REVIEW ARTICLE

The impact of transition metals on bacterial plant disease

Helen Fones[1] & Gail M. Preston[2]

[1]Institut für Biologie, Freie Universität Berlin, Berlin, Germany; and [2]Department of Plant Sciences, University of Oxford, Oxford, UK

Correspondence: Gail M. Preston, Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK. Tel.: 01865 275132; fax: 01865 275074; e-mail: gail.preston@plants.ox.ac.uk

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Abstract

Metals play essential roles in many biological processes but are toxic when present in excess. This makes their transport and homoeostatic control of particular importance to living organisms. Within the context of plant–pathogen interactions the availability and toxicity of transition metals can have a substantial impact on disease development. Metals are essential for defensive generation of reactive oxygen species and other plant defences and can be used directly to limit pathogen growth. Metal-based antimicrobials are used in agriculture to control plant disease, and there is increasing evidence that metal hyperaccumulating plants use accumulated metal to limit pathogen growth. Pathogens and hosts compete for available metals, with plants possessing mechanisms to withhold essential metals from invading microbes. Pathogens, meanwhile, use low-metal conditions as a signal to recognise and respond to the host environment. Consequently, metal-sensing systems such as *fur* (iron) and *zur* (zinc) regulate the expression of pathogenicity and virulence genes; and pathogens have developed sophisticated strategies to acquire metal during growth in plant tissues, including the production of multiple siderophores. This review explores the impact of transition metals on the processes that determine the outcome of bacterial infection in plants, with a particular emphasis on zinc, iron and copper.

Introduction

Plant diseases provide an important social and economic challenge, being responsible yearly for pre- and postharvest losses of 16–28% of crops (Chakraborty & Newton, 2011). It is increasingly understood that the concentration of transition metals in the environment and the availability of essential metals to support pathogen growth can have a significant impact on the outcome of plant–pathogen interactions. In this review we discuss the ways in which transition metals influence the outcome of plant–pathogen interactions, considering both their effects upon the plant, in terms of plant health, defensive signalling and alterations to the environment that the pathogen experiences *in planta*; and their effect upon the pathogen, in terms of mineral nutrition, regulation of virulence gene expression and toxicity. This review will focus on three metals that are of profound interest for their effects on plants and pathogens: iron, copper and zinc. Initially, we give a brief overview of the importance of transition metals for all forms of life, and the ways in which plants and microbes obtain and regulate their supply of these important but potentially toxic chemicals. We will discuss the ways in which pathogenesis can be dependent upon, or regulated by, metal availability; and how, in turn, this can influence the plants' response to the pathogen. Finally, we discuss the special case of metal hyperaccumulating plants, which maintain high foliar metal concentrations that appear to form an important part of their anti-pathogen defences. These unusual plants may not only provide insights into the role of metals in plant–pathogen interactions, but also genetic resources for the improvement of metal uptake by crops, and insights into the consequences of altering metal availability in crop plants for disease resistance.

Metals are essential for both plants and plant pathogens

To understand the effects that metals such as copper, zinc and iron have on plant–pathogen interactions, it is necessary to briefly consider their chemistry and the roles in

metabolism that they fulfil. Copper and iron, along with other transition metals, have incompletely filled d-orbitals, conferring strong redox activity; in aqueous solution, their ions may also participate in reactions as Lewis acids (see Box 1). These properties make these metals able to catalyse biochemical reactions, giving them an essential role in metabolism (Nies & Brown, 1998). Zinc is often included in discussions of transition metals, but it does not have an incomplete d-orbital, and it does not participate in redox reactions. Instead, its stability makes it an ideal cofactor for enzymatic reactions that require a stable metal ion to act as a Lewis acid during catalysis. Estimates suggest that as high a fraction as one third of all proteins require some metal cofactor (Waldron & Robinson, 2009). In illustration of the importance of these metals to all life forms, consider that iron has essential roles in oxygen metabolism, electron transport, lipid metabolism and the tricarboxylic acid (TCA) cycle; in fact, there are known to be over 100 metabolic enzymes with iron-based cofactors (Massé & Arguin, 2005; Miethke & Marahiel, 2007). So fundamental are some of its uses that bacteria have been found to have controls both at the gene expression and post-transcriptional level to ensure that iron is directed to the most essential proteins when it is in limited supply (Massé & Gottesmann, 2002; Massé & Arguin, 2005; Zaini *et al.*, 2008).

Zinc is necessary for the functioning of DNA/RNA polymerase enzymes and of ribosomes and in superoxide dismutase (SOD) (Zelko *et al.*, 2002). Zinc also has particular importance for plants, being found in carbonic anhydrase and in stromal processing peptide, and thus contributing to photosynthesis. Additionally, zinc is important for protein structure, with 4% of *Arabidopsis* proteins, for example, containing zinc finger domains (Hänsch & Mendel, 2009). Copper is needed in cytochrome oxidases (Waldron & Robinson, 2009), ascorbate oxidase (Santagostini *et al.*, 2004), SOD (Zelko *et al.*, 2002), polyphenol oxidase (Marusek *et al.*, 2006) and, in plants, in the receptor for the hormone ethylene, an important signal in plant development and disease resistance (Rodríguez *et al.*, 1999). These essential roles are by no means an exhaustive list of the functions of these metals *in vivo*.

Given the essential role of metals in living organisms, it is clear that either a lack, or an excess of essential metals may have a profound effect on a wide range of organisms, and by extension, their interactions. Add to this the fact that availability varies widely in natural situations– zinc, for example, is present at varying concentrations in different soil types (Reeves & Baker, 2000; Hotz & Brown, 2004), including growth limiting concentrations in many agricultural soils – and it becomes clear that the impact of these metals on plant–pathogen interactions must be taken into consideration in economically important agricultural settings. To gain more insight into the impact of varying metal availability on plant disease it will be necessary to consider in what ways these metals are available to both partners in this interaction.

Box 1 Chemical terms

Lewis acid: A molecule that is capable of taking part in reactions in which it forms a co-ordinate covalent bond by accepting a pair of electrons. Common examples are H^+ and metal cations:

(a) $H^+ + NH_3 \rightarrow NH_4^+$
(b) $H^+ + OH^- \rightarrow H_2O$
(c) $Cu^{2+} + 4NH_3 \rightarrow Cu(NH_3)_4^{2+}$

Fenton reaction: Generation of $OH^{\cdot}$ radicals from H_2O_2 by the reduction of $O_2^{\cdot -}$, catalysed by redox active metal ions, usually iron in biological systems:

(1) $Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$
(2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$

Sources of metals

Plants generally have one main source of mineral nutrients: the soil, although foliar fertilisation is also used in some crop management systems. The availability of mineral nutrients in the soil may be further affected by mycorrhizal symbioses and rhizosphere bacteria, detailed discussion of which is beyond the scope of this work. Although some opportunistic and soil-borne pathogens, such as *Ralstonia* spp., can obtain metals directly from the environment (Denny, 2007), pathogens that multiply inside the host are dependent on the host for their metal supply (Hancock & Huisman, 1981; Rico *et al.*, 2011). The importance of, and limitations imposed by, this dependency is at least partially determined by the mode of infection employed. Necrotrophs, such as soft rot pathogens belonging to the genera *Dickeya* and *Pectobacterium* (formerly *Erwinia*), have access to the full range of nutrients found within the colonised tissue, as they break down cell walls and membranes and release metals and other compounds that are sequestered in the vacuole or bound within the cell wall (Hugouvieux-Cotte-Pattat *et al.*, 1996). The tissue colonised may be of importance in determining metal availability, as certain metals may be retained within the roots of plants, particularly if toxic in excess (Lasat *et al.*, 1996; Prasad, 2004); or, if in short supply, will be translocated rapidly to sink tissues. Even within a tissue, there may be heterogeneity of availability as certain cell types may be used preferentially as stores of metals (Küpper *et al.*, 1999, 2004). The pathogens

likely to suffer the most restriction, however, may be xylem pathogens, such as *Xylella fastidiosa* (Hopkins, 1989) to which only those compounds translocated in the xylem are available, and where iron and other metals may be mainly available in chelated forms such as Fe-citrate. This means that metal availability to the pathogen depends both on metal availability to the plant and the plant's requirement for the nutrient in its aerial tissues.

Many biotrophic and hemibiotrophic bacteria, such as *Pseudomonas syringae*, colonise the apoplastic space between the cells of a plant's leaves (Preston, 2000). As these pathogens have no or limited access to intracellular stores such as the vacuole, they face particular challenges in obtaining essential but redox active metals, which are often sequestered in the vacuole for the plants' own protection (Küpper *et al.*, 1999). For hemibiotrophic strains, these difficulties may be obviated at later stages of infection, but they may be considered to be of importance during biotrophic growth.

Metal uptake, toxicity and homoeostasis

Given the importance of metals for life, it is unsurprising that organisms compete for them when they are in short supply, and that they have evolved specific systems that enable them to take up the metal ions that they require. This is of great importance in the case of iron, because, although an abundant metal, it exists most commonly as the insoluble and therefore nonbioavailable Fe^{3+} in aerobic environments (Miethke & Marahiel, 2007). For many plants and micro-organisms, the solution to this is to produce and secrete diffusible ligands, known as siderophores (Fig. 1), which have high affinities for this otherwise unavailable ferric iron (Romheld & Marschner, 1986; Braud *et al.*, 2009), in conjunction with the synthesis of dedicated uptake proteins for the siderophore-Fe^{3+} complex, such as the TonB-dependent uptake system (Cornelis & Matthijs, 2002). Certain bacteria, including the opportunistic pathogen *Burkholderia cenocepacia*, can obtain iron directly from host iron-chelating proteins such as ferritin (Whitby *et al.*, 2006). These abilities, however, are known to have become the subject of an evolutionary arms race between bacterium and host in vertebrate pathosystems (Skaar, 2010), an outcome which also seems likely in phytopathology. This makes the indirect pathway of siderophore production more stable, although there are also clear advantages for a bacterium in being able to assimilate siderophores produced by other organisms, including the host (Miethke & Marahiel, 2007).

