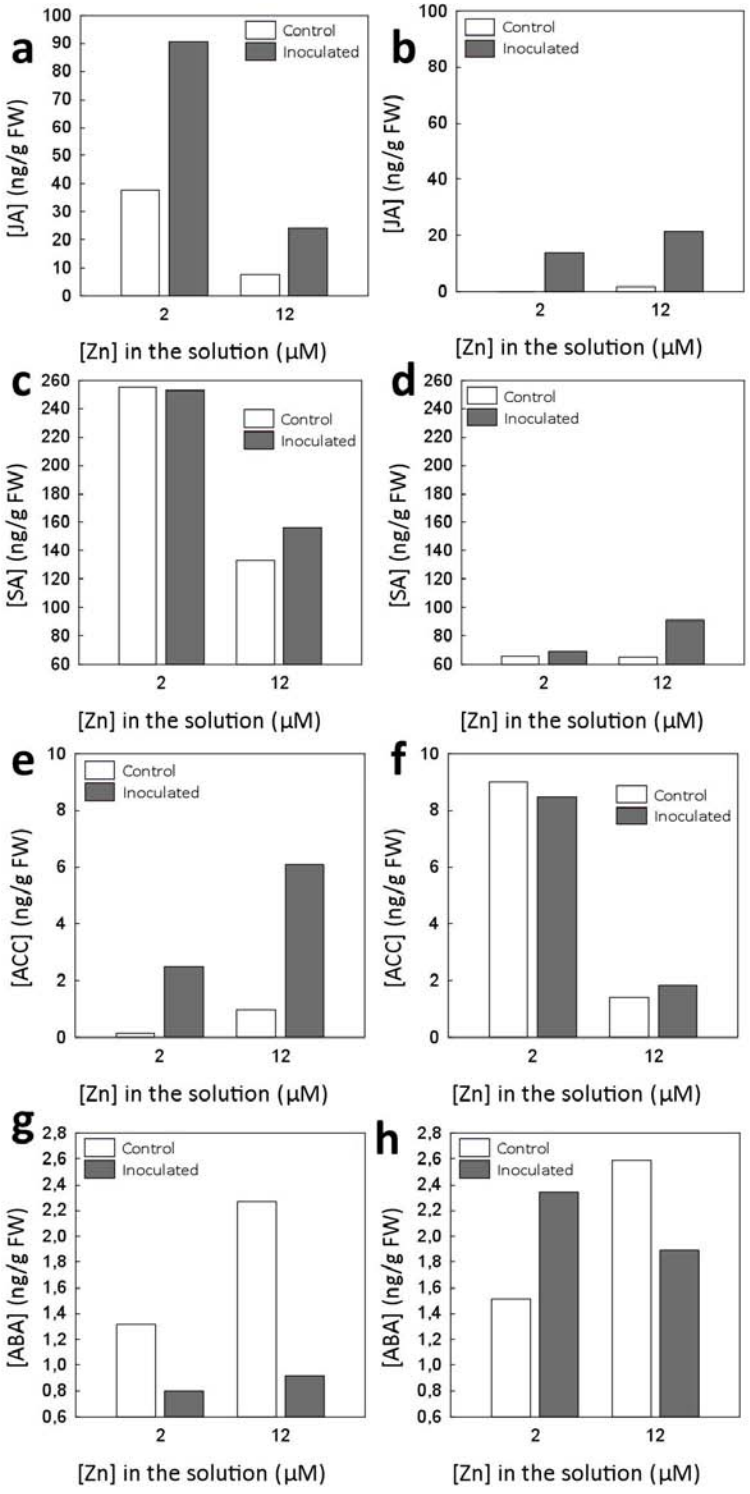


Figure 4.19: JA (a, b), SA (c, d), ACC (e, f) and ABA (g, h) concentrations in leaves of 3-month-old solution- cultured *A. thaliana* WT treated with 2 or 12 µM of Zn for 5 weeks. 4 weeks after Zn addition, half of the plants in each treatment were inoculated with *B. cinerea*. Data from pooled samples from two independent experiments where Zn was correlated with either improvement of plant resistance to *B. cinerea* (left column: a, c, e, g) or with favourising *B. cinerea* spread (right column: b, d, f, h) are presented.

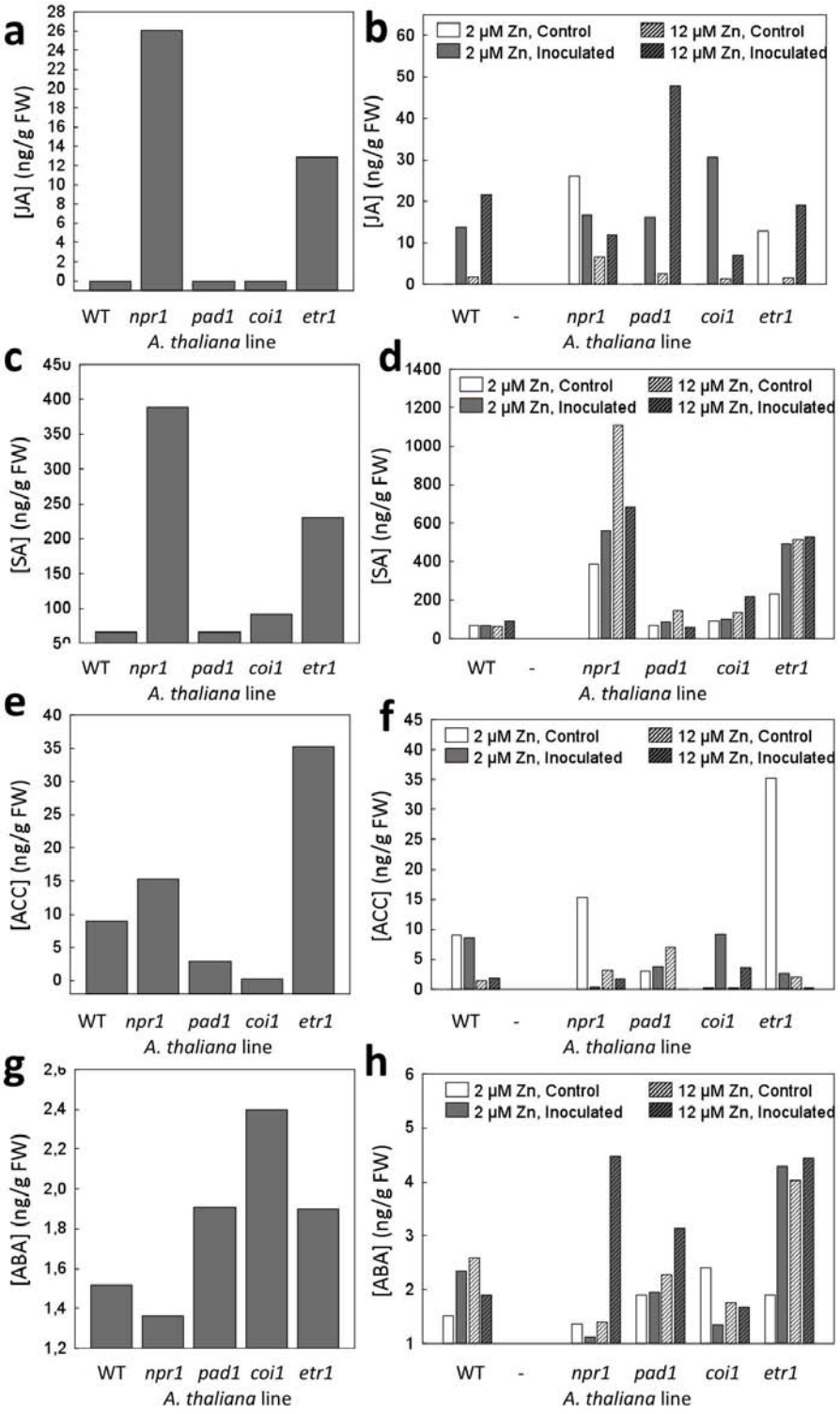


Figure 4.20: Hormone concentration of JA (a, b), SA (c, d), the Et precursor, ACC, (e, f) and ABA (g, h) in leaves of 3-month-old *A. thaliana* WT and four stress response defective mutants (*npr*1, *pad*2, *coi*1 and *etr*1) growing at 2 µM of Zn (left column: a, c, e, g) or treated with 2 or 12 µM of Zn for 5 weeks and half of the plants inoculated with *B. cinerea* in the 4th week (right column: b, d, f, h). Data from pooled samples from one experiment are presented.

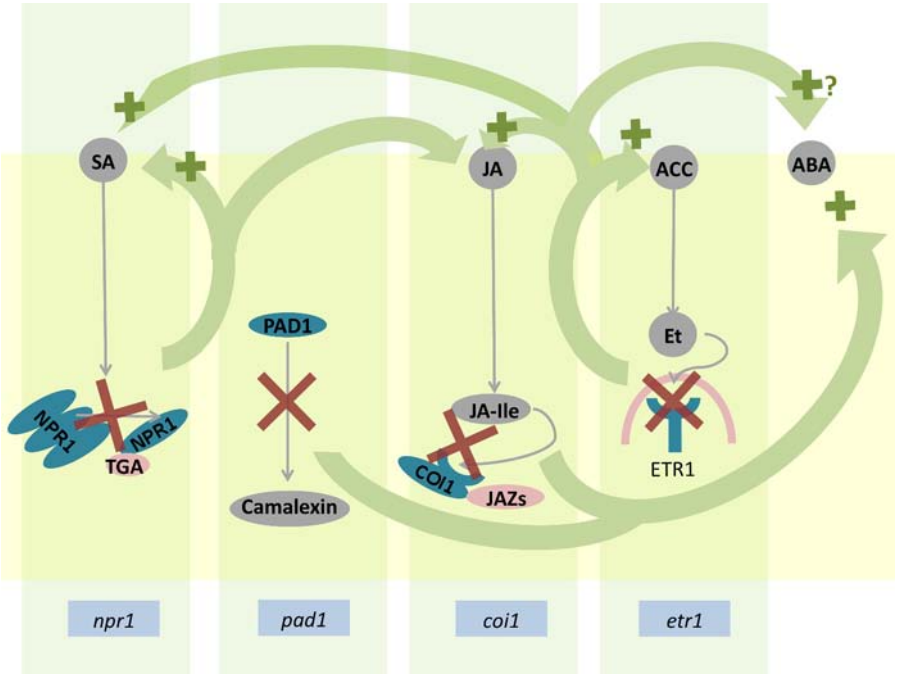


Figure 4.21: Putative effect of the stress response impairment over the SA, JA, ABA and ACC concentrations in four *A. thaliana* mutants submitted to Zn treatments of 2 or 12 µM of Zn for 5 weeks and inoculated in the last week with *B. cinerea*. SA: salicylic acid. *npr*1/NPR1: non-expressor of *PR* genes. TGA: Transcription factor. *pad*1/PAD1: phytoalexin-deficient 1. JA: jasmonic acid. JA-Ile: jasmonic acid conjugated with isoleucine. JAZs: jasmonate-zim domain (proteins). *coi*1/COI1: coronatine insensitive 1. ACC: 1-Aminocyclopropane-1-carboxylic acid. *etr*1/ETR1: ethylene receptor 1. ABA: Abscisic acid. (+) indicates an enhacement of the hormone (or ACC in the case of Et) synthesis.

able pattern is the higher concentration of SA in *npr1* and *etr1* and of ABA in *etr1*.

