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Metal hyperaccumulation in the Brassicaceae species *Arabidopsis halleri* reduces camalexin induction after fungal pathogen attack

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ABSTRACT

Metal hyperaccumulation from the soil by certain plant species can serve as a defence trait. Such hyperaccumulation might impact the expression of organic defences. Here, the induction of the phytoalexin camalexin after leaf infection with a pathogenic fungus was investigated in plants of the facultative hyperaccumulator *Arabidopsis halleri* grown on unamended and metal-amended soil. Reduced camalexin induction was expected in plants grown in metal-amended soil. Plants were grown for twelve weeks on soil, which was either amended with high concentrations of Zn and Cd or kept unamended, and the final leaf concentration of Zn and Cd was determined. Conidia of the pathogenic fungus *Alternaria brassicae* were applied on mechanically damaged leaves. Leaves were harvested after 24 h and 48 h, and the amount of induced camalexin was quantified. Plants grown on metal-amended soil hyperaccumulated Zn and Cd and induced significantly less camalexin than plants grown on unamended soils after pathogen infestation. This suggests a physiological trade-off between metal hyperaccumulation and an induced antifungal organic defence, which has, to our knowledge, not been observed before in *A. halleri* for other organic defence compounds. This phenomenon might partly be explained by limitations in sulphur pools and/or the interruption of (phytohormonal) defence signalling due to metal hyperaccumulation. This work highlights the importance of considering defences elicited by antagonists from various guilds when studying plant organic defence responses to specific abiotic environments.

1. Introduction

Metal hyperaccumulation is the ability of specific plant species to tolerate, take up and store unusually high amounts of a metal or a metalloid in their tissues (Krämer, 2010). This trait allows species to grow on natural and anthropogenically metal-enriched sites, which have expanded in the last 150 yr (Nriagu and Pacyna, 1988). One general hypothesis on the evolution of plant metal hyperaccumulation postulates that metals enriched in plant tissue serve as defence ('elemental defence hypothesis'; Boyd, 2007). Indeed, various studies found evidence for a high resistance of plants to both herbivores and pathogens, mediated by the (hyper)accumulation of metals (Boyd, 2007; Hörger et al., 2013). Apart from possible elemental defence in certain species, plants also contain a plethora of specialised organic defence metabolites to defeat antagonists (Schoonhoven et al., 2005). Because both metal hyperaccumulation and the production of organic chemical defence compounds are predicted to have physiological and ecological costs (Pongrac et al., 2010; Bekaert et al., 2012; Fones and Preston, 2013), negative correlations between concentrations of these defences can be expected ('trade-off hypothesis'; Martens and Boyd, 1994; Boyd, 2007). However, in ecological networks the estimation of costs and benefits can be highly complex (Boyd, 2013), and trade-offs might only be disentangled when investigating various traits with different defensive functions.

The specific environment of plant populations is likely to influence evolutionary adaptations following metal hyperaccumulation (Boyd, 2013). For example, species that regularly grow on metalliferous soils might become dependent on elemental defence while organic defences may be physiologically constrained by hyperaccumulation (Fones and Preston, 2013). In contrast, 'facultative' hyperaccumulator species (for differentiated terminology of non-obligatory hyperaccumulators see Stein et al., 2017) can also form populations on non-metalliferous sites (Fones and Preston, 2013; van der Ent et al., 2013) and thus need to maintain a high potential to express various organic defences. Organic plant defence compounds can be divided into phytoanticipins, that are constitutively present in significant amounts in plant tissues, and phytoalexins, that are synthesised *de novo* as a rapid local response to the attack of antagonists, especially to pathogens (Ahuja et al., 2012;

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Ribera and Zuñiga, 2012). Because the induction pathways of phytoanticipins and phytoalexins might be differentially altered by metal hyperaccumulation (Pongrac et al., 2010), both types of defences need to be considered for elucidating potential physiological trade-offs in defences. However, studies on phytoalexin induction in response to soil metal conditions are rare (but see Martellini et al., 2014).

About half of the known metal (hyper)accumulating plant species belong to the Brassicaceae (Krämer, 2010). Species of this family contain both phytoalexins (Conn et al., 1988; Pedras et al., 2011) and glucosinolates (GSs) as phytoanticipins, which are additionally inducible and act against a broad range of (generalist) antagonists (Halkier and Gershenzon, 2006). These types of defences differ in their activity against antagonists of different host specialisation. For example, the necrotrophic fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* can (partly) tolerate GS breakdown products, but can be fended-off by the phytoalexin camalexin (Kliebenstein, 2004; Glazebrook, 2005). Camalexin is the characteristic phytoalexin in *Arabidopsis thaliana* and related genera (Glawischnig, 2007; Bednarek et al., 2011), and shares the initial biosynthetic pathway steps with indole GSs using tryptophan as precursor (Glawischnig et al., 2004). Moreover, both camalexin and GSs are connected to sulphur metabolism, as is the synthesis of phytochelators required for metal hyperaccumulation (Rausch and Wachter, 2005; Ernst et al., 2008; Pongrac et al., 2010). The relationship between concentrations of metals and (classes of different) GSs in tissues has been addressed in various studies on different hyperaccumulator species (Pongrac et al., 2010), finding trade-offs (Tolrà et al., 2001; Jhee et al., 2006), simultaneous increases (Tolrà et al., 2006), or no consistent effects (Kazemi-Dinan et al., 2015b; Stolpe et al., 2017). Different from GSs, the induction of camalexin is stimulated by a rapid and complex signal transduction cascade which involves, for example, reactive oxygen species (ROS) (Glawischnig, 2007). These signalling molecules might be generally elevated when plants are exposed to high metal concentrations (Rascio and Navari-Izzo, 2011) and have been found to be uncoupled from pathogen attack signalling in the facultative hyperaccumulator *Noccaea* (*Thlaspi*) *caerulescens* (Fones et al., 2013). Thus, camalexin is an appealing target compound to study trade-offs between elemental and induced organic defences in metal hyperaccumulating plant species.