Siderophore production is regulated by iron availability as part of a complex system of iron homoeostasis. The topics of siderophore production, regulation and uptake have been extensively reviewed elsewhere (e.g. Neilands, 1993, 1995; Cakmak *et al.*, 1994; Chakraborty *et al.*, 2007), and will not be covered again herein. Interestingly, however, it has recently begun to emerge that some so-called secondary siderophores, which have much lower affinities for iron than other siderophores produced by the same organism, may be important for the acquisition of other metals, including zinc (Leach *et al.*, 2007). As will be discussed, siderophores and their regulatory systems have extensive influence on plant–pathogen interactions.

Redox active metals are not simply required for life. A delicate balance must be maintained, because these metals have the potential to become toxic at excess concentrations. Copper, for example, is able to displace other metals from complexes and to generate reactive oxygen species (ROS). Similarly, iron is a potent generator of ROS (Miethke & Marahiel, 2007), which is the necessary corollary of the redox activity that makes it so fundamentally useful. ROS may cause oxidative stress and damage to cells (Fones & Preston, 2012). The observation that the toxic metal, cadmium, kills cells by creating waves of H_2O_2 and superoxide illustrates the damaging potential of ROS (Garnier *et al.*, 2006). Additionally, excess metals may compete with required cofactors for binding sites in transport proteins and enzymes (Stohs & Bagchi, 1995; Hanikenne, 2003). This results in a need for stringently controlled metal ion homoeostasis, and mechanisms for tolerating elevated metal concentrations.

Metals are most toxic as free ions (Pollard *et al.*, 2002), so an important facet of tolerance is chelation of metal ions into complexes to reduce their toxicity. Such complexes are often sequestered into specific subcellular compartments (Küpper *et al.*, 1999; Krämer *et al.*, 2000; Cosio *et al.*, 2004). This strategy is common among plants; in barley, for example, metal toxicity is reduced by vacuolar and apoplastic sequestration (Brune *et al.*, 1994). Chelating agents such as phytochelatins and metallothioneins are also known to be involved in metal tolerance in many plants; for example, phytochelatins are essential for tolerance to cadmium in *Arabidopsis thaliana* (Fordham-Skelton *et al.*, 1998), while metallothioneins have been shown to be upregulated in response to zinc stress in *Populus alba* (Castiglione *et al.*, 2007). A metallothionein has also been implicated in cadmium and zinc tolerance in *Silene vulgaris*, but its role may be indirect, *via* copper homoeostasis, rather than directly chelating zinc or cadmium (Jack *et al.*, 2007).

Metal binding proteins are similarly important in bacterial metal tolerance. For example, the *copA*, *B* and *C* genes of *P. syringae* encode copper binding proteins that are found in the periplasm and outer membrane of the

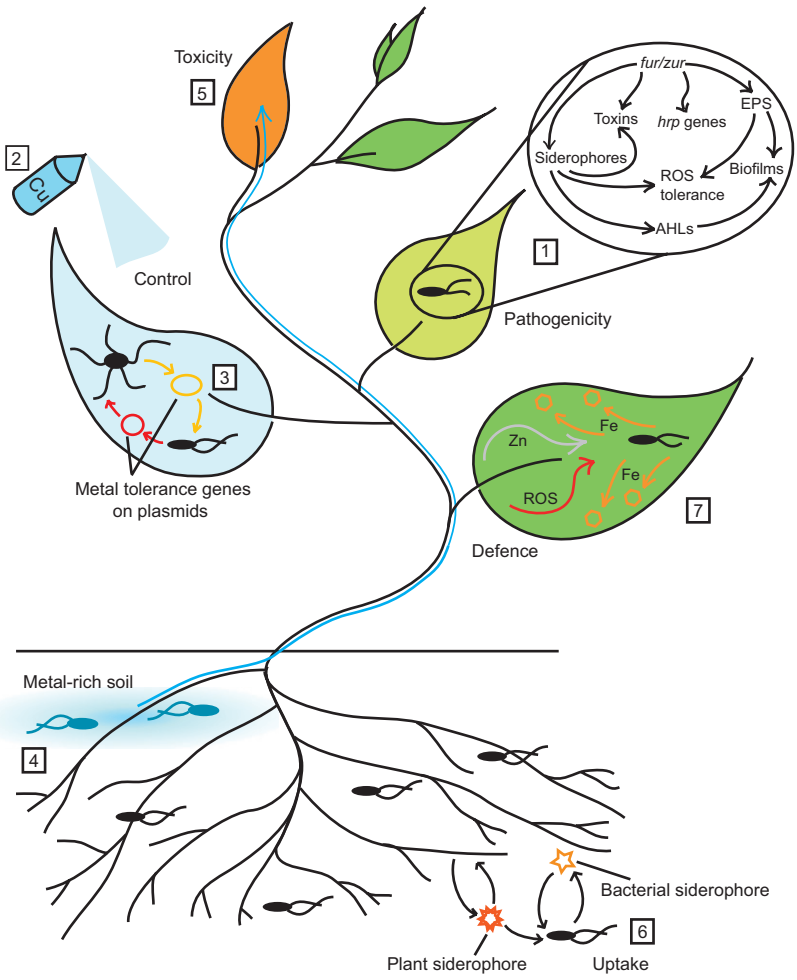

Fig. 1. Metals in plant-microbe interactions. Metals can influence the expression of various bacterial virulence factors, including toxins, EPS and *hrp* genes, most notably *via* signalling involving metal sensing systems including *fur* and *zur*, and metal uptake systems, particularly siderophores (1). Metals may also have roles in protecting the plant against infection and, especially in the case of copper, can be applied directly to crops as antimicrobials (2). This approach can lead to the development of metal resistant strains, accelerated by the horizontal transfer of resistance genes. Build-up of metals in the soil can affect microbial communities (4), with potential effects upon plant-microbe interactions, and can result in overexposure of the plant, causing toxicity symptoms such as russetting (5). Plants and microbes also compete for metals. Metal uptake often occurs *via* iron-chelating compounds such as siderophores, and soil-borne bacteria are frequently able to take up those produced by the plant, in addition to their own (6). Within the plant, competition for metals may also be important, with the withholding of metals, especially iron, being an important defence. Additionally, plants may use metals in defence, either as catalysts for ROS production, or more directly as antimicrobial toxins (7), as most clearly evident in the case of metal hyperaccumulating plants.

bacterium. These provide copper resistance by binding copper so effectively that the bacterial colonies can turn blue (Silver, 1996; Nies & Brown, 1998). This well characterised system is known to have parallels in many other bacteria (Cooksey *et al.*, 1990; Cooksey, 1994). Metallothioneins are also known in bacteria, having, in fact, first been discovered in the cyanobacteria *Synechococcus* (Silver, 1996). They are used particularly for zinc (Blindauer *et al.*, 2001) and also copper (Gold *et al.*, 2008). *Pseudomonas syringae* may also use histidine to bind copper (Cánovas *et al.*, 2003). Regulation of the expression of iron-binding proteins is thought to play an important role in iron homoeostasis. For example, transcriptomic studies of the xylem-limited pathogen *X. fastidiosa* showed that expression of the major iron storage protein, bacterioferritin, and other proteins containing iron-sulphur clusters decreased in iron-limited conditions (Zaini *et al.*, 2008).

However, the most common strategy for metal resistance in bacteria is the efflux of excess metal from the cell. For example, the *cueA* copper resistance gene of *Pseudomonas aeruginosa* encodes a P-type ATPase, which exports the metal (Schwan *et al.*, 2005). There are a number of well-studied metal resistance operons that enable efflux of metals from the cell. This efflux is mediated by a variety of transporters, the expression of which is regulated by metal ion sensing proteins (Busenlehner *et al.*, 2003). Efflux proteins include members of the Cation Diffusion Facilitator (CDF), Resistance-Nodulation-Cell Division (RND) and Major Facilitator superfamilies, as well as P-type and ABC ATPases (Mergeay *et al.*, 2003; Nies, 2003, 2007; Silver & Phung, 2005). There is evidence for the horizontal transfer of the genes encoding these metal-efflux systems (Mergeay *et al.*, 2003). The number of efflux pumps possessed by a bacterial species varies depending on lifestyle, with generalist bacteria typically possessing more, as they may be exposed to a greater variety of toxins. For instance, *Pseudomonas putida* and *P. aeruginosa* have twelve and ten efflux pumps, respectively, while *Escherichia coli* has four (Cánovas

et al., 2003). The mechanisms of bacterial metal tolerance are summarised in Fig. 2.

Interestingly, studies have also suggested a role for metal uptake systems in reducing metal toxicity, particularly the iron toxicity associated with bacterial responses to oxidative stress. Peroxide stress has been shown to induce a variety of adaptive changes in bacteria, including changes in metal ion homoeostasis such as increased expression of transporters involved in uptake of manganese and zinc. It has been speculated that increased cytosolic concentrations of these metals act to displace ferrous iron from enzyme targets, and a high Mn/Fe ratio has been correlated with oxidative stress tolerance (Faulkner & Helmann, 2011).