Nevertheless, the interpretation of these results is limited by the number of experiments and the variability in the resistance to *Botrytis*, yet a further analysis of the stress response marker genes expression, such as *PDF1.2*, *CHIB*, *PR1*, *BGL2*, *LOX2* and *VSP2*, would offer a more consistent approach.

4.3.2.5 *Concluding remarks*

Zn did not appear to have a protective effect in *A. thaliana* in response to *Botrytis* infection at the concentrations tested, as shown by the contradictory results on pathogen quantification in the leaves. The Zn concentration in leaves of *A. thaliana* plants growing at 12 µM Zn fluctuated around the *Botrytis* toxicity threshold for Zn, given as EC_{50}, what would explain the variability of the results in case Zn would be acting as an inorganic defence. Neither did Zn compensate any metabolic defect in the mutants, as first results indicate that Zn-treated plants were even more susceptible to the disease caused by *Botrytis*. First analysis on hormones related a higher concentration of the precursor of Et to a greater resistance to *Botrytis*, but a clear link with higher Zn concentration was not identified.

5

CADMIUM AND ZINC HYPERACCUMULATION IN NOCCAEA BRACHYPETALA POPULATIONS FROM NON-METALLIFEROUS SOILS IN THE EASTERN PYRENEES

The exact number of species within the genus *Noccaea* (Brassicaceae) is still not clearly established and is estimated in up to 120 (Al-Shehbaz 2012). Many hyperaccumulate Zn (35 species), some of them accumulate Ni (15 species), and a few have been described as Pb hyperaccumulators (Koch and German 2013). *Noccaea. caerulescens* and the closely related *N. praecox* (Vázquez et al. 1992; Vogel-Mikuš et al. 2005) have been described as Cd hyperaccumulators. Moreover, analysis of herbarium material has revealed hyperaccumulation of Zn and Cd in *N. brachypetala* (Reeves et al. 2001). *Noccaea caerulescens* is a model plant for studying Zn and Cd hyperaccumulation and huge amount of information on this species is available, especially on the Ganges and Prayon ecotypes (Assunção et al. 2003; Milner and Kochian 2008; Halimaa et al. 2014). Other *Noccaea* species have deserved less attention, especially in mechanistic studies.

Noccaea caerulescens is one of the most variable and taxonomically difficult species of the genus *Noccaea* (Koch and German 2013). These authors remark that the adequate taxonomical category of this species has been largely ignored by an important part of the European studies and its putative distribution and that of similar species is uncertain. The presence of *N. caerulescens* has been described both on metalliferous and on non-metalliferous soils with a widespread, but uncommon, distribution in Western and Central Europe (Clapham and Akeroyd 1993). France, with nearly 80 cited sites for *N. caerulescens*, has probably the best-known distribution of this species in one country (Reeves et al. 2001). Most of these sites are located in Southwest France and just two populations are indicated by the authors in the Northern Pyrenees. Spain is the southern limit of this species and three Spanish databases (see Materials and Methods) locate all the known populations in the northern part of the Peninsula (from the west to east: Galicia, Cantabria, Basque Country, Aragon and Catalonia). All the eastern Spanish locations are in the Pyrenees. According to Koch and German (2013) the limit between *N. caerulescens* and *N. brachypetala* is unclear, and some specimens from Central and Eastern France are either considered as synonyms or as subspecies. Nevertheless, Al-Shehbaz (2014) recognizes both taxa as separate species. Meyer (1973) accepted three subspecies for *N. brachypetala* (subsp. *brachypetala*, subsp. *tatrensis*. and subsp. *huteri*, but the concept

should be revised when considering populations from Central France and Spain (Koch and German 2013).

A first aim of this study was to contribute to accurately identify the populations of *Noccaea* from the Eastern and Central Pyrenees based on constant and conspicuous differences involving reproductive features amongst plants, finally identified as *N. brachypetala* (Jord.) F.K. Mey., and other related species: *N. caerulescens* J. Presl & C. Presl subsp. caerulescens and *N. occitanica* (Jord.) F.K. Mey. These differences were confirmed by a close examination of herbarium specimens. Evidences supporting that the plants in this work should be referred to as *N. brachypetala* are presented below. Moreover, a genetic analysis of 4 different regions was developed to support the morphological study. Some authors have used nuclear ribosomal DNA or chloroplast DNA to clarify the phylogenetic relationships within the Brassicaceae family (Koch et al. 1998a,b; Franzke et al. 1998;Bailey and Doyle 1999;Koch and Mummenhoff 2001; Koch and Al-Shehbaz 2004; Koch et al. 2007), as well as in the genus *Thlaspi* (Mummenhoff and Koch 1994; Zunk et al. 1996; Mummenhoff et al. 1997). However, taxonomies within Brassicaceae based on two or more datasets combining nuclear and chloroplast information are not generalized. Currently, more common is the use of ITS, sometimes with unclear results. The nuclear and chloroplast DNA databases used seek to ensure the identity of the populations sampled in this study within the three *Noccaea* species reported from the Pyrenees.

Previous studies reported mineral analysis of field collected samples indicating hyperaccumulation of Zn in this species. However no confirmation from hydroponically –grown plants under controlled-environmental conditions has been reported. With the purpose to unequivocally establish the hyperaccumumulaton character of *N. brachypetala* both field and hydroponic studies were performed. Plant growth and the differential expressions of key metal transporters under Cd and Zn exposure were compared both among the *N. brachypetala* populations and with *N. caerulescens* to link metal tolerance to the molecular bases of metal transport and compartmentation.

In this regard, our study addresses the lack of combined taxonomical and physiological studies highlighted by Koch and German (2013). To our best knowledge this is the first study demonstrating the ability of *N. brachypetala* to hyperaccumulate Zn and Cd under both field and hydroponic conditions and the first report on Zn- and Cd-induced expression of key genes involved in metal hyperaccumulation and hypertolerance of this species.

5.1 MATERIALS AND METHODS

5.1.1 *Plant and soil sampling*

In order to localize *Noccaea* species in the southern slope of the Eastern Pyrenees, a survey based on the information of different databases was performed: 1) Anthos program from the Spanish Royal Botanical Garden (http://www.anthos.es/), 2) Biodiversity Data Bank of Catalonia (BDBC; http://biodiver.bio.ub.es/biocat) and 3) Flora Atlas of Aragon (http://www.ipe.csic.es/floragon/).

According to this databases information, the distribution of *Noccaea* in the Catalonian Pyrenees was transferred on cartography marking all the localizations where the genus has been cited. The sampling was performed in spring 2011 when 16 out of the 38 described localizations were visited. During the sampling, it was observed that the original distribution of *Noccaea* is currently reduced and plants of this genus could not be found at most previously cited localizations. Finally, three *Noccaea* populations were located (Figure 5.1). Throughout the text these populations will be named as Aneu, Mauri, and Freser. In 2014 another population was detected 11 km north from Freser, in Núria. This population was only included in the genetic analysis.

Fruit bearing plants from Aneu and Mauri populations were excavated together with adjacent soil and transferred to culture pots. After transport to the lab, potted plants were located into a growth chamber and regularly watered until seed ripening. The field-collected plants were used for morphological studies and mineral content analysis. As the Freser population was very small only some seeds were collected and no plants were removed for not disturbing this fragile population. At each site soil close to the plants was collected in triplicate using an Edelman drill, universal type of 7 cm of diameter (Eijkelkamp, Giesbeek, Netherlands) at a depth of 10 cm.

5.1.2 *Morphological study*

Positive identification of the three Pyrenean populations was based on morphological and anatomical observations of collected plants and herbarium specimens from two herbaria of the Botanical Institute of Barcelona (BC) (http:// www.ibb.bcn-csic.es/ html/ herbari_ang .html) and the University of Barcelona (BCN) (http:// crai.ub.edu/ es/
conoce-el-crai/ CeDocBiV). Representative herbarium material examined is listed in the supplemental material (v on page 131). Morphological characters traditionally used in floristic or taxonomic treatments were examined (Bolos and Vigo 1990; Pujadas 1993; Clapham and Akeroyd 1993; Bolos et al. 2005; Al-Shehbaz 2014). Features of

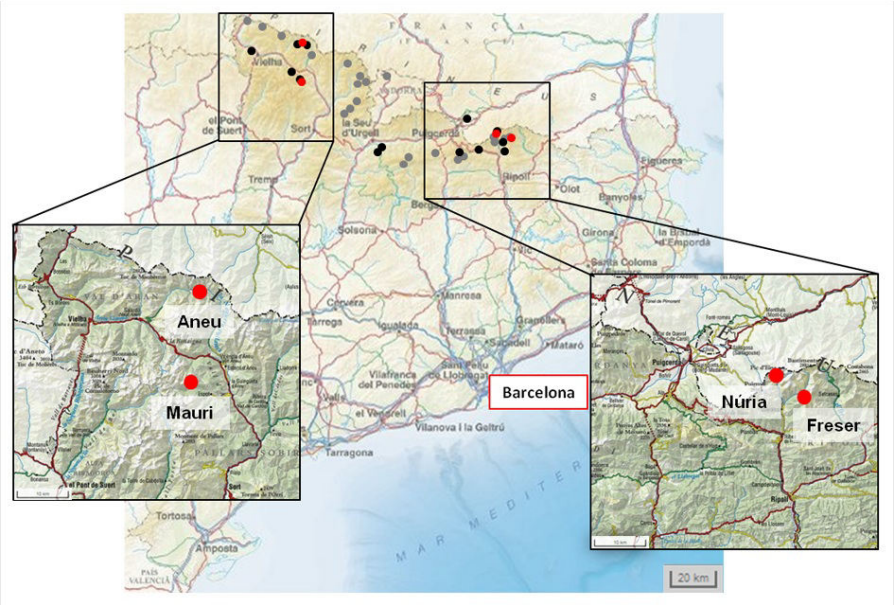


Figure 5.1: Map of distribution of *Noccaea* species in the Catalonian Pyrenees. Points indicate the 38 sites where *Noccaea* (*N. brachypetala*, *N. caerulescens* and *N. occitanica*) are reported in the databases. Black and red points indicate the 16 sites visited during this survey; red points indicate sites where *N. brachypetala* populations were found; black points are sites where no *Noccaea* species could be found,

Table 5.1: Date of sampling, analyses performed and parameters measured (x) in the different *N. brachypetala* populations and *N. caerulescens*.

