Can adaptation to metalliferous environments affect plant response to biotic stress?

Insight from Silene paradoxa L. and phytoalexins.

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Abstract

This work was performed to evaluate if metal adaptation can affect the response to biotic stress in higher plants. Three populations of *Silene paradoxa*, from a noncontaminated soil, a serpentine soil and a copper mine soil respectively, were cultivated in the presence/absence of nickel or copper and then were exposed to pathogen-associated molecular patterns (PAMP). In particular, the non-catalytic fungal protein cerato-platanin, secreted by the parasitic Ascomycete *Ceratocystis platani*, was used, because of its well documented ability to act as a PAMP, and the production of phytoalexins was assayed. Cerato-platanin exposition determined phytoalexin production in a population- and treatment-dependent way. Particularly, an over-production of phytoalexins was recorded for the copper mine population grown in the presence of copper, suggesting that, in particular cases, the adaptation to metalliferous environments can effectively affect plant response to biotic stress. Nevertheless, this supposition cannot be generalized to all the types of metalliferous environments and of metals studied; however, this work can be considered one of the first example of positive interaction between abiotic and biotic stimuli.

Keywords: biotic interactions, cerato-platanin, heavy metals, phytoalexins, *Silene paradoxa*
1. Introduction

Plants are exposed to varying environmental conditions that generally include the combination of biotic and abiotic factors. Detrimental stressors, such as pathogens or herbivores, populate almost each kind of environment, therefore plants not only have acquired mechanisms to contemporaneously cope with biotic and abiotic stress, but probably also to exploit their simultaneous presence to a own possible advantage (Aktinson and Erwin 2012). Each stress elicits a complex cellular and molecular response system implemented by the plant in order to prevent damage and ensure survival. However, when examining the effects of an abiotic stress with simultaneous impact of a pathogen or herbivore, both positive and negative interactions have been observed depending on the timing, nature, and severity of each stress (Aktinson and Erwin 2012).

In this context, an interesting and appropriate model system to investigate such intriguing topic can be represented by the study of responses of heavy metal adapted plants to pathogen attack. To our knowledge, literature on this topic is still extremely scarce.

In metal enriched soils, the high level of metal contamination is a powerful selection factor for more-resistant genotypes of plants, animals and microbes (Ernst, 2000, Baath et al., 2005, Vogel-Mikus et al., 2005) and the consequences for interactions between plants and other organisms can be complex. The effect of mycorrhiza and rhizosphere bacteria on plant metal accumulation and toxicity have attracted most attention (Whiting et al., 2001, Idris et al., 2004, Sell et al., 2005, Pongrac et al., 2007, Sousa et al., 2012, Ma et al., 2013), whereas there is much less information on the influence of excess heavy metals on plant-pathogen relationships. Some information is present in the case of non metal adapted plants, where decreased host susceptibility is consistently observed in most plant-fungal interactions (Poschenrieder et al., 2006). Metal ions can confer pathogen resistance (“metal-induced fortification”) eliciting defense reactions (Mithofer and al., 2004, Walters et al., 2005). For example, in wheat metal-induced proteins are thought to be responsible for cadmium-induced resistance against Fusarium infection (Mittra et al., 2004) and in Arabidopsis cadmium concentrations close to the toxicity threshold were found to induce defense signaling pathways which potentiate the plant response against Botrytis infection (Cabot et al., 2013). The triggering of defense signals and the synthesis of defense related secondary metabolites was instead found to have a relationship with metal-induced reactive oxygen species (Walters et al., 2005, Jonak et al., 2002).

Some metals are known to be fungistatics, able to kill or inhibit the growth and development of the pathogen in the soil or on the plant surface, according to the so-called “phytosanitary effect” (Poschenrieder et al., 2006). Among them, a well known example is the fungistatic effect of copper sulfate, widely used since the 19th century to prevent fungal infections in vineyards.

The self-defense against biotic stress is thought to be increased by the accumulation of high levels of metals in the plant tissues themselves and it is called “elemental defense”, possible if the metal is less toxic to the plant than to the parasite (Poschenrieder et al., 2006). Such hypothesis was initially formulated for metal hyperaccumulators, a group of metal adapted plants that can accumulate metals to exceptionally high concentrations in their shoots (Reeves and Baker, 2000).
and that were noted to be less chewed by insects than nonaccumulating plants (Martens and Boyd, 1994). Regarding an effective metal protection against fungi, further investigations reported an actual role for high levels of Ni, Zn, Cd or Se concentrations, supported also by the observation that hyperaccumulators, when grown on a substrate with low levels of metal, are highly sensitive to biotic stress. Such effect can be supposed to be highly improbable, or at least of a lower importance, in the majority of metal adapted plants, that tend to actively exclude the metals from their tissues. Such nonaccumulator plants, or excluders, generally have root and shoot metal concentrations higher than the same plant species grown on non contaminated soil, but probably not high enough to trigger the elemental defense effect.