From the perspective of plant pathogenesis it is interesting to note that the regulation or expression of a number of metal tolerance systems alters during host-bacteria interactions. For instance, the *cueA* gene is upregulated in the plant growth promoting rhizobacterium *Pseudomonas fluorescens* SBW25 when growing on the surface of sugar beets (Zhang & Rainey, 2008), and it has been found that this gene is important for colonisation of plant hosts (Zhang & Rainey, 2007). The *copRSCD* genes present in a strain of *P. fluorescens* isolated from a diseased fish have been implicated in spread and survival in host tissues, as well as copper resistance (Hu *et al.*, 2009). In plants as well, metal resistance systems appear to play a role during interactions with pathogens. For instance, metallothioneins are known to be upregulated in *Arabidopsis*, tobacco and velvetleaf during infection (Dauch & Jabaji-Hare, 2006), while phytochelatin synthesis has been linked to basal defence responses (Clemens & Peršoh, 2009). This interplay between metal usage, metal stress and plant–pathogen interactions will be explored in more detail in the following sections of this review.

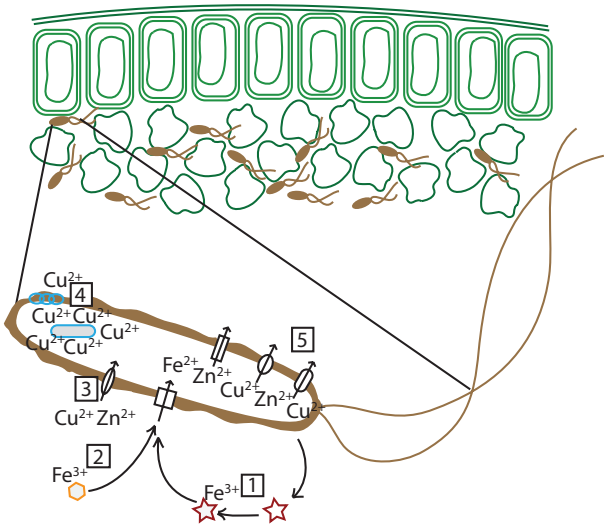

Fig. 2. Bacterial metal uptake and homoeostasis. Iron is often limiting in plant-associated environments. Iron-chelating compounds such as siderophores are produced to maximise uptake (1). Bacterial pathogens may also be able to obtain iron from host iron-chelating compounds such as ferritins, which may function to restrict iron availability to the pathogen (2). Metals such as zinc and copper are imported by membrane transporters (3). The presence of high concentrations of individual metals in the environment may restrict uptake of other essential metals through competition for metal ion binding sites in transport mechanisms. Toxic excesses of metal ions are often tolerated through chelation and sequestration, either by compounds within the EPS, periplasm or outer membrane, such as the CopABC copper binding system of *Pseudomonas syringae*, or compounds such as metallothioneins, found within the cell (4). The final and frequently most important layer of defence employed by bacteria against toxic excesses of metals is efflux from the cell (5), mediated by a number of types of membrane transporter including P-type and ABC ATPases, as well as CDF-, RND- and major facilitator family proteins.

Metal-dependent regulation of virulence

Fur and *zur*: metal sensing in virulence

There are a number of ways in which metals can influence the ability of a micro-organism to be pathogenic on a plant host. Their most obvious impact is in terms of metal availability to support bacterial growth in plant tissues. However, metals can also be involved in the regulation of pathogenesis and virulence genes, both directly and indirectly (Fig. 1). An important example of this is the transcriptional regulator, *fur*. *fur* controls the expression of iron uptake and iron storage systems in an iron-dependent manner (McHugh *et al.*, 2003; Butcher *et al.*, 2011). The mode of action of the Fur protein has been well studied in *E. coli*, where it is known to form a complex with Fe^{2+}, which binds to a 'fur box' sequence in the promoter of various genes. In the absence of iron, Fur cannot bind and repression of transcription of these genes is released (Bagg & Neilands, 1987). As well as this negative regulation, Fur can activate genes by repressing the transcription of the small RNA, RyhB, which, when expressed, causes degradation of mRNAs encoding iron-utilising enzymes (Massé & Gottesman, 2002). However, the role of Fur is not restricted to effects upon iron metabolism. Instead, Fur behaves as a global regulator of gene expression, with involvement in acid tolerance, oxidative stress tolerance, toxin production and the expression of virulence factors (Kitphati *et al.*, 2007), making iron and *fur* critical for pathogenesis (Ratledge & Dover, 2000).

Unsurprisingly, *fur* mutants have been found to have reduced virulence and pathogenicity. For example, a *fur* mutant of *P. syringae* pv. tabaci 11528, the causal agent

of wildfire disease of tobacco, was found to have reduced virulence and population growth *in planta*, which may be linked to *fur* regulation of iron uptake and siderophore synthesis, but may also be linked to reduced production of the toxin, tabtoxin, in *fur* mutants (Cha *et al.*, 2008). Tabtoxin is a monocyclic β-lactam, which is cleaved in a zinc-dependent manner (Durbin & Uchytil, 1985; Levi & Durbin, 1986) in plant cells to generate the toxin tabtoxinine-β-lactam (TβL). TβL inhibits glutamine synthesis by glutamine synthetase, which may cause chlorotic symptoms due to the build-up of ammonia (Barta *et al.*, 1992; Bender *et al.*, 1999). Similarly, a spontaneous *fur* mutant of *Xanthomonas campestris* pv. *campestris* was reported to show high intracellular iron, reduced virulence and increased sensitivity to ROS (Jittawuttipoka *et al.*, 2010). In some bacteria, including the plant symbionts *Rhizobium leguminosarum* and *Sinorhizobium meliloti*, *fur* has been found to regulate manganese uptake, while losing its role in iron homoeostasis (Johnston *et al.*, 2007). This is also the case in the tumour-inducing pathogen *Agrobacterium tumefaciens*, where the gene is renamed *mur*. Despite regulating a different metal, *mur* remains essential for full virulence (Kitphati *et al.*, 2007).

A homologue of *fur*, named *zur*, has been discovered which controls zinc homoeostasis by repressing zinc uptake and up-regulating zinc efflux (Hantke, 2001, 2005; Huang *et al.*, 2008). Zur behaves as a zinc responsive global transcription regulator, and, like *fur*, can be shown to be involved in pathogenicity and virulence. A *zur* mutant of *Xanthomonas oryzae* grows slowly in both rich medium and in rice leaves, and shows reduced virulence on rice (Yang *et al.*, 2007). A *zur* knock-out mutant in the related bacterium *X. campestris* shows reduced zinc tolerance and reduced virulence (Tang *et al.*, 2005). It is possible, however, that the virulence effect in this instance may be attributable to the fact that the mutant produces lower quantities of extracellular polysaccharides (EPS), which are important for virulence (Yu *et al.*, 1999; Fones & Preston, 2012).

It has been shown that *zur* is involved in the regulation of *hrp* (hypersensitive response and pathogenicity) genes in *X. campestris* (Huang *et al.*, 2009). This provides a direct link between this gene and the pathogenicity of the bacterium: the *hrp* genes, in concert with genes named 'hypersensitive response conserved' (*hrc*) produce the 'syringe-like' type three secretion system (T3SS) (Alfano & Collmer, 1997; Collmer *et al.*, 2000) through which 'effector' proteins are injected into host cells to enhance pathogenicity (Preston, 2000; Grant *et al.*, 2006). Additionally, *fur* is known to regulate the expression of T3SS genes in animal pathogens such as *Salmonella enterica* (Ellermeier & Slauch, 2008), suggesting that this link between metal regulation and the expression of the *hrp* cluster is unlikely to be confined to *Xanthomonas*. Indeed, it is known that iron nutrition can affect *hrp* gene expression in *P. syringae* (Bronstein *et al.*, 2008; Kim *et al.*, 2009, 2010). Other plant colonisation and virulence factors shown to be regulated by iron include type IV pili in *X. fastidiosa* (Zaini *et al.*, 2008) and exoenzyme production in soft rot pathogens such as *Dickeya dadantii* (Franza *et al.*, 2002).

Siderophores: metal binding in virulence

Links between siderophore production and pathogenesis have been clearly demonstrated in mammalian systems, in which it is understood, for example, that the siderophore yersiniabactin is essential for the pathogenicity of *Klebsiella* and *Yersinia pestis* and pyoverdine for *P. aeruginosa* on mouse hosts (Meyer *et al.*, 1996; Bearden *et al.*, 1997; Lawlor *et al.*, 2007). In plants, a large body of work performed by Expert and coworkers using the model system of *Erwinia chrysanthemi* (now renamed *D. dadantii*) and *Saintpaulia ionantha* has provided much of our current knowledge in this area, demonstrating a clear link between siderophore production and pathogenicity in this model system (Enard *et al.*, 1988; Expert, 1999). *Dickeya dadantii* produces two kinds of siderophore involved in high-affinity Fe uptake: achromobactin and chrysobactin. Both are needed for survival and full virulence *in planta* (Expert, 1999). The importance of these siderophores in mediating plant-microbe competition for iron can be illustrated by the finding that chrysobactin production by *D. dadantii* leads to iron deficiency in the plant host, as measured by the amount of iron bound to plant ferritins (Neema *et al.*, 1993).