	N. brachypetala populations				
	Aneu	Mauri	Freser	Núria	*N. caerulescens*
Year of sampling	2011	2011	2011	2014	
Genetic analyse	x	x	x	x	x
[Ion] soil	x	x	x		
[Ion] plant *in situ*	x	x			
[Ion] plant hydroponic	x	x	x		x
Photosynthesis	x	x	x		x
Growth	x	x	x		x
Root scanning	x	x	x		x

gross morphology were studied under a Zeiss binocular stereoscopic microscope.

5.1.3 *Genetic data*

To establish the genetic affinities of the three Pyrenean populations to *Noccaea* species described in the region and to other close members of the Brassicaceae fresh leaves from the collected Pyrenean plants, dried caulinar leaves from the herbarium specimens, and seeds from our personal collection or purchased from a commercial supplier (B & T World Seeds, Aigues-Vives, France) were used . Total genomic DNA was extracted using the NucleoSpin ® Plant II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Amplifications of four non-coding regions (ITS1-4, trnL-trnF, rpl32-trnL(UAG,) and trnQ- 5'rps16) were conducted using a MJ Mini TM Gradient Thermal Cycler (Bio-Rad, California, USA) in a 20 µl reaction with IQ TM supermix (Bio-Rad Laboratories, California, USA). The ITS region was amplified and sequenced using primers ITS1 and ITS4 (White et al. 1990) and the protocol of Likar et al. (2010). The trnL-trnF region was amplified and sequenced using the primers c and f (Taberlet et al. 1991). The PCR procedure started at 95 °C for 4 min, followed by 35 cycles of denaturation at 93°C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. The amplification and sequencing of the rpl32-trnL(UAG) region was performed using the rpl32F and trnL(UAG) as forward and reverse primers, respectively (Shaw et al. 2007). The last chloroplast region, trnQ-5'rps16, was amplified and sequenced using the trnQ(UUG) as the forward and rpS16x1 as the reverse primer, respectively (Shaw et al. 2007). The last two regions were amplified by using the program "rp116" (Shaw et al. 2005). Primer sequences can be found at v on page 125. All PCR amplicons were checked on 1% agarose, TBE 0.5x gels stained by SYBR ®safe. Previous to sequencing, PCR products were purified by enzyme digestion of exonuclease I and Antarctic phosphatase (New England Biolabs, Massachussetts, USA). Sequencing was performed at Bioarray, S.L. (Parque Científico y Empresarial de la UMH, Elche, Alicante, Spain) on a BigDye Terminators v3.1 Cycle Sequencing kit (Applied Biosystems, California, USA) and analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, California, USA).

The sequences were edited and aligned using Bioedit Sequence Alignment Editor (version 7.0.7.0) (Hall 1999); alignment was also checked visually by sequential pair wise comparison (Swofford and Olsen 1990). Phylogenetic analyses were carried out using the Bayesian method and the software MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The best-available model of molecular evolution required for Bayesian estimations of phylogeny

was selected using the Akaike information criteria (AIC) and Bayesian information criterion (BIC), as implemented in the software jModeltest 0.1.1 (Guindon and Gascuel 2003; Posada 2008), which considers nucleotide substitution models that are currently implemented in MrBayes 3.1.2. The GTR model , with variable base frequencies, was assumed to follow a discrete gamma distribution and was selected as the best-fit model of nucleotide substitution for the cpDNA and ITS-cpDNA combined dataset. For the ITS alignment, the symmetrical model with equal base frequencies and rate variation among sites (SYM+G) was selected (Zharkikh 1994). Bayesian inference analyses were initiated with random starting trees and were run for 106 generations. Four Markov Chains were run using Markov Chain Monte Carlo (MCMC) principal sample trees. One tree out of every 100 generations was saved, which resulted in 10,000 sample trees. Data from the first 2500 generations were discarded as the "burn-in" period, until values had stabilized prior to the 2500th generation. All sequences are deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases.

5.1.4 *Analysis of field-collected soil and plant samples*

Cd, Co, Cu, Pb and Zn in soils from the three sampled locations were extracted with aqua regia and analyzed by ICP-MS (Perkin Elmer Inc., ELAN 6000, MA, USA) (Bech et al. 2012). Prior to root and shoot metal concentration analysis field collected plants from Aneu and Mauri populations were carefully washed and processed as described below. The metal concentrations in plants from Freser were not analyzed as the original population was too small to be sampled.

5.1.5 *Hydroponic studies*

To verify metal hyperaccumulation in the three Pyrenean populations identified in this study the well-characterized *N. caerulescens* J. & C. Presl (ecotype Ganges) was hydroponically grown along with the Pyrenean populations. Germination of seeds of *N. caerulescens* (Ganges) (B&T World Seeds, Aigues-Vives, France) and field-collected seeds from the three Pyrenean populations was synchronized by treatment with 10-5 M gibberellin at 4 °C for 4 days. Seeds were germinated and seedlings were first grown on vermiculite irrigated with half strength Hoagland solution (pH 5.5) in a growth chamber (day night temperature 25/18 °C, photoperiod 12h light/12 h darkness; PAR 135 µE m^{-2} s^{-1}). After two weeks the seedlings were individually transferred to plastic pots (100 ml capacity) filled with continuously aerated nutrient solution which was renewed weekly. When plants were one-month old, 100 µM of $ZnSO_4$ or 1.5 µM of $CdCl_2$ were added to the solution and plants were grown for a further four-

week period. Fifteen plants per species or population and treatment were used in each experiment.

5.1.6 *Growth, photosynthesis, and mineral analysis*

Growth was assessed by recording rosette and root dry weight after the plant tissues had been oven dried at 60 °C. To reveal possible metal toxicity effects leaf chlorophyll concentrations (CCM-300 chlorophyll content Meter, Opti-Sciences, NH, USA) and chlorophyll fluorescence (Fv/Fm ratio) (JUNIOR-PAM, Heinz Walz GmbH, Germany) were measured. Both parameters were quantified as the average of three independent measurements on fully-expanded leaves. Non-destructive parameters (roots scanning, fluorescence and content of chlorophyll) were measured at the beginning of the treatment (t=0) and every two weeks (t=2nd and 4th week).

After four weeks of metal exposure, plants were separated into roots and shoots, carefully washed with distilled water and oven dried (60 °C). The dry material from ten plants per population and treatment was homogenized to fine powder followed by acid digestion (HNO_3 : H_2O_2 69 % : 30 %, 5:2 v/v) in a hot-block digestion system (SC154-54-Well Hot BlockTM, Environmental Express, SC, USA). The concentrations of the selected elements were determined by ICP-OES (Thermo Jarrell-Ash, model 61E Polyscan, England). Quality control was assured as previously described (Bech et al. 2012). The data shown are means of three independently analysed samples from the homogenized powder.

5.1.7 *Expression of metal transporter genes*

The expressions of selected metal transporter genes were analysed in hydroponically grown plants treated with Cd or Zn in hydroponics for one week as described above. Five plants from each *N. brachypetala* population and from *N. caerulescens* (Ganges) were divided into roots and shoots. Parts from each population were collected together and then directly immersed into liquid nitrogen, homogenized to fine powder and stored at -80°C until use. Total RNA of each pool (around 100 mg) was extracted and quantified as described in 3.1.6 on page 39.

The effects of Zn and Cd on the expression of metal transporters *HMA*3, *HMA*4 and *MTP*1 were studied. A trial with the primers described for the close relative *N. caerulescens* was successful and were used for the expression studies. The primer sequences for *HMA*3, *HMA*4, *MTP*1 and the housekeeping gene used for *N. caerulesces*, *Tubulin*, are detailed in Appedix A, v on page 125.

Treatment influence on relative gene expression was calculated by the ratio (Efficiency of the target gene) ΔCT, target (calibrator – test) / (Efficiency of the reference gene) ΔCT, reference (calibrator – test)

(Pfaffl, 2001). The calculated amplification efficiencies were 90.6 % (*HMA*3), 95.9 % (*HMA*4), 99.5 % (*MTP*1), and 93.2 % (*Tubulin*). The results are means of three independent samples from the homogenized powder.

5.1.8 *Statistics*

Continuous data were analyzed by the software Statistica 7.0 (Stat Soft, Inc. OK, USA). Normal distribution was checked and data non-adjusting to normal distribution were transformed with logarithm and sinus corrections, where necessary transformed in order to apply parametrical tests. ANOVAs followed by Tukey HSD tests were performed. Morphometric data were used in principal component analysis.

5.2 RESULTS AND DISCUSSION

5.2.1 *Localization and identification of Pyrenean* Noccaea *populations*

During the 2011 survey only three *Noccaea* populations were found in the Catalonian Pyrenees. From the west to the east, the populations are located close to Estany de Sant Maurici (42°57′80.70″N, 10°08′98.4″E; altitude 1,950 m, called "Mauri"), in the Vall de l'Àneu (42°76′63.05″N, 1°05′39.98″E; altitude 1,500 m; called "Aneu") and in the Vall del Freser (42°38′20.78″N, 2°21′55.15″E; altitude 1,940 m, called "Freser") (Figure 5.1 on page 98). Aneu and Mauri populations developed below trees on subalpine meadows, with each population constituted by more than 15 specimens. In contrast, at Freser only 2 plants growing on a boundary path were found. This population was not stable throughout the years, as confirmed by later visits to the area. This year, 2014, a small population was detected in Núria, 11 km north from Freser. This population was only included in the genetic study. In the available databases, *N. caerulescens* is classified as an occasional species and correspondingly it should be widespread in the Pyrenees; but this survey revealed that the current distribution of Noccaea in the Pyrenees is reduced compared to previous information.