Arabidopsis halleri (L.) O'Kane & Al-Shebaz (Brassicaceae) is a hyperaccumulator of Zn and Cd that can grow on both contaminated and non-contaminated soils (Stein et al., 2017). Metal accumulation in leaves of *A. halleri* provides an effective elemental defence against various biting-chewing and piercing-sucking herbivores (Kazemi-Dinan et al., 2014, 2015a; Stolpe et al., 2017). However, no consistent indications for a negative correlation between leaf metal concentrations and GS concentrations were found (Kazemi-Dinan et al., 2015b; Stolpe et al., 2017). In response to fungal infestation, the species induced less camalexin and other compounds from the pathogen-inducible tryptophan and indole GS metabolism than *A. thaliana* (Bednarek et al., 2011), pointing to an evolutionary trade-off compared to the non-hyperaccumulating sister species. However, it is unknown how metal accumulation influences induction of camalexin within *A. halleri*.

To investigate the influence of soil metal conditions on plant camalexin induction after fungal infestation, we cultivated *A. halleri* plants on soil without or with amendment of Zn and Cd. Conidia of the necrotrophic ascomycete *Alternaria brassicae* (Berk.) Sacc. (Pleosporaceae) were applied on mechanically damaged leaves. Leaves were harvested after 24 and 48 h and the amount of induced camalexin was measured. To quantify metal accumulation, concentrations of Zn and Cd in leaves were analysed. We expected that the synthesis of camalexin in leaves is reduced in plants grown on metal-amended soil compared to plants grown on unamended soil.

2. Material and methods

2.1. Plant and pathogen rearing

Experimental *A. halleri* plants were produced by taking cuttings from plants originating from a metal-contaminated site in Langelsheim (Germany; N51.942839, E10.348911, accession LAN 3.1), which had been kept for several years in the greenhouse on low-metal soil. Cuttings were transferred to 250-mL plastic pots (one cutting per pot) into a substrate containing a mixture of 1:2 sand and low organic loamy soil (H. Lauterbach, Schwabach, Germany). For half of all pots, the substrate was amended with final concentrations of 127 ppm Zn ($ZnCl_2$; Arcos Organics, Fair Lawn, NJ, USA) and 1.7 ppm Cd ($CdCl_2$; Arcos Organics) as described by Stolpe et al. (2017), whereas the remaining half was kept metal-unamended, with 21 pots per soil treatment. After ten weeks, plants were transferred with their entire soil into 600-mL cups (polyethylene terephthalate; Wimex, Náchod, Czech Republic) with four holes in the bottom and placed in separate dishes for watering. In these cups plants were cultivated for two further weeks. All plants were arranged in a randomised order in a climate cabinet at 20 °C, 70% relative humidity and a 8:16 h light:dark cycle and watered three times per week. When the plants were used for experiments, they were non-flowering rosettes (about 8 cm diameter) and had developed about 20 leaves.

Mycelium of the fungus *A. brassicae* (strain CBS 102.24) was obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). The species is a generalist phytopathogen on different Brassicaceae species (Farr and Rossman, Fungus-host distribution database, retrieved April 2018), and is more sensitive to camalexin and other phytoalexins than to GSs (Conn et al., 1988; Giamoustaris and Mithen, 1997; Sellam et al., 2007). Cultures were maintained on a medium containing 30 g L^{-1} potato dextrose agar (Carl Roth, Karlsruhe, Germany) as low strength substrate with 10 µg L^{-1} $ZnSO_4 \times 7H_2O$ (Merck, Darmstadt, Germany) and 5 µg L^{-1} $CuSO_4 \times 5H_2O$ (Applichem, Darmstadt, Germany) as additional trace nutrients. Cultures were kept in a climate cabinet under a 1:1 mixture of white light (Lumilux Deluxe Cool Daylight, L36W/965; Osram Licht AG, Munich, Germany) and black light (TL-D, 36W/BLB; Philips GmbH, Hamburg, Germany) (8:16 h light:dark cycle) at 20 °C to promote production of conidiospores.

2.2. Infection procedure

Conidia were harvested from cultures with a growth medium containing 26.5 g L^{-1} potato dextrose broth (Carl Roth) for nutrition and 0.03% [w/v] Triton X-100 (Sigma-Aldrich, Steinheim, Germany) as detergent in millipore water. For this, 10 mL of the medium was added to the plate, the culture was gently suspended using a glass rod, and the conidia suspension filtered through two layers of Miracloth tissue (Millipore Corp., Billerica, MA, USA) to remove mycelium fragments. At each of the four days within two weeks (10–12 plants per day) at which plants were infected, a conidia suspension was freshly prepared and the concentration adjusted to 2000 conidia per µL using a hemocytometer (Neubauer, Marienfeld, Lauda-Königshofen, Germany).

For standardised infection, four young leaves from the inner rosette of each plant were mechanically damaged using a tool of four combined needles (insect pins, 0.4 mm diameter, arranged in a square with 3 mm edge length). Subsequently, 10 µL of conidia suspension were applied on the damaged area and immediately covered with 1.5% [w/v] agar discs (Carl Roth; in millipore water) of 6.7 mm diameter and 1.5 mm height (Fig. 1a). Agar discs were used to attach the conidia suspension to the damaged area and to moisten the infection sites. Each cup was closed with two lids (200 mL volume) with a central hole of 25 mm diameter, in which nine layers of Miracloth tissue (50 × 50 mm) were fixed between the lids, serving as a filter impermeable for conidia (Fig. 1b,c).