In addition to those of *D. dadantii,* siderophores of related enterobacteria have been extensively studied. *Erwinia carotovora* (now *Pectobacterium carotovorum*) produces the high-affinity hydroxamate siderophore, aerobactin (Ishimaru & Loper, 1992), and a high-affinity class of siderophores, desferriodoxamines, is conserved among *Erwinia* and *Pantoea* species (Smits & Duffy, 2011). *Erwinia amylovora* infects the blossoms of pear and apple trees, which are known to provide an iron-limited environment (Temple *et al.*, 2004), a fact that supplies a possible role for these conserved high-affinity siderophores.

The transcriptional regulator Fur, discussed in the preceding section, regulates siderophore synthesis in many plant pathogenic bacteria, including *D. dadantii* (Franza *et al.*, 2005), *P. syringae* pv. *tabaci* (Cha *et al.*, 2008) and *P. syringae* pv. *tomato* (Jones *et al.*, 2007). Although *fur* mutants generally show constitutive siderophore production, it is interesting to note that nonlethal Fur missense mutants of *P. aeruginosa* have been reported to show con-

stitutive synthesis of the siderophores pyochelin and pyoverdine, but reduced iron uptake (Hassett *et al.*, 1996), which may provide one explanation for the reduced ability of some *fur* mutants to grow in the host environment. However, as noted above, *fur* does not regulate siderophore synthesis in all bacteria. A *mur (fur)* mutant of the tumourigenic plant pathogen *A. tumefaciens* did not show altered siderophore synthesis, although it did show increased manganese uptake, reduced virulence, reduced growth in iron-limiting conditions and increased sensitivity to oxidative stress (Kitphati *et al.*, 2007).

Evidence of a role for siderophore synthesis and iron uptake in the pathogenesis of *P. syringae* was suggested in work by Bronstein *et al.* (2005), which showed that mutants of *P. syringae* pv. *tomato* DC3000 lacking the twin-arginine translocation (tat) system had reduced siderophore synthesis, reduced iron uptake and decreased virulence. More direct evidence was provided by a study showing that siderophore-deficient mutants of *P. syringae* pv. tabaci were impaired in their ability to infect tobacco (Taguchi *et al.*, 2010). *Pseudomonas syringae* pv. *tomato* produces two siderophores, yersiniabactin and pyoverdine, and is also able to use citrate as an iron chelator (Buell *et al.*, 2003; Jones *et al.*, 2007; Jones & Wildermuth, 2011). However, siderophore-deficient mutants of *P. syringae* pv. *tomato*, although impaired in iron uptake *in vitro*, remained fully pathogenic in tomato. Similarly, siderophore mutants of the bean pathogen *P. syringae* pv. *phaseolicola* 1448A and the sweet cherry pathogen *P. syringae* pv. *syringae* B301D were shown to retain virulence when inoculated into bean pods and cherry fruit, respectively (Cody & Gross, 1987; Owen & Ackerley, 2011). Studies of a number of other plant pathogenic bacteria, including the rice pathogen *X. oryzae* pv. *oryzae*, the gram-positive plant pathogen *Streptomyces scabies*, the wilt pathogen *Ralstonia solanacearum* and *A. tumefaciens* have also failed to demonstrate a role for siderophore synthesis in plant pathogenesis (McQueen & Schottel, 1987; Bhatt & Denny, 2004; Rondon *et al.*, 2004; Pandey & Sonti, 2010; Seipke *et al.*, 2011).

Several explanations have been put forward to explain why siderophore-deficient mutants of some plant pathogenic bacteria remain fully virulent in plant hosts. It has been suggested that pathogens are able to acquire iron from plant iron compounds such as heme/hemin or iron-nicotiamine, or that iron levels in plant tissues are sufficient to support pathogen growth in the absence of siderophore synthesis (Bhatt & Denny, 2004; Jones & Wildermuth, 2011). Iron complexes with phytic acid (myo-inositol hexakisphosphate, InsP6) and other myo-inositol trisphosphate and tetrakisphosphate regio-isomers have also been suggested to be a source of iron for plant pathogenic bacteria (Smith *et al.*, 1994; Hirst *et al.*, 1999). Genome sequence analyses have revealed a greater degree of functional diversity and functional redundancy in many plant pathogens than previously anticipated, with pathogens such as *P. syringae* pv. *actinidiae* containing genes for the production of multiple siderophores, including pyoverdine, enterobactin and yersinabactin (Scortichini *et al.*, 2012). Finally, it has been suggested that some of these bacteria have novel iron chelation systems that act as alternative mechanisms for iron uptake in the absence of siderophore synthesis. For example, Grinter *et al.* (2012) have recently shown that two ferredoxin containing bacteriocins produced by the soft rot pathogen *P. carotovorum* can enhance bacterial growth in the presence of spinach ferredoxin under iron-limiting conditions, suggesting that plant ferredoxins can be used as an additional source of iron. The activity of iron chelation systems may be enhanced by the activity of compounds such as phenazine-1-carboxylic acid, produced by the opportunistic pathogen *P. aeruginosa*, which can act to reduce Fe^{3+} to Fe^{2+} increasing iron availability in host tissues (Wang *et al.*, 2011).

Despite the lack of evidence for a role for siderophore synthesis in the growth of many plant pathogenic bacteria inside plant tissues, siderophores have been shown to have an important role in the earliest stages of the *P. syringae* infection cycle. *Pseudomonas syringae* pv. *syringae* B728a and *P. syringae* pv. *phaseolicola* 1448A produce two siderophores, pyoverdine, in common in with *P. syringae* pv. *tomato* and other pseudomonads, and achromobactin, in common with *D. dadantii* (Berti & Thomas, 2009; Owen & Ackerley, 2011). Mutants of the epiphytic strain *P. syringae* pv. *syringae* 22d/93 deficient in either pyoverdine or achromobactin synthesis showed reduced epiphytic growth on soybean leaves when spray inoculated onto the leaf surface, with a double mutant lacking both siderophores showing an even greater reduction in growth (Wensing *et al.*, 2010). This reduction was not evident when bacteria were wound inoculated into leaves, indicating that wound sites leaked sufficient iron to support bacterial growth. It is interesting to note that for opportunistic pathogens such as *P. aeruginosa* and *B. cenocepacia* the requirement for siderophore synthesis differs between plant and animal hosts, with siderophores playing a more central role in animal models of infection. For example, pyoverdine and ornibactin-deficient mutants of *B. cenocepacia* showed only a slight reduction in the ability to infect alfalfa, but were strongly attenuated in their ability to infect *Galleria mellonella* and *Caenorhabditis elegans* (Uehlinger *et al.*, 2009). It has been shown that some plant pathogenic bacteria, including *P. syringae*, *Pantoea stewartii* and *D. dadantii* can be disseminated by insect vectors such as aphids (Grenier *et al.*, 2006; Stavrinides *et al.*, 2009, 2010), and it is possible that siderophore-mediated iron

uptake plays an important role in these interactions. Finally, it is also important to note that there may be counter-selection against the use of certain siderophores during plant colonisation, as the pseudomonad siderophore pyoverdine can be recognised and assimilated by *Arabidopsis*, thus both alerting the plant to the presence of the invader and providing it with an iron source (Vansuyt *et al.*, 2007). Indeed, the enterobactin genes of closely related *P. carotovorum* strains have been shown to be highly polymorphic (Bull *et al.*, 1994), suggesting selection for diversity among these enzymes and their products, which may be driven by plants or other microorganisms.

Beyond their role in iron acquisition, siderophores are implicated in the regulation of many essential virulence functions; for example, pyoverdine can, *via* the TonB-dependent siderophore receptor, induce both itself and the important virulence factors exotoxin A and endoprotease in *P. aeruginosa* (Vasil, 2007). *Pseudomonas syringae* pv. *tabaci* mutants deficient in pyoverdine synthesis were found to be deficient in production of tabtoxin and EPS, and to show severely reduced virulence following both infiltration and spray inoculation (Taguchi *et al.*, 2010). Greenwald *et al.* (2012) used RNAseq analysis to show that the extracytoplasmic sigma factor AcsS regulates achromobactin synthesis in *P. syringae* pv. *syringae* B728a, and found that AcsS-deficient mutants showed altered expression of 287 genes, including genes associated with motility, toxin synthesis and EPS synthesis.

Dickeya dadantii seems to be relatively unusual in requiring full siderophore functionality for growth in plant tissues; neither *P. syringae* pv. *tomato*, *P. aeruginosa*, *A. tumefaciens* nor even the more closely related *P. carotovorum* show compromised virulence or growth *in planta* when their siderophore synthesis systems are mutated (Leong & Neilands, 1981; Ishimaru & Loper, 1992; Jones *et al.*, 2007; Nadal Jimenez *et al.*, 2010; Jones & Wildermuth, 2011). One possibility is that siderophores might be used *in planta* to help protect *D. dadantii* from oxidative stress caused by excess iron released during tissue maceration (Expert *et al.*, 1996), explaining why multiple siderophores are of greater importance in this soft rot pathogen than in more biotrophic pathogens. Consistent with this, Boughammoura *et al.* (2008) found that strains of *D. dadantii* lacking the main iron storage ferritin FtnA showed increased sensitivity to oxidative stress and reduced virulence *in planta*. Both oxidative stress and iron regulate siderophore production in the plant-associated bacterium *Azotobacter vinelandii*, suggesting that here, too, siderophores might function to protect the bacterium from oxidative stress induced by excess iron (Tindale *et al.*, 2000). However, as noted earlier, in *D. dadantii*, several genes involved in infection, including pectinases essential for soft rot symptoms, are also controlled *via* iron availability, and siderophores are directly implicated in modulation of plant signal transduction pathways to promote bacterial growth, creating a far more complex picture than simply the use of siderophores to 'mop up' excess iron (Franza *et al.*, 2002; Dellagi *et al.*, 2009). The iron-binding activity of chrysobactin and deferrioxamine have been shown to induce salicylic acid sigalling in *Arabidopsis*, thereby antagonising jasmonate signalling and impairing plant defences (Dellagi *et al.*, 2009).