Morphological studies (Appendix D, v on page 131) comparing the sampled populations with herbarium specimens revealed that three species are recognized in the Pyrenees: *N. brachypetala*, *N. caerulescens* and *N. occitanica*. This is consistent with the taxonomic treatments given by the 'Flora ibérica' (Pujadas 1993), the 'Euro+Med PlantBase' (Marhold 2012) and the synopsis of the genus *Noccaea* by Al-Shehbaz (2014). The number and quality of the discriminating morphological characters found so far (see Appendix D, v on page 131) and the lack of intermediate specimens linking the extreme morphotypes strongly

supports the view that these three separate species are present in the Pyrenees. Taking the obtained data together with previously published studies a detailed morphological description of *N. brachypetala* is provided as follows: Biennial or perennial; glabrous, glaucous. Flower stalks 13-55 cm long, erect, and usually unbranched. Basal leaves rosulate; petiole 5-20 mm; leaf blades oblanceolate, ovate or elliptical, 5-24 × 2-9.5 mm, base cordate, margins entire or denticulate, apex obtuse. Cauline leaves 3-20; blade ovate to lanceolate, 5-30 × 2-9 mm, base auriculate, margins entire or denticulate, apex obtuse to acute. Racemes 3-22 cm, considerably elongated in fruit. Fruiting pedicels horizontal, straight, 2-8 mm. Sepals 1-1.3 mm, oblong, light green to yellowish-green; petals obovate to oblanceolate, 1-1.5 mm, white, erect. Stamens slightly tetradynamous, filaments 1.5-2 mm; anthers 0.3-0.4 mm, violet, green or whitish. Style 0.3-0.5 mm included within the notch of the ripe fruit. Fruits 3.5-8 × 2-3.5 mm, obcordate, not winged or narrowly winged basally, broadly winged above (up to 2 mm width), with rounded apical lobes and a deep notch; base cuneate. Seeds 1.5-1.7 x 1-1.2 mm, slightly compressed, brown to reddish. According to these morphological data the plants from the three populations located in this study are clearly referable to *N. brachypetala*.

With regard to genetic data, the aligned matrix for ITS consisted of 21 sequences of 677 bp and 107 parsimony-informative characters. The cpDNA matrix consisted of 21 sequences of 1976 bp and 180 parsimony informative characters. The number of variable sites amongst *N. brachypetala*, *N. caerulescens* subsp. caerulescens and *N. occitanica*, using the ITS-cpDNA combined dataset, ranges from 0 for *N. brachypetala* (populations Freser, Mauri and Núria) and *N. occitanica*, to 3 for *N. brachypetala* (Freser, Mauri and Núria) and *N. caerulescens* subsp. *caerulescens* Prayon, and to 8 between *N. brachypetala* (Aneu) and the rest of the clade containing *N. brachypetala*, *N. caerulescens* subsp. *caerulescens* and *N. occitanica*.

Bayesian analyses of ITS, cpDNA and ITS-cpDNA combined datasets, provided similar topologies except for a significantly supported topological incongruence involving *N. rotundifolia* (L.) Moench, which was clustered in the ITS tree at the same clade as *N. brachypetala* and *N. caerulescens* but out of this clade in cpDNA tree (trees not shown). Nevertheless, the matrices of ITS and cpDNA were combined due to the improvement in the phylogenetic signal and because the phylogenetic reconstruction of genus *Noccaea* is out of the aim of the work and *N. rotundifolia* is not a key species in the study. The phylogenetic tree for ITS-cpDNA, with Bayesian posterior probabilities (PP), is shown in Figure 5.2 on the next page.

Although *N. brachypetala*, *N. caerulescens* and *N. occitanica* are more or less sympatric in the Eastern Pyrenees, the three populations localized by this survey were recognized as *N. brachypetala*. This iden-

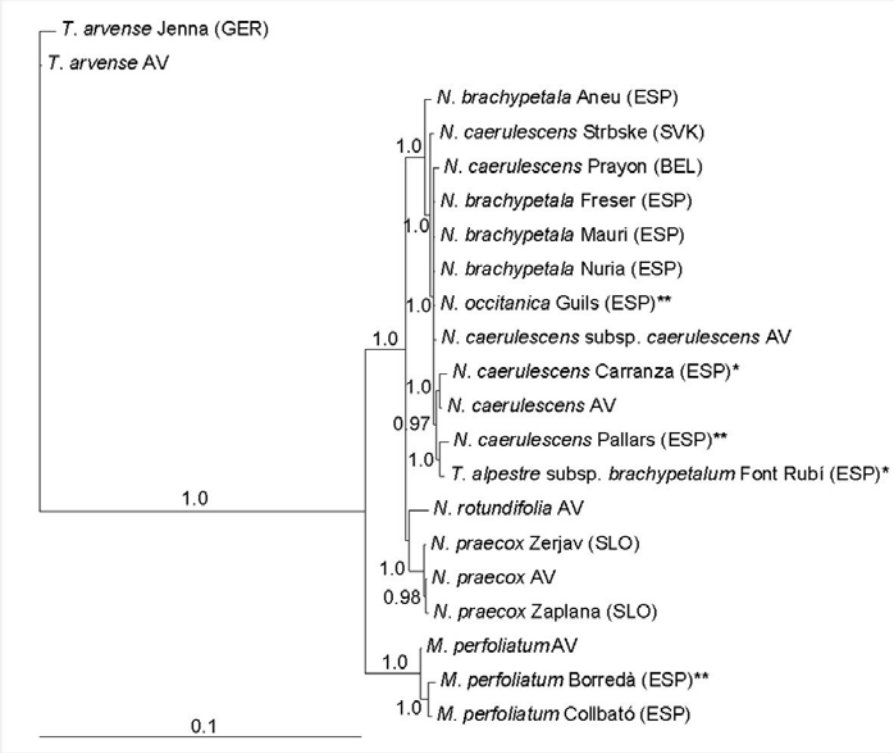


Figure 5.2: 50% majority-rule consensus tree obtained from the Bayesian analysis of the combined ITS and cpDNA dataset indicating supported clades (PP > 0.95). Numbers indicate the posterior probabilities (PP). Country names are given as the ISO standard. AV, seeds purchased from B&T World Seeds, Aigues-Vives, France. * indicates individuals from Herbarium BC. ** indicates individuals from Herbarium BCN

tification is strongly supported by vegetative and reproductive characters (Appendix D, v on page 131) consistent with the taxonomical treatments done by Pujadas (1993), Marhold (2012), and Al-Shehbaz (2014). In contrast to the cues from the morphological data and despite the efforts made in this study to clarify the relationships between *N. brachypetala*, *N. caerulescens* subsp. *caerulescens* and *N. occitanica* on molecular grounds, using species-level phylogenetic markers (Mummenhoff and Koch 1994; Koch and Al-Shehbaz 2004), there are not enough phylogenetic signals to separate them. The poor genetic variation could be explained by a recent or a rapid species radiation event, as denoted by the presence of short branches in the phylogenetic trees (Figure 5.2). Moreover, incomplete lineage sorting or the presence of interspecific gene flow, mediated by weak genetic barriers, could be hampering the phylogenetic reconstruction as they act as homogenizing forces. The presence of these phenomena is demonstrated by the divergent positions where different accessions of *N. brachypetala* and *N. caerulescens* were recovered in the phylogenetic tree (Figure 5.2). The hypothesis of incomplete lineage sorting may be a better explanation since gene flow is unlikely in putatively inbreeding plants (Lombi et al. 2000; Besnard et al. 2009), although, it cannot be discarded especially when out-crossing rates in *N. caerulescens* can be much higher than originally thought (Koch et al. 1998a). Nonetheless, higher inbreeding coefficients have been observed in non-metallicolous than in metallicolous populations of *N. caerulescens* (Dubois et al. 2003).

Noteworthy, Mauri and Freser, non-metallicolous populations of *N. brachypetala*, were in the same clade as *N. caerulescens* Prayon, a metallicolous ecotype from heavily polluted soil. In contrast, Aneu from a soil with only slightly enhanced Cd and Zn concentrations was on a close, but different clade (Figure 5.2 on the preceding page). Our data are in contrast with the high degree of genetic differentiation found in several studies for *N. caerulescens* (Koch et al. 1998b; Dubois et al. 2003; Basic and Besnard 2006; Jiménez-Ambriz et al. 2007). However, these studies apply population genetics approach, using AFLP/isoenzymes and several individuals per population and, therefore they are not fully comparable. Future work should address this issue using more variable markers, such as AFLP or SSR, and focusing sampling effort on the closest relatives: *N. brachypetala*, *N. caerulescens* subsp. *caerulescens* and *N.occitanica*.

In the Pyrenees the presence of these three *Noccaea* species is usually described in grasslands, meadows, prairies and occasionally in forests (Pujadas 1993). According to the BDBC database, *N. caerulescens* is present in 28 UTM grids (10x10 km). Surprisingly, during our sampling campaign in 2011 searching 16 out of 38 described sites in databases for the occurrence of *Noccaea* and in further visits in 2012 and 2014, *N. brachypetala* was only located at Aneu, Mauri, Freser,

and Núria, while neither *N. caerulescens* nor *N. occitanica* were found. This becomes especially relevant as in the herbarium material studied, only 4 specimens are dated in the XXI Century; while the rest of them, 16, were between 40 and 140 years old. Therefore, based on the herbarium material collected dates and on our experience in sampling in the Pyrenean range, it can be suggested that *Noccaea* may be declining in this zone. Besides increasing tourism and urban activities and enhanced herbivore pressure due to cattle raising, this apparently recent decline of *Noccaea* could be caused by a low resilience to confront climate change, as this zone of the Pyrenees mountain range is on the southern limit of these species distribution area. There is clear evidence that the number of cold days per year has been declining during the last 40 years in the Eastern Pyrenees (Morán-Tejeda et al. 2013) and this expectedly may have affected the reproductive fitness of *Noccaea* species which require vernalization for flowering.

5.2.2 *Metal concentrations in soils and* N. brachypetala

Concentrations of Cd, Zn, Co, Cu and Pb in the soil surface layer at Aneu, Freser and Mauri (Figure 5.3 on the facing page a, b, e) were within the common range (Kabata-Pendias 2011). No differences in the low soil metal concentrations between Mauri and Freser were observed, while soils from Aneu had higher contents. Cd and Zn concentrations in the shoots of field-collected plants were extraordinarily high (Figure 5.3 on the next page b, d). Aneu plants accumulated 70 times higher Cd and 18 time higher Zn concentrations than those found in the soil, and Mauri plants had 56 and 19 times higher Cd and Zn concentrations, respectively. In contrast, concentrations of Co, Cu, and Pb were lower in plants than in soils (Figure 5.3 on the facing page f, e). The bioaccumulation index relating the metal concentrations in the shoots to that of soils revealed the extreme ability of *N. brachypetala* to hyperaccumulate Zn and Cd (Figure 5.5 on page 109 a) from these unpolluted soils. Shoots from Aneu contained significantly higher Zn and Cd concentrations than those from Mauri (Figure 5.3 on the facing page b, d). However, no differences between the bioaccumulation factors of Aneu and Mauri were found (Figure 5.5 on page 109 a) due to the higher soil metal concentrations at Aneu.