Anyway, also metal excluders show a general difficulty of cultivation in soils with low metal concentrations. For example, cuprophytes, plants that are adapted to copper-rich environments, are very sensitive to pathogens of soil (Paton and Brooks, 1996; Chipeng et al., 2010). A relaxed pressure of pathogenic fungi on metal-rich soils has been advocated as the cause of such effect (Tadros, 1957), but it has never been clearly demonstrated.

Regarding the response to pathogen attacks, plants have evolved at least two lines of active defense. The first line provides basal defense against all potential pathogens and is based on the recognition of conserved pathogen associated molecular patterns (PAMPs), by so-called PAMP recognition receptors (PRRs) that activate PAMP-triggered immunity (PTI) and prevent further colonization of the host (De Wit, 2007; Jones and Dangl, 2006).

Therefore, microbe can secrete in the culture medium or localize on their cell wall molecules that can prime the defense in plants. The first step in the induction of the primary plant defense response towards biotic stress is the recognition of certain molecules derived by potentially pathogenic microbes and known as elicitors or MAMPs/PAMPs (microbe/pathogen-associated molecular patterns). Upon PAMP recognition, downstream signal transduction cascades of primary defense responses become activated leading to events that negatively affect pathogen colonization, such as cell wall alterations, production of reactive oxygen species (ROS), synthesis and activation of mitogen-activated protein kinase (MAPK) cascades, and accumulation of defense-related proteins (Jones and Dangl 2006, De Wit et al. 2009, Zipfel 2009, Thomma et al. 2011).

Several PAMPs from bacteria have been identified, but little is known about fungal PAMPs. Chitin, β-glucans and other cell wall components such as galacto-glucomannans have been shown to possess PAMP activity. More recently, some non-catalytic proteins secreted by Ascomycetes and Basidiomycetes have been proposed to act as PAMPs, because they are involved in various aspects typical of primary defense.

Many of these proteins belong to the cerato-platanin family whose members are involved in the host microbe interaction acting as inducer of systemic acquired resistance, hypersensitive response and inducers of enhanced resistance (Frias et al 2011; Vargas et al 2008; Yang et al. 2009). Cerato-platanin (CP), the core member of CP family, is secreted by Ceratocystis platani, an Ascomycete which is the causal agent of the canker stain disease of the plane tree (Pazzagli et al. 1999, Scala et al. 2004). CP is a double psi-beta barrel protein that is able to bind chitin and to weak
cellulose fibers; being the last activity probably related to its role as a PAMP in the host interaction (Oliveira et al. 2011; Baccelli et al. 2013). As a PAMP, CP induces mitogen-activated protein kinases (MAPKs) phosphorylation, production of reactive oxygen species and nitric oxide, overexpression of defense related genes, phytoalexin synthesis, restriction of conidia growth and, finally, programmed cell death with apoptotic features in various host and non-host plants (Fontana et al. 2008; Comparini et al. 2009, Bernardi et al. 2011; Lombardi et al. 2013).

Among the biotic stress induced compounds that enable the plant defense towards pathogens, phytoalexins are a heterogeneous group of low molecular mass secondary metabolites with antimicrobial activity (Ahuia et al., 2012). Such activity has been tested in vitro on several species of bacteria, oomycetes and fungi, but the mechanisms by which phytoalexins exerts their toxicity are still unknown and until now only disruption of microbial membranes and induction of fungal apoptotic-like programmed cell death have been proposed (Ahuia et al., 2012). Phytoalexins have been found to be induced also by abiotic stresses, such as UV-B, UV-C, organic chemicals and heavy metal ions (Zhao et al., 1998, Tierens et al., 2002). For example, mercury and copper have been found to induce phytoalexin production in a variety of plant species, but there is still no convincing explanation for this finding (Mithofer et al., 2004), even though a role in metal detoxification through chelation has been hypothesized (Matsouka et al., 2011).

Therefore, to shed light on the intriguing and yet unexplored topic of the interaction between heavy metal stress and biotic stress in the case of metal adapted plants, we investigated phytoalexin production, induced by CP exposure, in metallophilous and non metallophilous populations of *Silene paradoxa* L. grown in presence and in absence of metals.