The challenge of untangling the links between iron uptake and oxidative stress can be further illustrated by work on the iron regulators RirA and IrrA in *A. tumefaciens.* RirA is a repressor of iron uptake and siderophore synthesis and *rirA* mutants produce increased levels of siderophore and take up excess iron in iron-sufficient media, resulting in increased sensitivity to ROS and reduced virulence gene expression (Ngok-Ngam *et al.*, 2009). IrrA is regulated by degradation when bound to iron-containing heme, and represses expression of *rirA*. As might be expected, a mutant lacking *irrA* contains decreased iron, and shows increased ROS tolerance. However, a double *rirA irrA* mutant has slightly increased iron and decreased ROS tolerance (Hibbing & Fuqua, 2011). The authors of this study speculate that this discrepancy in the correlation between iron concentrations and ROS tolerance could be explained if IrrA acted as a negative regulator of a ROS defence mechanism. Another example of cross-talk between iron uptake and oxidative stress mechanisms in plant pathogenic bacteria has been reported in *P. syringae* pv. *tomato*, in which genes associated with siderophore synthesis were found to be co-regulated with azurin, a periplasmic protein predicted to be associated with responses to oxidative stress (Swingle *et al.*, 2008).

Metals and siderophores in bacterial quorum sensing and virulence

When considering the diverse roles of siderophore synthesis in plant pathogenesis, it is interesting to note that in some bacteria siderophore-deficient mutants have been shown to produce reduced levels of acyl-homoserine lactones (AHLs), signals that are known to be important in bacterial quorum sensing (Dong *et al.*, 2001). A reciprocal observation is that quorum sensing–deficient mutants of *P. aeruginosa* produce reduced levels of the siderophore pyoverdine (Stinzi *et al.*, 1998). Quorum sensing is important for the virulence of many bacterial pathogens and also controls the formation of biofilms (Williams *et al.*, 2000; Waters & Bassler, 2005). The importance of biofilms for pathogen survival is particularly well-studied

in the case of human pathogens (e.g. Parsek & Singh, 2003). Under the control of quorum-sensing systems, iron can influence biofilm formation, leading to a connection between loss of siderophore synthesis and reduced virulence for *P. aeruginosa* PAO1 (Banin *et al.*, 2005; Diggle *et al.*, 2007). Consequently, iron chelators are currently being explored as therapeutic agents to limit *P. aeruginosa* infection (Moreau-Marquis *et al.*, 2009; Hurley *et al.*, 2012).

Recently, parallels have been found in plant pathogens, showing that siderophore production and iron-based regulation of quorum-sensing genes can also be important for biofilm formation and virulence *in planta* (Cha *et al.*, 2008; Taguchi *et al.*, 2010). An intriguing example of the links between iron availability, quorum sensing and virulence was reported by Dulla *et al.* (2010), who found that the ability of epiphytic bacteria to inhibit quorum sensing in *P. syringae* strongly correlated with their ability to compete for iron with *P. syringae* through the production of iron-chelating siderophores. However, co-inoculation of these iron/quorum sensing–limiting strains with *P. syringae* increased the number of disease lesions. Experiments using nonmotile *P. syringae* indicated that this increased virulence could be due to an increase in motility in iron-limited conditions. Additionally, Cha *et al.* (2008) found that AHL synthesis is suppressed in the *fur* mutant of *P. syringae* pv. *tabaci*, indicating that a similar link between iron levels and quorum sensing may exist in this bacterium. These studies, together with studies discussed in previous sections, support the idea that there are two distinct phases to *P. syringae* infection, an epiphytic phase in which iron is limited and quorum–sensing signals are present in low concentrations, promoting motility towards stomata and wounds, and an endophytic phase in which iron is more available, quorum–sensing signals increase, and bacteria switch from planktonic to sessile growth.

In *P. aeruginosa*, Bollinger *et al.* (2001) have shown that iron limitation induces SOD, an essential part of the bacterium's response to ROS stress, in a manner dependent upon quorum–sensing systems, which may promote *in planta* survival and thus pathogenicity. At low iron concentrations, *P. aeruginosa* has also been shown to express an acylase, PvdQ, which degrades AHLs (Nadal Jimenez *et al.*, 2010), thus quenching the quorum signal. The expression of *pvdQ* is essential for biofilm formation, swarming motility and full virulence under low iron conditions. AHL acylases similar to *pvdQ* are also present in genome-sequenced strains of *P. syringae*, providing a potential mechanism linking iron availability with quorum sensing in this pathogen. Consistent with this, a strain of *P. syringae* pv. *syringae* B728a lacking two AHL acylases named HacA and HacB was found to be deficient in siderophore production and to exhibit a distinctive colony morphology (Shepherd & Lindow, 2009).

Links between iron availability and quorum sensing are not limited to AHL-based quorum-sensing systems. The *rpfB* gene of *X. fastidiosa*, which encodes a long chain fatty acid coenzyme A ligase important in the production of diffusible signal molecules by xanthomonads and *Xylella*, was found to be down-regulated in response to both excess iron and iron-limiting conditions (Zaini *et al.*, 2008).

Intriguingly, considering these links between iron, quorum sensing and virulence, it appears that there may also be a role for siderophores and redox active phenazines such as pyocyanin in cell–cell communication in some plant pathogenic bacteria. In *P. aeruginosa*, pyoverdine is involved in the regulation of secreted virulence factors and of itself. At low iron, the secretion of pyoverdine is increased, and the secreted siderophore is then able to regulate virulence factor expression in other bacteria by which it is taken up (Lamont *et al.*, 2002; Beare *et al.*, 2003). Pyocyanin induces expression of efflux pumps and the iron storage protein bacterioferritin, but downregulates certain genes associated with ferric iron uptake and zinc uptake, including genes involved in siderophore-mediated iron uptake and signal transduction (Dietrich *et al.*, 2006; Shirley & Lamont, 2009). As discussed above, the AcsS sigma factor of *P. syringae* pv. *syringae* B728a regulates the synthesis and secretion of the siderophore achromobactin, but also the iron response, EPS production and motility of these bacteria (Greenwald *et al.*, 2012). It would therefore be of great interest to know whether achromobactin can itself influence the expression of AcsS in a manner parallel to the auto-regulation of pyoverdine, and whether similar regulatory mechanisms exist for other siderophores produced by plant pathogenic bacteria, with iron availability acting not only as a property of the host environment, but also an indicator of the number of competing con-specifics in that environment.

Additional roles for metals in pathogenicity

Iron is not the only metal involved in regulating virulence gene expression. In the potato pathogen, *S. scabies*, zinc is known to regulate the expression of esterase, an important enzyme for the virulence of this bacterium (McQueen & Schottel, 1987; Schottel *et al.*, 1992). There are also other potential roles for metals in pathogenesis. Bacterial NRAMP manganese transporters are known to be important for virulence, but their precise role is not known (Papp-Wallace & Maguire, 2006). Similarly, a copper transporter is necessary for the pathogenicity of the fun-

gal phytopathogen *Colletotrichum gloeosporioides*. In this case, the copper is required by the pathogen for the production of copper-SOD in order for the pathogen to tolerate ROS produced during the plant's defence response, but also for fungal spore germination (Barhoom *et al.*, 2008). *Pseudomonas syringae* strains also possess a Cu-Zn-SOD, which is absent from most nonpathogenic pseudomonads, along with a Mn-SOD and Fe-SOD that are more broadly conserved (Fones & Preston, 2012). All three SODs are predicted to have a periplasmic location, placing these metal-dependent enzymes in the front line of defences against oxidative stress. Also important in protecting the pathogen from ROS stress is the aconitase enzyme of *X. campestris* pv. *vesicatoria*, an iron-sulphur protein that is involved in the TCA cycle, and also in sensing of iron and reactive oxygen. Mutants lacking functional aconitase are more susceptible to superoxide toxicity, and show impaired *in planta* growth and symptom development on pepper (Kirchberg *et al.*, 2012). One of the most direct links between metal and pathogenicity is in the alfalfa pathogen, *Corynebacterium insidiosum*, which has been found to produce a copper-containing phytotoxic glycopeptide that induces wilt by blocking xylem vessels (Ries & Strobel, 1972).

In some cases it may be necessary for a pathogen to resist high concentrations of metal in the host. A well-studied example of this is the human pathogen *Helicobacter pylori*, which requires metal-efflux systems providing resistance to cadmium, zinc and nickel to colonise the human stomach (Stähler *et al.*, 2006). There is also evidence that plant disease symptoms can be associated with increased or unbalanced metal concentrations in plant tissues, which may lead to a requirement for increased metal tolerance in plant pathogens. For example, high levels of iron, zinc and manganese have been recorded in chlorotic leaves of citrus plants infected with *X. fastidiosa* (Silva-Stenicio *et al.*, 2009). The following sections consider the various ways in which metal resistance can be important for plant pathogens.