The three populations identified in this study are located in cell N29E01 of the Global Terrestrial Network (GTN) of Forges countries in the Geochemical Atlas of Europe (http://weppi.gtk.fi/publ/foregsatlas/) where soil concentrations between 0.06 and 3.03 µg g^{-1} for Cd and between 97 and 398 µg g^{-1} for Zn are reported in the Spanish site of the cell.

This considerable variability for Zn and Cd soil concentration was also reflected in this study. While soils from Mauri and Freser had the same typically low Zn and Cd concentrations of non-metalliferous

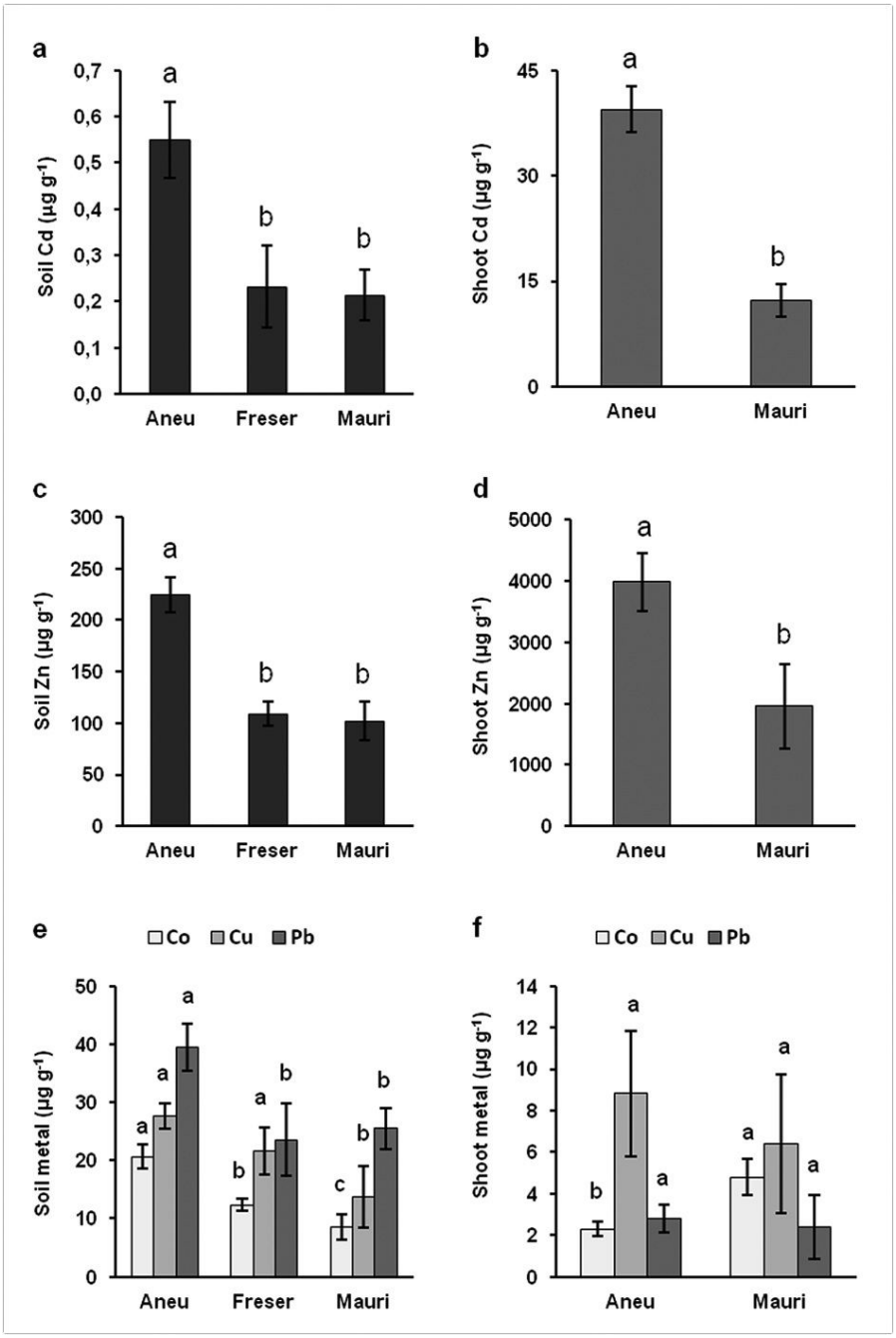


Figure 5.3: Concentrations (µg g-1 dry weight) of Cd (a, b), Zn (c, d) and Co, Cu and Pb (e, f) in soils (a, c, e) and *N. brachypetala* populations (b, d, f) sampled on the southern slope of the Eastern Pyrenees.

Figure 5.4: *N. brachypetala* plants from populations Aneu, Mauri and Freser growing hydroponically.

soils, at Aneu, two times higher concentrations of both metals were observed along with somewhat higher Pb concentrations. This slightly enhanced metal level is not due to industrial or mining activities in this zone but of geochemical origin as soils at Aneu are derived from shales.

5.2.3 *Growth, Zn and Cd accumulation in hydroponics*

The hydroponic experiments confirmed the high capacity of *N. brachypetala* to hyperaccumulate Zn and Cd and revealed the high Zn and Cd tolerance of these plants. All plants exhibited translocation factors greater than 1.0 (Figure 5.5 on the facing page b). No differences in the Zn and Cd translocation factors among the three *N. brachypetala* populations were found. Surprisingly, however, the *N. brachypetala* populations had higher translocation factors for Zn and Cd than *N. caerulescens* ecotype Ganges. *N. caerulescens* accumulated lower root and shoot Zn concentrations than *N. brachypetala* (Figure 5.5 on the next page d). Shoot Cd concentrations were highest in *N. brachypetala* population Freser, followed by Aneu (Figure 5.5 on the facing page c). Mauri and Ganges had the lowest shoot Cd concentrations, but clearly above the hyperaccumulation threshold.

The hyperaccumulation of Cd did not affect the plant biomass in any population (Figure 5.6, a). However, high Zn accumulation significantly reduced root and shoot growth in plants from Freser. In contrast, biomass production was stimulated by the high Zn supply in plants from the Mauri population (Figure 5.6, b). Neither chlorophyll concentrations nor chlorophyll fluorescence parameters were affected by the metal treatments (data not shown). Differences amongst the populations were more patent that differences within a population as a consequence of the treatments with Cd or Zn. A reflection of it, is the phenotypical diversity in leaf and rosette shape in the populations, found in plants growing hydropobically (Figure 5.4).

This natural range in soil Zn and Cd concentrations is of special interest for the investigation of the mechanisms of metal accumulation and tolerance in the potential metal hyperaccumulating species occur-

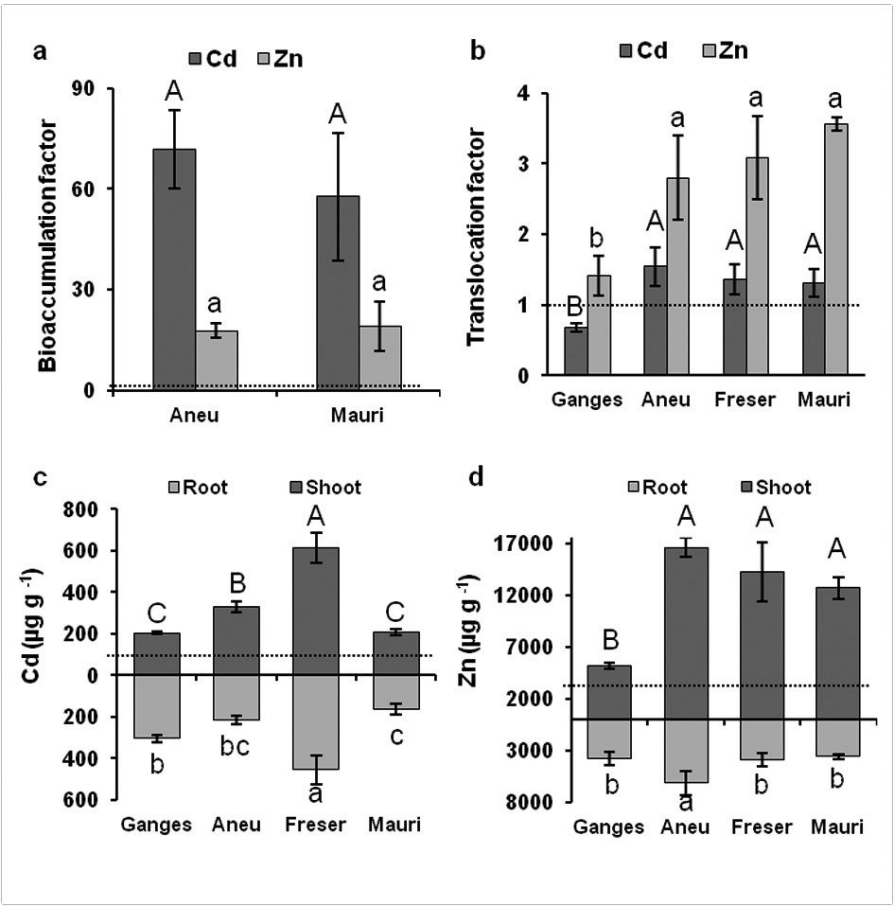


Figure 5.5: Metal bioaccumulation factors (µg g-1 shoot/µg g-1 soil) in field sampled *N. brachypetala* populations (a); translocation factors (µg metal shoot/ µg metal root) (b) and Cd (c) and Zn concentrations (d) in hydroponically-grown *N. brachypetala* populations and *N. caerulescens* (Ganges). Error bars on columns in (a) and (b) are standard deviations based on three different ICP analyses from a pool of 5 collected plants or 5 soil samples; columns with the same letters (capitals for Cd and lower case for Zn) are statistically not different (Tukey test; $p < 0,05$). Error bars on (c) and (d) are standard deviations based on three independent ICP analyses from a pool of 10 plants; columns with the same letters (capitals for shoots and lower case for roots) are statistically not different (Tukey test; $p < 0,05$) Dotted lines indicate threshold for hyperaccumulation.