Use of metals in disease control

The fact that metals can behave as toxins when present in excess means that they can be used as protective, anti-microbial agents in agriculture. For instance, copper is part of a fungicide application known as 'Bordeaux mixture', used in orchards and vineyards since the 19th century to guard these crops against mildew (Russell, 2005). Indeed, Bordeaux mixture, commercialised after its accidental discovery by Milardet in the 1880s, represents the first large scale use of fungicide (Floyd, 1991), although copper is recorded as being used as a protectant for cereal crops as long ago as 1761 (Van Zweiten *et al.*, 2007). Today, copper-based fungicides remain popular, largely because they are one of the few permitted anti-microbial applications in organic farming. A recent report conducted by the Australian government listed an impressive diversity of crops, from macadamia nuts to cucumber to mangoes, of which a variety of diseases including leaf spot, late blight, canker, anthracnose and mildew are treated with copper-based fungicides (Van Zweiten *et al.*, 2007). Copper is often one of a very limited number of options available for bacterial disease control, and in Florida, frequent use of copper sprays for control of canker has increased since the citrus canker eradication programme was suspended in 2006 (Behlau *et al.*, 2011).

There are, however, a number of problems with the use of copper as a crop protectant. Firstly, copper is not only toxic to pathogens, but is also phytotoxic, and, when used excessively, causes russetting of fruits (Montag *et al.*, 2006). An additional concern is that copper-based products can build up in soils where they are used over long periods, eventually becoming toxic to the crops they are intended to protect. This is a particular problem in viticulture and in orchard settings (Rusjan *et al.*, 2007). Copper in soil can also have a detrimental effect on mycorrhizal symbionts (Van Zweiten *et al.*, 2007). When used in the treatment of citrus canker, copper has been shown to induce a viable but nonculturable state in *Xanthomonas axonopodis* pv. *citri*, potentially creating a reservoir of the pathogen in the soil (del Campo *et al.*, 2009). Copper has also been reported to induce expression of the virulence-associated Xcs (type II) secretion system in *X. axonopodis* pv. *citri* (Palmieri *et al.*, 2010). As a result, methods are being developed to minimise the use of copper in these crop systems (Kuflik *et al.*, 2009) and the European Union has considered a ban on the use of copper-based pesticides and fungicides due to their environmental persistence (Houlton, 2009).

Another concern, from the perspective of disease control, is the problem of pathogen resistance to metals, which often develops as the result of prolonged exposure. The development of copper resistance has been well documented. For example, Bordeaux mixture is used widely in Portugal and Spain to treat apical necrosis of mangoes, caused by *P. syringae* pv. *syringae* (Cazorla *et al.*, 2002). The efficacy of this treatment is declining, in common with that of copper-based treatments of other diseases caused by pseudomonads and xanthomonads (Schenk & Pscheidt, 1998). Resistant strains of both species have been discovered (Marco & Stall, 1983; Andersen *et al.*, 1991) and there is evidence that extensive horizontal transfer of the copper resistance determinants, which are largely plasmid borne (Sundin *et al.*, 1989) occurs, both among pseudomonads and between pseudomonads and

xanthomonads (Voloudakis *et al.*, 1993). This process can be rapid, with newly resistant strains appearing within 1 year of the application of Bordeaux mixture to mango crops (Cazorla *et al.*, 2002). In Japan, strains of *P. syringae* pv. *actinidiae*, the causal agent of bacterial canker of kiwi fruit, were found to have *copA* and *copB* genes at the onset of disease outbreaks, but following repeated treatment with copper-based bactericides strains were also found to have acquired *copS* and *copR* (Nakajima *et al.*, 2002). Furthermore, when copper is used in concert with the antibiotic streptomycin, a common strategy to prevent tip-dieback disease, resistance to both of these anti-microbials can quickly become linked in pathogenic pseudomonads (Sundin & Bender, 1993).

Interestingly, despite the long-term use of copper as an antimicrobial, its mode of action has not been fully elucidated. One recent study determined that toxicity towards the fungus *Venturia inaequalis* does not occur as a result of external ROS production, but requires that the copper be taken up by the organism (Montag *et al.*, 2006). It is known that contact with copper surfaces can be bactericidal, causing loss of membrane integrity, DNA damage and respiratory inhibition (Grass *et al.*, 2011; Warnes *et al.*, 2011). The exact mechanism underlying this appears to vary depending upon the bacterial species, although ROS are known to be involved (Warnes *et al.*, 2011). It has also been proposed that copper sulphate is lethal to *E. amylovora* through enzyme inhibition severe enough to prevent growth and promote autolysis (Geider, 1999). What is understood is that copper resistant pathogens have a number of different mechanisms for tolerating the metal. One is the over-production of oxalate, which complexes with copper to form a harmless precipitate (Clausen & Green, 2003). The *Pseudomonas* and *Xanthomonas* strains discussed above possess several different resistance determinants (Cazorla *et al.*, 2002) and it has been speculated that these may have roles in addition to copper tolerance, contributing to the persistence of copper resistance in the absence of copper use. This is the case for a copper resistance gene in *Aeromonas*, which is known to also function as a pathogenicity gene (Francki *et al.*, 2000).

Although used to a lesser degree than copper, zinc is also used as an anti-microbial which can be applied to crops; for example, zinc has been shown to prevent fungal spore germination *via* the production of toxic levels of ROS (Montag *et al.*, 2006), while zinc sulphate and zineb (zinc ethylene bisdithiocarbamate) were found to inhibit growth of the bacterial pathogen *X. campestris* pv. *vesicatoria* (Adaskaveg & Hine, 1985). However, again there is evidence that strains can rapidly become resistant to zinc compounds (Adaskaveg & Hine, 1985; Fones *et al.*, 2010). Copper, streptomycin and zinc compounds have been used in combination to control bacterial plant disease, and there is evidence that bacteria can acquire resistance to all three compounds (Ward & O'Garro, 1992).

Metals in plant defence

The involvement of metals in plant defence is not restricted to their artificial application in agricultural systems: as already discussed, metals are an integral component of living systems and thus play a number of roles in plant defence. There are several ways in which plants use or rely on metals to influence the outcome of infection. Firstly, plants may disrupt the pathogen's supply of essential metals (Bullen, 1981; Hammer & Skaar, 2012). Secondly, the plant may attempt to overwhelm the pathogen's metal homoeostasis and tolerance mechanisms by oversupplying a potentially toxic metal (Fones *et al.*, 2010; Yuan *et al.*, 2010). There are a number of metalloenzymes that are important for plant defence (Wu *et al.*, 2011), meaning that the availability of these metals to the plant can be an important factor in the outcome of the plant–pathogen interaction. Metal-induced ROS have a number of important roles both in defensive signalling and as antimicrobials (eg Wojtaszek, 1997). Finally, metal stress, either as deficiency or excess, can act as a 'priming' stimulus for plant defence, *via* the overlapping pathways by which plants signal biotic and abiotic stress (Mithöfer *et al.*, 2004).

Graham (1983) states that the balance of an interaction between plant and pathogen can be tipped by changes in micronutrition, especially when the plant is deficient in, or has access to excess levels of, a trace element. This may be most important in cases where the pathogen has not had the opportunity to co-evolve with the plant; for example when it has newly arrived in the plant's environment. Such a pathogen would, of course, be likely to lack tolerance mechanisms for the lack or excess of an element, allowing the plant to turn this contingency to its advantage.

Withholding of metals in defence

The disruption of the pathogen's supply of metal is an obvious strategy for disease resistance, considering that microorganisms and host organisms are in competition for metal ions (Bullen, 1981; Hammer & Skaar, 2012). Thus it is to the plant's advantage to ensure that metals are diverted from the pathogen to plant cells (Fig. 1). In this context, the most relevant metal to consider is iron. Iron is one of the most abundant elements in the world (Expert *et al.*, 1994), but in aerobic environments it mostly exists as the insoluble Fe^{3+} ion, so that its bioavailability is comparatively low (Touati, 2000). This makes iron one of the most intensely competed ions during host-pathogen interactions (Payne, 1993; Weinberg,

1993; Johnson, 2008; Nairz *et al.*, 2010). The host strategy of withholding iron to limit pathogen growth has been particularly well documented in mammalian systems (Bezkorovainy, 1981; Ward & Connelly, 2004; Nairz *et al.*, 2010). Iron withholding *via* sequestration by storage proteins such as transferrin and lactoferrin is common in vertebrates and invertebrates, and can be effective, as iron is needed for bacterial growth, pathogenicity and biofilm formation (Ong *et al.*, 2006). The last of these is of great importance clinically, as the restriction of pathogen iron supply by lactoferrin can prevent necessary signalling for the development of drug-resistant biofilms (Singh *et al.*, 2002; Banin *et al.*, 2005).

More recently, evidence has been uncovered that indicates that plants also employ a strategy of pathogen iron-deprivation. In plants, a ferredoxin-like protein has been demonstrated to be involved in defence against *P. syringae pv. tomato* DC3000, *X. campestris* and *D. dadantii*, a pathogen known to depend on iron scavenged from host tissues during the progression of soft rot disease, as discussed previously. The defensive function of this protein was found to rely on its ability to bind iron (Huang *et al.*, 2006). Perhaps inevitably, there are also iron-binding proteins produced by pathogens, so that the final outcome depends on the interaction between host and pathogen iron-chelating agents (Boughammoura *et al.*, 2007). *Arabidopsis* has been found to upregulate ferredoxin in response to the detection of iron-uptake siderophores from pathogens (Dellagi *et al.*, 2005). Other chemicals that may be used to bind and withhold iron from pathogens include polyphenols, shown to be effective against *D. dadantii* mutants with reduced iron-uptake capacity (Mila *et al.*, 1998), and to inhibit the growth of *P. syringae* pv. *syringae* B728a on leaf surfaces (Karamanoli *et al.*, 2011).