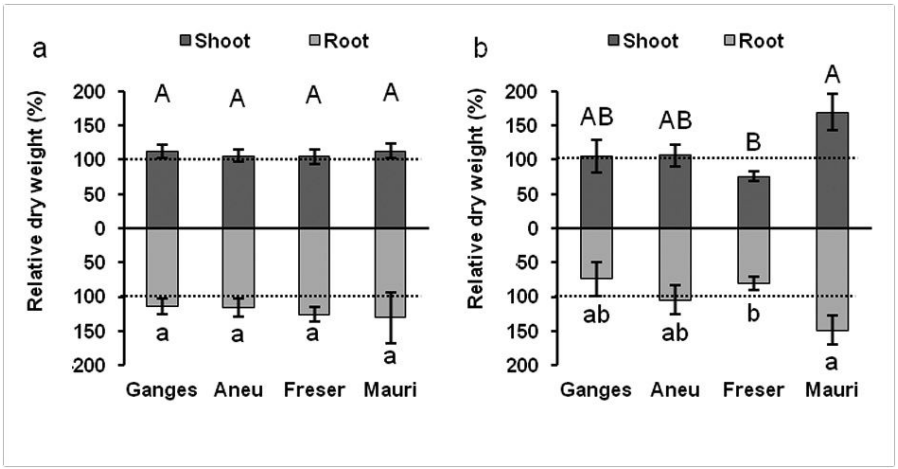


Figure 5.6: Influence of exposure to 1.5 µM Cd (a) or 100 µM Zn (b) on plant dry biomass (% values relative to controls without Cd (a) or with 2 µM Zn (b) of different *N. brachypetala* populations and *N. caerulescens* (Ganges). Values are means ± SD (n=10). Columns with the same letter are statistically not different ($p < 0.05$). Dotted lines mark 100%.

ring here. While intense research has been performed in *N. caerulescens* and enormous population differences in the ability to accumulate and tolerate Zn and Cd have been described in this species (Halimaa et al. 2014), *N. brachypetala* has been ignored for mechanistic studies. Hyperaccumulation of Zn, Cd, Pb, and/or Ni in *N. brachypetala* from field or herbarium observations has occasionally been mentioned (Reeves and Brooks 1983; Reeves et al. 2001; Koch and German 2013). However, as far as we know this is the first study addressing population differences in Zn and Cd hyperaccumulation and tolerance of *N. brachypetala* in relation to differences in metal transporter gene expression. In fact, the shoot Zn and Cd concentrations of *N. brachypetala* plants (Figure 5.3, b, d) growing in their natural habitat on soils with normal metal concentrations (Figure 5.3, a, c) did not hit the threshold concentrations of 10,000 mg kg^{-1} Zn and 100 mg kg^{-1} Cd usually considered for hyperaccumulator species (Baker and Brooks 1989).Only plants from the Aneu population surpassed 3000 mg kg^{-1} Zn, the concentration more recently proposed as Zn hyperaccumulation threshold by several authors (van der Ent et al. 2013). However, these thresholds are typically reached or even widely surpassed in hyperaccumulator species growing on metalliferous soils. Here we report abnormally high shoot Cd and Zn concentrations in plants from normal soils, displaying bioaccumulation factors far above unity (Figure 5.5, a). This along with the results from the hydroponic experiments, where plants from all three *N. brachypetala* populations exceeded the shoot hyperaccumulation thresholds for Zn and Cd and accumulated higher shoot than root Cd and Zn concentrations, clearly demonstrates the hyperaccumulation character of *N.brachypetala* ac-

cording to the more rational criteria recently established by van der Ent et al. (2013).

A further clear demonstration of the high efficiency of these *N. brachypetala* populations to hyperaccumulate Cd and Zn is the fact that when grown under the same hydroponic conditions these plants accumulated as high (Mauri for Cd) or even higher Cd and Zn shoot concentrations than *N. caerulescens* Ganges, an ecotype that is considered to be highly efficient in Cd and Zn hyperaccumulation (Ueno et al. 2011). Also root to shoot translocation factors for Zn and Cd were higher in the *N. brachypetala* populations than in *N. caerulescens* (Ganges) (Figure 5.5, b). *Noccaea caerulescens* Ganges is highly adapted to metalliferous soils rich in Zn, Pb and Cd (Lombi et al. 2000). Previous investigations have shown that *N. caerulescens* populations from non-metalliferous soils may accumulate higher Zn and Cd concentrations than those from metalliferous habitats (Escarré et al. 2000). This may be due to lower capacity for root vacuolar storage of metals leading to less exclusion from the transpiration stream. Metal hyperaccumulation and metal tolerance are independent traits (Bert et al. 2003). In this regard, *N. caerulescens* populations from Zn-rich soil exhibited Zn tolerance, while a population from non-metalliferous soil registered chlorosis when exposed to 50-100 µM of Zn (Assunção et al. 2003). Enhanced expression of metal transporter genes in combination with constitutively high amounts of metal chelating substances are key factors for metal hyperaccumulation and hypertolerance (Tolrà et al. 1996; Krämer 2010; Kozhevnikova et al. 2014).

Despite the fact that *N. brachypetala* populations developed on soils with less than 1 mg kg^{-1} Cd, all their offspring when hydroponically grown tolerated the accumulation of more than 200 µg g-1 Cd dry weight in their shoot tissues without any growth reduction, chlorophyll decrease or influence on photosynthetic capacity. Root development was also not affected in *N. brachypetala* (data not shown). All *N. brachypetala* populations, excepting Freser, were as Zn tolerant as *N. caerulescens* (Ganges) (Figure 5.6). Freser was the most Zn sensitive population. Accumulation of more than 12,000 µg g^{-1} dry weight Zn in the shoots and more than 3000 µg g-1 Zn in the roots caused a significant decrease in biomass production in Freser.

5.2.4 *HMA4, HMA3 and MTP1 expression*

One week after the Zn or Cd treatment was initiated, *N. caerulescens* (Ganges) and the three *N. brachypetala* populations showed quite different patterns in the expression of metal transporter genes *HMA4*, *HMA3* and *MTP1* (Fig. 6). Zinc treatment enhanced root expression especially of *MTP1* and *HMA3* in Ganges, but not in Mauri. In Aneu Zn exposure induced a slight enhancement of *HMA3* expression in roots. In contrast, in roots from Zn treated plants from Freser more

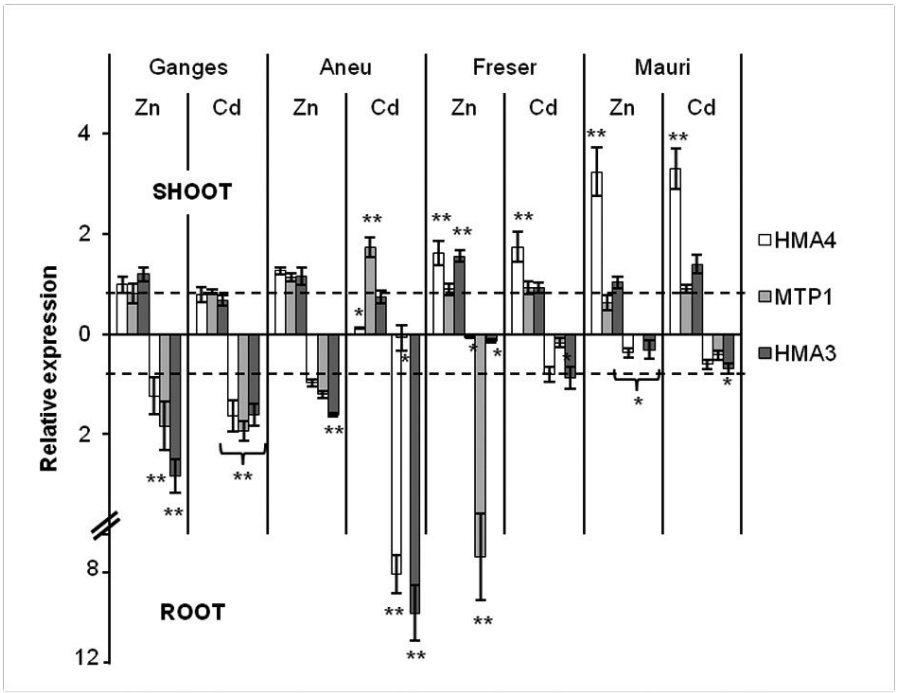


Figure 5.7: Relative expression analysis of three metal transporters according to Pfaffl method. The expression is expressed as the fold change compared to non-treated plants and normalized by tubulin gene. Shoot markers expression is represented on the top part of the graphic and root markers expression on the bottom part. Dotted lines mark similar expression (fold change around 1) for treated and control plants; double asterisk indicates over-expression ($\geq$ 1.5) and simple asterisk indicates down-regulation ($\leq$ 0.5)

than a 7 fold increase in the expression of *MTP*1 was observed, while expressions of *HMA*4 and *HMA*3 decreased. However, *HMA*4 expression showed a more than 2-fold increase in shoots of Mauri. In Freser the Zn treatment caused a small, but statistically significant enhancement of the expression of *HMA*4 and *HMA*3 in shoots. In *N.caerulescens* Ganges Cd induced a close to two fold increase in the root expression of *HMA*4, *HMA*3 and *MTP*1. Cd-induced up-regulation of *HMA*4 and *HMA*3 was strongest in roots of Aneu achieving values close to 8-fold increase, while *MTP*1 expression decreased in the roots and increased in the shoots. *MTP*1 expression was also decreased in the roots of Cd- treated Freser and Mauri, while its shoot expression was substantially enhanced in plants from both populations (Figure 5.7).

Differences in the relative expression of the three metal transporter genes *MTP*1, *HMA*3 and *HMA*4 also support the view of differences in Zn tolerance amongst the studied species and populations (Figure 5.7). *NcHMA*4 that is expressed throughout the vascular system with highest expression in the crown has been identified as a key

player in both hyperaccumulation and hypertolerance of Zn and Cd (Hanikenne et al. 2008; Craciun et al. 2012). Compartmentation of Zn and Cd into vacuoles is achieved by the tonoplast transporters *MTP*1 (Assunção et al. 2001) and *HMA*3 (Ueno et al. 2011), respectively. Increased copy number of genes involved in Zn and Cd transport and compartmentation seem to be responsible for the different expression levels and the ecotype differences in metal uptake, transport, and tolerance in *N. caerulescens* and *Arabidopsis* halleri (Krämer 2010; Craciun et al. 2012).

Constitutive shoot expression of *HMA*4 and *HMA*3 were substantially higher in *N. caerulescens* Ganges than in *N. brachypetala* (Figure 5.7). This is not surprising as *N. caerulescens* ecotype Ganges is highly adapted to metalliferous soils heavily polluted by Zn, Pb and Cd, while our *N. brachypetala* populations were from unpolluted sites. Noteworthy, Freser displayed higher root expression of *HMA*4 under control conditions than Ganges (Figure 5.7). However, exposure to moderately high concentrations of Zn or Cd induced considerable differences in the expression patterns. In *N. caerulescens* Ganges root vacuolar storage of Zn and Cd was apparently favoured by up-regulation of *MTP*1 and *HMA*3 in the roots while the constitutively high shoot expression did not change under the relatively mild stress conditions of this study (Figure 5.7). Amongst the *N. brachypetala* populations the high constitutive expression of *HMA*4 in roots of Freser indicates high xylem loading; this high root expression level is maintained under Cd exposure and considerably enhanced in the shoots. In fact, Freser accumulated and tolerated the highest Cd shoot and root concentrations (Figure 5.5). Contrastingly, this population was sensitive to Zn (Figure 5.5) so that the strong up-regulation of the Zn vacuolar transporter *MTP*1 observed in the roots of Freser exposed to excess Zn for one week (Figure 5.7) did not lead to an efficient detoxification and restriction of Zn transport to the shoots in the longer term as shown by the tissue concentration values determined after 4 weeks of exposure. In contrast, Mauri, was Zn tolerant as indicated by the Zn-induced growth enhancement (Figure 5.5). Opposed to the other specimens, Mauri maintained or even reduced the root expression of *HMA*3, *HMA*4 and *MTP*1 and exhibited the highest up-regulation of shoot *HMA*4 under both Zn and Cd exposure (Figure 5.7). Zinc-induced changes in expression patterns in Aneu were similar to those found in Ganges. However, Cd supply caused a strong up-regulation of *HMA*3 and *HMA*4 in roots and of *MTP*1 in shoots, while expression of *MTP*1 was significantly reduced in the roots. These changes may favour Cd storage in the roots and Zn transport to the shoots under Cd stress. Taken together our results suggest differences amongst the hyperaccumulating *N. brachypetala* populations in the strategies for handling enhanced Cd and Zn availability: restriction of Cd uptake and enhanced Cd and Zn shoot xylem

loading in Mauri with Zn-induced growth stimulation; activation of root vacuolar storage of excess Zn, but insufficient for both restriction of shoot Zn accumulation and avoidance of Zn toxicity in Freser; in Aneu high Zn accumulation in both roots and shoots, high root vacuolar storage of Cd, and decreased root Zn vacuolar storage under Cd exposure favouring Zn translocation to the shoot.

5.3 CONCLUDING REMARKS

In conclusion, *N. brachypetala* was clearly identified based on morphological traits, while usual specific genetic markers failed to differentiate the three *Noccaea* species reported in the Eastern Pyrenees. This study confirms for the first time the Zn and Cd hyperaccumulation character of *N. brachypetala* both in the field and in hydroponics according to criteria by van der Ent et al. (2013). Different expression patterns of metal transporter genes in response to Zn and Cd supply indicate different strategies for handling excess metal ions. *Noccaea* species seem to be in regression in the southern slope of the Eastern Pyrenees and further investigations to quantify this risk of extinction should be promoted to avoid irreparable loss of this important germplasm.

Table 5.2: Summary of results. Approximate values of metal concentrations are given in $\mu g g^{-1}$ of dry weight and bioaccumulation factors in $\mu g g^{-1}$ shoot / $\mu g g^{-1}$ root. Differences in expression are given as "-" and "+" when expressions were lower or higher than the control, respectively, and as "++" when the expression was remarkably higher than in the control.

Parameter	Aneu	Mauri	Freser	Ganges
Soil [Zn]	Higher (225)	Same (100)	Same (100)	-
Soil [Cd]	Higher (0,5-0,6)	Same (0,2-0,3)	Same (0,2-0,3)	-
Soil [Co], [Cu], [Pb]	Slightly higher	Same	Same	-
Shoot [Zn] (field)	4000	2000	-	-
Shoot [Cd] (field)	40	10	-	-
Shoot [Co], [Cu], [Pb]	Similar	Similar	-	-
Bioaccumulation factor	Same (50-70)	Same (15)		-
Translocation factor	Same	Same	Same	Lower
Shoot [Zn] (hydroponic)	Same (12000-17000)	Same (12000-17000)	Same (12000-17000)	Lower (5000)
Root [Zn] (hydroponic)	Higher (6000)	Same (3000)	Same (3000)	Same (3000)
Shoot [Cd] (hydroponic)	Middle (300)	Lower (200)	Higher (600)	Lower (200)
Root [Cd] (hydroponic)	Middle (200)	Lower (<200)	Higher (500)	Middle (300)
Relative Dry Weight (Cd)	Same	Same	Same	Same
Relative Dry Weight (Zn)	Middle	Higher	Lower	Middle
*HMA*3 expression (Zn)			- Root	+ Root +Shoot
*HMA*4 expression (Zn)		+ Shoot	- Root	+ Shoot
*MTP*1 expression (Zn)			++ Root	+ Root + Shoot
*HMA*3 expression (Cd)	++ Root -Shoot			
*HMA*4 expression (Cd)	++ Root -Shoot			
*MTP*1 expression (Cd)	+ Shoot - Shoot			

Part IV

GENERAL CONCLUSIONS

6

GENERAL CONCLUSIONS

1. ZN IMPROVES NOCCAEA CAERULESCENS RESISTANCE TO ALTERNARIA BRASSICICOLA, LEADING TO A TRADE-OFF BETWEEN THE METAL AND SOME OF THE ORGANIC-BASED DEFENSES.

1.1. Zn accumulation protects *Noccaea caerulescens* from *Alternaria brassicicola*.

1.2. *Alternaria* is sensitive to Zn concentrations above 0.5 m.M, a concentration that is overpassed in leaves from 102 μM Zn-treated *Noccaea* if total, bound and free, Zn is considered.

1.3. *Alternaria* infection does not favor Zn uptake by the roots and its translocation to the shoots; neither the expressions of the Zn-transporter genes *HMA3* and *HMA4* are enhanced.

1.4. Zn accumulation is inversely correlated with leaf Ca, Mg, S and Mo concentrations but it does not affect negatively *Noccaea* resistance to *A. brassicicola.*

1.5. JA, SA and ABA and the expression of the JA/Et, JA and SA signalling pathways marker genes *PDF1.2*, *CHIB*, *LOX2*, *PR1* and *BGL2* are evenly induced in infected plants from all Zn treatments 24 hours after the inoculation with *Alternaria*.

1.6. One week after the inoculation there is a trade-off between leaf Zn concentration and the concentration of JA, SA and ABA and the related marker genes.

1.7. Leaf total glucosinolates also show a negative relationship with leaf Zn concentration, but, in contrast, one week after the inoculation, glucosinolates concentration increases in the infected leaves of plants receiving the higher Zn treatment. The glucosinolates synthesis and degradation dynamic requires further study.

1.8. In part, the results support the trade-off hypothesis between inorganic and organic defenses in *N. caerulescens*, as SA, JA and ABA hormonal pathways are downregulated one week after inoculation in high-Zn-treated plants but not earlier, at 24 hours, and more glucosinolates concentration is present in high-Zn, infected leaves, but not in the healthy ones.

2. A. THALIANA WT ZN-TREATED PLANTS ARE MORE RESISTANT TO A. BRASSICICOLA ATTACK IN A POSSIBLE MECHANISM THAT COMBINES ZN ACTING AS AN INORGANIC DEFENCE AND ENHANCEMENT OF ORGANIC DEFENSES. A PROTECTIVE EFFECT OF ZN AGAINST B. CINEREA IS NOT CLEARLY PATENT.

2.1. *A. thaliana* WT plants treated with 12 μM of Zn are more resistant to *A. brassicicola* attack, but it is not clear if they are also more resistant to *Botrytis*.

2.2. *Botrytis* toxicity threshold for Zn is approximately two times higher than that of *Alternaria*, what could be a reason for the plant enhanced protection to *Alternaria* by Zn.

2.3. *A. thaliana* WT and the stress signaling defective mutants accumulate in their tissues similar leaf Zn concentration in plants growing at 12 μM of Zn, a concentration that it is accounted to be potentially toxic for *Alternaria*, but not for *Botrytis*.

2.4. In *A. thaliana* WT plants, Zn amplifies the expression of genes related to the SA and JA/Et signaling pathway, such as *PR*1 and *BGL*2 for SA and *CHIB* and *PDF*1.2 for JA/Et. *PDF*1.2 is the gene that more strongly responds to *Alternaria* attack and its expression is greatly amplified by Zn during the infection.

2.5. Changes in the endogenous concentrations of SA, JA, ABA and the precursor of Et, ACC, cannot be made responsible for the differences in the resistance to *Alternaria* in the Zn-treated WT and mutant plants.

2.6. In contrast to *A. thaliana* WT plants, the Zn-treated mutant lines do not present increased resistance to *Alternaria* or even more susceptibility to *Botrytis*. As Zn did not compensate the metabolic defect in the mutants infected by *Alternaria*, it is suggested that the plant organic defenses activated by the enhanced Zn levels play a more important role than Zn as a direct inorganic defense against *A. brassicicola*.

2.7. Zn concentration in leaves of treated *A. thaliana* is potentially toxic for *Alternaria* in our model, although Zn supply is unable to compensate immune defects in mutant plants. Moreover, Zn interacts with the organic defences, enhancing the expression of marker genes from the SA and JA/Et signalling pathways, especially the gene encoding for the defensine PDF1.2. A joint effect between Zn as an inorganic defence and an enhancement of the plant organic defences could be accounted for the better resistance of *A. thaliana* to *Alternaria* attack. Nevertheless, the role of Zn would be better understood if further studies in stress signalling gene expression in the mutant lines would be performed.

3. NOCCAEA BRACHYPETALA FROM NON-METALLIFEROUS SOILS IN THE EASTERN PYRENEES HYPERACCUMULATES CADMIUM AND ZINC.

3.1. *Noccaea brachypetala* can be clearly distinguished from other *Noccaea* species by morphological characteristics, while molecular studies analyzing the differences of four non-coding regions does not reveal clear differences

3.2. Four populations of *Noccaea brachypetala*, presumably at risk of extinction, are present in the southern slope of the Eastern Pyrenees. The fragility of the populations and the accessibility limit their use for field studies in metal hyperaccumulation and defence.