More evidence for the importance of iron withholding in plant–pathogen interactions comes from investigations into the role of NRAMP metal transporting proteins in *Arabidopsis.* These proteins are, again, known from mammalian systems where they appear to have a role in regulating the concentrations of iron, zinc and manganese to which pathogens are exposed within macrophages (Gunshin *et al.*, 1997; Forbes & Gros, 2001; Goswami *et al.*, 2001). NRAMPs occur throughout the diversity of life and have recently been characterised in plants, including *Arabidopsis*, where they have been shown to function as uptake pumps for iron and manganese (Curie *et al.*, 2000). Certain of these NRAMPs are upregulated under biotic stress and iron starvation, and NRAMP3 and 4 have been found to be involved in basal resistance to *D. dadantii* in a manner independent of other defence-associated signals such as salicylic acid and jasmonate (Segond *et al.*, 2009). As noted previously, there is evidence that in some interactions pathogen invasion causes iron starvation, and the presence of bacterial siderophores can induce the iron storage protein FER1 (Dellagi *et al.*, 2005; Boughammoura *et al.*, 2007). Thus, bacteria-induced iron deficiency may provide the link between NRAMP proteins and defence. Additionally, it was found that a double knockout mutant, *nramp3nramp4*, has an attenuated oxidative burst, providing the suggestion that these transporters might be involved in providing Fe^{3+} for Fenton reactions (See Box 1) for the generation of ROS (Segond *et al.*, 2009).

Iron is not the only metal which may be withheld as a form of defence. In mammalian systems, the protein calprotectin is used by neutrophil cells to prevent bacterial growth by preventing pathogens from acquiring zinc (Clohessy & Golden, 1995). Not only can this prevent the growth of bacteria, but it can also disable zinc-dependent SOD, rendering the bacteria more susceptible to bactericidal ROS production by neutrophils (Kehl-Fie *et al.*, 2011). As yet, there is limited evidence for zinc-withholding as a means of pathogen defence in plants, although it is thought that metal-chelating chemicals such as polyphenols may restrict the availability of multiple metal ions, including zinc and copper (McDonald *et al.*, 1996; Mila *et al.*, 1998).

Iron withholding in antagonism and biocontrol

In addition to competing with host plants for metal ions, plant pathogens compete directly with other plant-associated microorganisms for essential metals, a process that is of increasing interest as a means of enhancing biocontrol. In many well studied cases, siderophores produced by biocontrol bacteria are known to limit fungal growth, as they often have a higher affinity for iron than fungal siderophores and may even remove bound iron from these (Duffy *et al.*, 2003; Compant *et al.*, 2005). Similarly, the anti-mycoplasma factor, micacocidin, initially identified in *Pseudomonas* sp. No. 57-250, and subsequently found to be produced by the plant pathogen *R. solanacearum,* is expressed under iron-limited conditions, binds metals, and may itself be a siderophore (Kobayashi *et al.*, 1998; Kreutzer *et al.*, 2011). Interest in the impact of metal on biocontrol activity is also driven by studies showing that both the fitness (Hartney *et al.*, 2011), and biocontrol activity (Duffy & Défago, 2000; Ownley *et al.*, 2003) of biocontrol bacteria can be impacted by iron and zinc availability.

The biocontrol strain *Pantoea agglomerans* C9-1 produces desferrioxamine siderophores (Smits *et al.*, 2010), which are known to sequester iron and thus inhibit the growth of bacteria unable to take them up (Dellagi *et al.*, 1998). Similarly, *Burkholderia* species such as the rice rhizosphere bacterium, *Burkholderia vietnamiensis* produce a

siderophore, ornibactin, which cannot be taken up by pseudomonads (Meyer *et al.*, 1995). Thus, biocontrol of bacterial disease using strains that compete for essential metals by producing different siderophores may be possible. In contrast, Wensing *et al.* (2010) found that siderophore production by the biocontrol strain *P. syringae* pv. *syringae* 22d/93, which produced the same siderophores as the target strain *P. syringae* pv. *glycinea* 1a/96, had no significant impact on biocontrol activity under the conditions tested. Although detailed consideration of the biocontrol field is beyond the scope of this review, it is clearly an area meriting further research. The various potential interactions between biocontrol bacteria, pathogens and host plants are summarised in Fig. 3.

Metals as toxins in defence

In contrast to the metal-withholding mechanisms discussed above, there are instances where the plants' defence mechanism exposes the pathogen to an excess of a metal. An example is the bacterium *X. oryzae* pv. *oryzae*, which, when growing in the xylem of rice, can be limited by high concentrations of copper (Yuan *et al.*, 2010). The importance of this for the outcome of this plant–pathogen interaction is illustrated by the evolution of a bacterial TAL effector protein that initiates transcription of *Xa13*, a rice gene encoding a transmembrane transporter that works in concert with two additional rice proteins to remove copper from the xylem (Yuan *et al.*, 2011). Animals, too, are known to use copper as an antimicrobial (reviewed in Samanovic *et al.*, 2012). It has been suggested that a novel *Arabidopsis* MFS-family zinc transporter, an orthologue of which is induced by pathogen infection in maize (Simmons *et al.*, 2003), may release zinc from the vacuole in infected tissues, thus playing a role in defence (Haydon & Cobbett, 2007). Zinc is also known to have a role in defence in humans, with zinc-deficient humans being more susceptible to *Staphylococcus pneumonia* infection. It has been suggested that zinc acts to limit bacterial growth by competing for manganese transporters (McDevitt *et al.*, 2011). In this context, it is of interest that the NRAMP proteins discussed above may also function to transport manganese (Segond *et al.*, 2009).

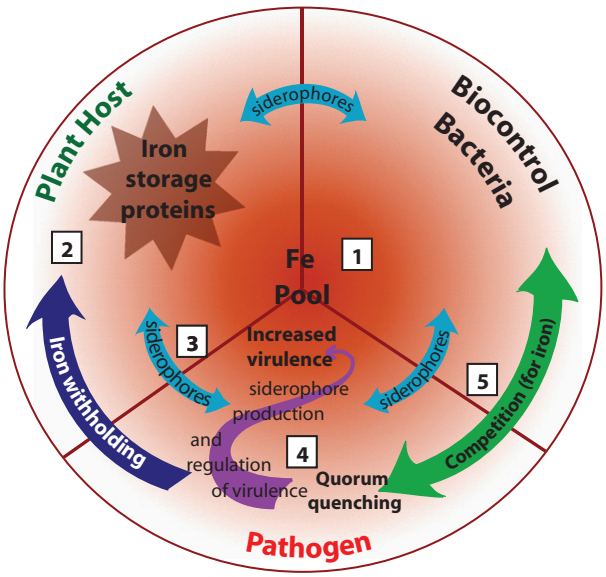

Fig. 3. The importance of iron in the interactions of pathogen, host and biocontrol agents. The finite amount of iron available in the environment may be thought of as an 'iron pool' for which different organisms compete (1). An important example of such competition is the withholding of iron from pathogens by hosts (2), using both iron storage proteins to sequester the metal and competitive uptake by high-affinity siderophores (3). Both iron itself and siderophores produced by the pathogen can have extensive effects upon the regulation of the pathogen's virulence, with virulence often, although not exclusively, increasing with higher iron availability (4). One mechanism through which iron can affect virulence is through its effects upon quorum-sensing systems, with low iron conditions leading to quenching of the quorum signal. Suppression of virulence and quorum sensing regulated gene expression through competition for iron may be one mechanism through which biocontrol bacteria can reduce pathogen virulence (5).

Indirect effects of metals in defence

As well as affecting pathogens directly through direct deficiency or toxicity, metals can affect plant–pathogen interactions indirectly *via* their inclusion in metalloenzymes. An exhaustive review of this topic is beyond the scope of this review, but a few examples of particular note are discussed here. Two metalloenzymes of particular importance in plant–pathogen interactions are SOD, which exists as three isoforms that can be separated according to their metallic cofactors of Fe, Mn and Cu/Zn (Kliebenstein *et al.*, 1998; Wu *et al.*, 2011), and catalase, which relies for its activity on four heme groups (Reid *et al.*, 1981; Willekens *et al.*, 1995). During the oxidative burst, plants produce such high concentrations of ROS that their own anti-oxidant defences are temporarily overwhelmed (Vanacker *et al.*, 1998). To prevent escalating damage to plant cells, it is essential that the plant possesses functional antioxidant enzymes to regain control of ROS levels at the appropriate time. Indeed, it is known that control of ROS levels is important in the plant's response to both biotic and abiotic stresses (Mittler, 2002; Prashanth *et al.*, 2008).

Some plant pathogens attempt to manipulate ROS production by the host as part of their pathogenicity (Fones & Preston, 2012). For example, the phytotoxin coronatine, employed by many strains of *P. syringae* and by the

soft rot pathogen *Pectobacterium atrosepticum*, causes light-dependent upregulation of ROS production and a concomitant suppression of SOD activity, and is essential for full virulence of these pathogens (Bender *et al.*, 1987; Ishiga *et al.*, 2008, 2009; Uppalapati *et al.*, 2008). Similarly, *P. aeruginosa* produces a redox active phenazine toxin called pyocyanin, which induces ROS production and systemic resistance in plants and animals, but enhances susceptibility to the fungal pathogen *Rhizoctonia solani* in rice (Mahajan-Miklos *et al.*, 1999; O'Malley *et al.*, 2003; De Vleesschauwer *et al.*, 2006).

Another enzyme which is important in plant defence is the pathogen-related protein, PR-10, of *Theobroma cacoa*. This protein is a ribonuclease, which, when released during pathogen-induced programmed cell death, is internalised by fungal cells and can act as a fungicidal toxin against *Moniliophthora perniciosa*, the causal agent of witches' broom disease (Pungartnik *et al.*, 2009). Although the exact mechanism of toxicity is unclear, it has been demonstrated that toxicity is reduced if a fungal high-affinity copper transporter is not expressed, suggesting that either copper is needed for PR-10 enzyme activity, or that the toxin works by disrupting copper homoeostasis in the pathogen (Pungartnik *et al.*, 2009). In a separate study, over-expression of maize ZmPR10.1 in *A. thaliana* was found to cause increased susceptibility to *P. syringae* pv. *tomato*, and Cu^{2+} was identified as an inhibitor of ZmPR10 and ZmPR10.1 RNase activity (Xie *et al.*, 2010).

The final two aspects of the involvement of metals in plant defence we will cover in this section concern their role in the generation of ROS. ROS are understood to have a number of pivotal roles in plant defence, both as antimicrobials produced at the site of infection (Peng & Kuc, 1992; Lamb & Dixon, 1997; Wojtaszek, 1997) and as signals for further defence responses (Alvarez *et al.*, 1998; Love *et al.*, 2005; Torres *et al.*, 2006; Choi *et al.*, 2007; Van Breussegem *et al.*, 2008). The importance of metals for ROS production has already been noted. Redox active metals, particularly iron, can participate in Fenton reactions by which hydroxide radicals may be generated from H_2O_2 (Pierre & Fontecave, 1999). Cereal crops traffic large vesicles containing H_2O_2 and Fe^{3+} to the site of *Blumeria graminis* infection, where they appear to participate in the oxidative burst; similarly, the ABC transporter *ATPEN3*, which is involved in the translocation of the redox active metal, cadmium, has been found to be involved in resistance to *P. syringae* and *Phytophthora infestans* (Hückelhoven, 2007). Iron appears to be involved in a feed-forward mechanism controlling ROS generation, for as well as participating in the generation of ROS, its efflux can also be signalled by H_2O_2 (Lui *et al.*, 2007).

In addition to this direct role in the defensive production of anti-microbial ROS, metals can induce ROS in plants by acting as stressors (Boominathan & Doran, 2003; Garnier *et al.*, 2006). Since ROS are important signals in the plant response to pathogen invasion, it is perhaps unsurprising that there is evidence of cross-talk between metal stress and pathogen resistance, (Mithöfer *et al.*, 2004). ROS, whether generated by biotic or abiotic stress, can induce the production of oxylipins, which also have signalling roles in plant defence (Bleé, 2002; Mithöfer *et al.*, 2004). Although the mechanism has not been fully elucidated, low-intensity spraying of crops with nickel can induce phytoalexin production and protect against fungal infection (Wood & Reilly, 2007). It is logical to speculate that this effect may also be due to common signalling pathways between nickel and pathogen stress. There is, however, also evidence of a synergistic effect of metal and pathogen stress, in which each may increase the susceptibility of the plant to the other (Stroniski & Floriszak-Wieczorek, 1990; Miteva *et al.*, 2001).

A special case – metal hyperaccumulating plants

Some plants found growing on metal-rich soils have an unusual interaction with metals, actively taking up and storing the metals in their aerial tissues. These are 'hyperaccumulators' (Jaffré *et al.*, 1976), defined as plants that take up 'exceptionally high concentrations of an element in the above ground parts of a plant under field conditions' (Pollard, 2000). For example, the brassica, *Noccaea* (formerly *Thlaspi*) *caerulescens*, can accumulate up to 30 000 $\mu g\ g^{-1}$ zinc and over 1300 $\mu g\ g^{-1}$ cadmium (Brown *et al.*, 1995). Around 400 plant taxa are classed as hyperaccumulators (Baker & Brookes, 1989; Freeman *et al.*, 2005). Nickel is the most commonly accumulated metal (Küpper *et al.*, 1999; Reeves & Baker, 2000; Assunção *et al.*, 2003), but zinc hyperaccumulation is also relatively common (Prasad & de Oliveira Freitas, 2003). Copper, too, can be hyperaccumulated, although the number of plants that do so is limited to around 25 species (Jiang *et al.*, 2004).

The reason for the evolution of metal hyperaccumulation is, at present, unknown, but the hypothesis that hyperaccumulated metals provide a defence against herbivores or pathogens (Boyd & Martens, 1992; Poschenrieder *et al.*, 2006), has received much attention. For herbivores, it is clear that deterrence does occur, although this depends on many factors (Boyd, 2007; Vesk & Reichman, 2009). There are a number of ways in which metals could protect plants; the so-called elemental defence hypothesis postulates that the metals act directly to deter or kill pests and pathogens, but it is possible that protection could occur in other ways. Poschenrieder *et al.* (2006) use the term 'metal

fortification' to describe the possibility that hyperaccumulated metals induce responses in the plant that might normally occur in response to a pathogen attack, thus indirectly rendering the plant less susceptible to disease. This is plausible, since plant responses to biotic and abiotic stress share a number of common features and signalling pathways (Piffanelli *et al.*, 2002; Mithöfer *et al.*, 2004; Freeman *et al.*, 2005; Chmielowska *et al.*, 2010).

As this review has shown, the ways in which metals can influence or potentially influence plant–pathogen interactions are extremely diverse, and can be complex or indirect. However, the idea of simple protection of hyperaccumulators against bacteria by direct metal toxicity has received support from work which demonstrated that zinc levels in the *N. caerulescens* were sufficient to account for observed inhibition of the bacterial pathogen, *P. syringae* pv. *maculicola*, *in planta*, and that bacterial survival in these plants was correlated to zinc tolerance (Fones *et al.*, 2010; Figs 1 and 4). These observations provide one of the clearest cases for the importance of metals in hyperaccumulator-pathogen interactions to date.

Concluding remarks

In this review, the ways in which metals can influence plant–pathogen interactions have been explored. Metals, especially those with redox activity, are essential for life *via* their indispensable roles in various enzymes. Because such metals have the capacity to elicit the production of ROS, they can be toxic in excess and their uptake and homoeostasis must be closely controlled. Some of these metals are also subject to limited availabillity in biotic environments. These factors combined ensure that the manipulation of their availability is a key strategy for both microbial pathogens and their plant hosts. This occurs *via* competition for essential metals and also *via* host attempts to overwhelm the pathogen with a toxic excess of metal, a technique that may have been adopted as a constitutive form of defence in metal hyperaccumulating plants. It is also common for metals to be applied directly for crop protection against disease.

As well as applying metals as anti-microbials, there are incentives to either supplement soils with mineral nutrients or to breed or engineer plants with increased ability to assimilate and accumulate the nutrients already available to them, an approach known as biofortification of crops. This is an increasingly important ideal in 21st century agriculture: for example, zinc deficiency is an important problem throughout the world, affecting around 33% of people, rising to 74% in some countries (Sillanpää, 1982; Hotz & Brown, 2004; Singh, 2008) and causing, in severe cases, important physical and psychological difficulties in humans and livestock (Hotz & Brown, 2004; Graham, 2008). Iron is also an element that many cultivated soils lack in a bioavailable form, with the World Health Organisation (WHO) estimating that 30% of the world's human population are anaemic. To date, successful attempts have been made to increase the iron and zinc content of rice, pineapple and banana by expression of soybean ferritin (Goto *et al.*, 1999; Kumar *et al.*, 2011; Mhatre *et al.*, 2011). Approaches such as biofortification may also allow the cultivation of additional land; as one common problem that affects land use and crop yield is a lack, or toxic excess, of mineral nutrients (Lal, 2009; Beddington, 2010). With an expanding world population, it is of increasing importance that we find ways of exploiting agricultural land to the fullest effect, while limiting malnutrition (Beddington, 2010), however the impact of biofortification strategies on plant disease resistance remains to be explored.

As we have seen, metals and the systems that regulate their uptake and homoeostasis are often implicated in the regulation of virulence and pathogenicity genes, including the T3SS; they are also essential components of the enzymes used in the production and tolerance of ROS, another key feature of the battle between plants and pathogens. Thus, to maximise future productivity, while minimising losses from crop disease, researchers must endeavour to understand all the ways in which metals can influence the interactions between plants and pathogens.

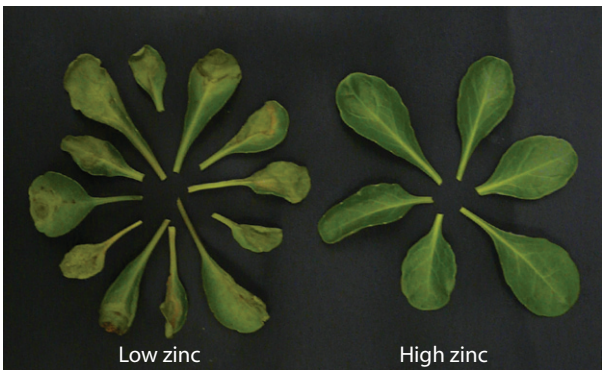

Fig. 4. The effect of zinc hyperaccumulation on susceptibility of *Noccaea caerulescens* to infection by *Pseudomonas syringae*. Plants shown were grown hydroponically for 10 weeks with either 0.04 (low) or 300 (high) μM Zn in the nutrient solution, and inoculated with bacteria at 10^8 CFU mL^{-1}. Photographed leaves show the development of symptoms 7 days after inoculation.

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