3.3. This study for the first time confirms the Zn and Cd hyperaccumulation capacity os *Noccaea brachypetala* in hydroponics under controlled lab conditions, confirming previous field studies

3.4. Different expression patterns of metal transporter genes in response to Zn and Cd supply in the populations indicates different strategies for handling excess metal ions.

Part V

SUPPLEMENTARY MATERIAL

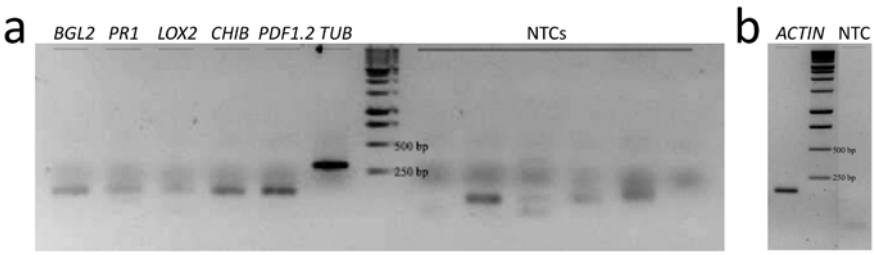
APPENDIX A: PRIMER SEQUENCES

Table A.1. Primer sequences used for gene quantification in *N. caerulescens* ([a]), *A. thaliana* ([b]), *N. brachypetala* ([c]), *A. brasscicicola*([d]) and *B. cinerea* ([e])

Type of gene	Gene	Primer 5′-3′ (F/R)	References
Stress markers	*PR1*[a,b]	GTTGCAGCCTATGCTCGGAG /	Abe et al. 2008
		CCGCTACCCCAGGCTAAGTT	
	BGL2[a,b]	GCCGACAAGTGGGTTCAAGA /	Abe et al. 2008
		AACCCCCCAACTGAGGGTT	
	PDF1.2[a,b]	CCATCATCACCCTTATCTTCG /	Abe et al. 2008
		TGTCCCACTTGGCTTCTCG	
	CHIB[a,b]	ACGGAAGAGGACCAATGCAA /	Abe et al. 2008
		GTTGGCACCAAGGTCAGGGT	
	LOX2[a,b]	TTGCTCGCCAGACACTTGC /	Abe et al. 2008
		GGGATCACCATAAACGGCC	
	VSP2[b]	GTTAGGGACCGGAGCATCAA /	Abe et al. 2008
		AACGGTCACTGAGTATGATGGGT	
Transporters	*HMA3*[a,c]	TTAAAGCTGGAGAAAGTATACCGA /	Ueno et al. 2011
		GCTAGAGCTGTAGTTTTCACCT	
	HMA4[a,c]	GTGGCAGAAGAGTTACTTCGACG /	Iqbal et al. 2013
		TTTGGAACGGGGAGATGAGG	
	MTP1[a,c]	AGAGACCGAGAGAGCAAAGG	Klein et al. 2008
		TTGCGTTCTTTGGTATCCCC	
Housekeepings	*Tubulin*[a,c]	CTACGCACCAGTCATCTCT /	Wu et al. 2009
		CGAGATCACCTCCTGGAACA	
	Tubulin[b]	AAGCTTGCTGATAACTGTACTGGT	Wu et al. 2009
		GGTTTGGAACTCAGTGA CATCA	
	AbActin[d]	GGCAACATTGTCATGTCTG /	Cho et al. 2007
		GAGCGAAGCAAGAATGGAAC	
	ActinA[e]	TGTCCTTGAGACCTTCAACGC /	Choquer et al. 2007
		GGTGCAATGATCTTGACCT	

Table A.2. Primer sequence for *N.brachypetala* taxonomical study

Region	Primers	Primer sequence (5′-3′)	Reference
5.8S rDNA	ITS1/ITS4	TCCGTAGGTGAACCTGCGG /	White et al. 1990
		TCCTCCGCTTATTGATATGC	
trnL – *trnF*	c/f	CGAAATCGGTAGCGCTACG /	Taberlet et al. 1991
		ATTTGAACTGGTGACACGAG	
*rpl*32 – *trnL*(*UAG*)	rpl32F / trnL(UAG)	CAG TTC CAAAAAAACGTACTTC /	Shaw et al. 2007
		CTG CTT CCT AAG AGC AGC GT	
trnQ – 5′*rps*16	trnQ(UUG) / rpS16x1	GCG TGG CCA AGY GGT AAG GC /	Shaw et al. 2007
		GTT GCT TTY TAC CAC ATC GTT T	



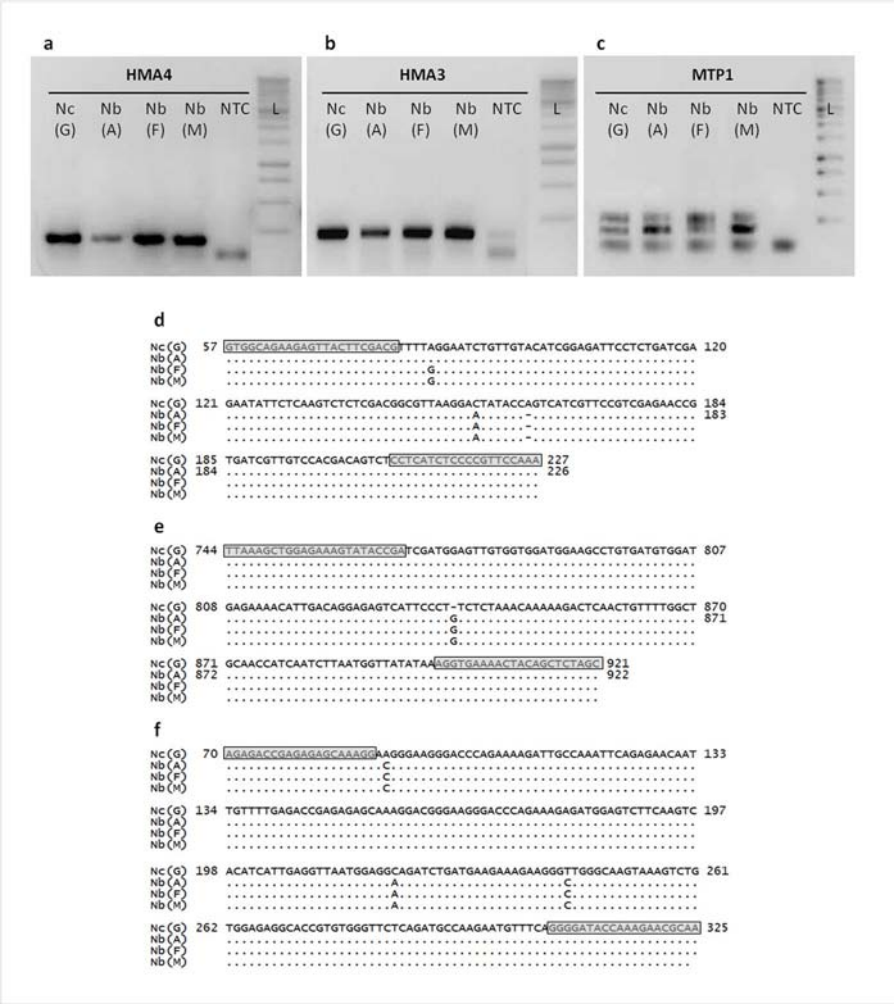
B.1. 2% agarose gel showing bands from qPCR products of 5 defense genes and the housekeeping *Tubulin* from *N. caerulescens* (a) and *Actin* from *A. brassicicola* (b), using primers from *A. thaliana* (Table 3.2 on page 40).

B.2. Percentage of identity with closely realted species of the amplified sequences from the *N. caerulescens* leaf RNA using the *A. thaliana* and *A. brassicicola* (in inoculated leaves) primers. The high percentage of identity with the regions of the same genes confirm the suitability the *A. thaliana* primers for *N. caerulescens* and the correct amplification of the fungal RNA within the extracted plant RNA.

Gene	BLAST Identity (%)	Species
BGL2	87	*Camelina sativa*
	82	*Arabidopsis thaliana*
PR1	74	*Arabidopsis thaliana*
LOX2	90	*Brassica rapa*
	83	*Camelina sativa*
	81	*Arabidopsis thaliana*
CHIB	88	*Camelina sativa*
	85	*Arabidopsis thaliana*
PDF1.2	86	*Arabidopsis thaliana*
	86	*Arabidopsis halleri*
AbActin	99	*Alternaria alternate*
	91	*Pyrenophora teres f.maculate*

APPENDIX C: PRIMERS SUITABILITY II

Suitability of use of *N. caerulescens* (Nc(G)) primers for amplification of regions from the *HMA*3, *HMA*4 and *MTP*1 genes in *N. brachypetala* cDNA from three populations: Aneu (Nb(A)), Freser (Nb(F)) and Mauri (Nb(M)). The correct amplification of the cDNA regions were checked in a 1,5% agarose gel (a, b, c; NTC: non-template control; L: DNA ladder) and their sequences compared to that of *N. caerulescens* (d: *HMA*3, e:*HMA*4 and e: *MTP*1).



APPENDIX D: TAXONOMICAL CHARACTERS

Main morphological differences between *Noccaea brachypetala*, *N. caerulescens* and *N. occitanica*.

	Noccaea brachypetala	*N. caerulescens*	*N. occitanica*
Flower stalk length (cm)	13-55	5-40	4-19
Inflorescencein fruiting stage	lax, rarely dense	lax	dense
Fruiting pedicels length (mm)	2-8	3-10	5-20
Sepals length (mm) and colour	1-1.3 ligth green to yellowish-green	1.5-2.5 ligth green, usually violet stained	1.3-2 green and violet stained
Petals length (mm) and colour	1-1.5, violet or white	2-4, white, sometimes pale rose or pale violet	1.8-3.2, white or pale rose
Stamen filament length (mm)	1,5-2	2-4,2	2,1-3,5
Anthers length (mm)	0,3-0,4	0,4-1	0,3-0,5
Style length (mm)	0.3-0.5 included within the notch of the ripe fruit	1-2 mm equaling or exceeding the notch of the ripe fruit	0.7-1.7 included within the notch of the ripe fruit
Fruit size (mm) and shape	3.5-8 × 2-3.5 obcordate, not winged or narrowly winged basally, broadly winged apically	5-8 × 3-4oblong-cuneate to obcordate, narrowly winged basally, broadly winged apically	6-10 x 4-8 broadly obcordate, broadly winged throughout its length
Seeds size (mm)	1.5-1.7 x 1-1.2	1.4-1.6 x 0.8-1.1	1.5-2.1 x 0.9-1.4

Part VI

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