The species *Silene paradoxa* is an excluder pseudometallophyte, generally found in non-contaminated dry areas and occasionally evolving metal tolerant populations on metalliferous soils (Chiarucci et al., 1995). The present work was performed by studying three populations of such species with contrasting metal tolerance phenotypes (Gonnelli et al., 2001), one from a calcareous soil (metal sensitive population), one from a serpentine outcrop (nickel tolerant population) and one from a copper mine dump (copper tolerant population). We compared PAMP-induced phytoalexin production in these populations, hypothesizing a different response depending on the metal status of the site of origin. Particular attention was addressed to the role of the presence of the metal in the culture medium in a possible differentiation of the response in the different populations.

2. Materials and Methods

2.1 Plant material and experimental conditions

*Silene paradoxa* L. seeds were collected from plants living on non-contaminated soil (Colle Val D’Elsa, CVD), serpentine soil (Pieve Santo Stefano, PSS) and a copper mine deposit (Fenice Capanne, FC) in Tuscany (Italy). Sites and populations were described in Chiarucci et al., (1995), Gonnelli et al., (2001), Pignattelli et al., (2012). Seeds were sown in peat soil and after six weeks seedlings of the three populations were transferred to hydroponic culture, in 1-L polyethylene pots (three plants per pot) containing a modified half-strength Hoagland’s solution composed of 3 mM
KNO₃, 2 mM Ca(NO₃)₂, 1 mM NH₄H₂PO₄, 0.50 mM MgSO₄, 20 µM Fe(Na)-EDTA, 1 µM KCl, 25 µM H₃BO₃, 2 µM MnSO₄, 2 µM ZnSO₄, 0.1 µM CuSO₄ and 0.1 µM (NH₄)₆Mo₇O₂₄ in milliQ-water (Millipore, Billerica, MA, USA) buffered with 2 mM 2-morpholinoethanesulfonic acid (MES), pH 5.5, adjusted with KOH (Hoagland and Arnon, 1950) and different copper (CuSO₄) or nickel (NiSO₄) concentrations. Nutrient solutions were renewed weekly and plants were grown in a growth chamber for eight weeks (24/16°C day/night; light intensity 75 µE m⁻² s⁻¹, 12 h d⁻¹; relative humidity 60-65%). At the end of incubation in test solutions, root samples were desorbed with ice-cold (4°C) Pb(NO₃)₂ (10 mM) for 30 min. Plants were then divided into roots and shoots and the dry weight of the organs was recorded after drying at 70°C for 1 day.

Measurements of plant biomass were performed on twelve replicates, the determination of copper and nickel concentration was made on six replicates. Phytoalexin production and MAPK activation was evaluated on three replicates. Each replicate was measured three times.

2.2 Determination of element concentration

Element concentrations were determined by digesting oven-dried plant material in a 5-2 (v/v) mixture of HNO₃ (Romil, 69%) and HClO₄ (Applichem, 70%) in 25 ml beakers at 120-200 °C and afterwards the volume was adjusted to 10 ml with milliQ-water. Elements were determined by atomic absorption spectrometry (Analyst 200, Perkin Elmer).

2.3 Leaf treatment with cerato-platanin

CP was heterologously expressed in the yeast Pichia pastoris, from InVitrogen, transformed with the pPIC9-cp plasmid according to Pazzagli et al 2009. The recombinant protein (25 mg) was purified from 1L of cultured medium by RP-HPLC chromatography and assayed for it secondary structure, molecular weight and biological activity according to Carresi et al 2006. Protein concentration was determined by the Bicinchoninic acid assay (BCA, Pierce).

Silene paradoxa leaves were removed from plants and placed directly into boxes containing moist filter paper. 10µL droplets of water (control) and 3x10⁻⁴ M CP were applied on the lower surfaces of leaves. On each leaf, 3 droplets were applied on the right side (water) and 3 droplets on the left side (CP). Leaves were incubated for 6h, 24h and 48h in a moist chamber at 25°C in presence of light.

After the incubation time, the droplets were recovered and the spots of application were washed twice with 10 µL of water. Both droplets and the washing fractions were recovered and brought to a final volume of 500 µL. Samples were stored at -20°C until the measurement of phytoalexin. The detection of phytoalexins in the droplets instead of in the tissue avoids the interference of phenols naturally present in leaves (Pazzagli et al 1999, Scala et al 2004).
2.4 Phytoalexin assay

The recovered droplets were assayed for phytoalexin production according to Lombardi et al., 2013. Phytoalexins were detected taking advantage of their intrinsic fluorescence that enable a rapid and quantitative measurement of the total phenol concentration in the sample (El Modafar et al 1995). Since cerato-platanin contains two residues of triptophan and it is naturally fluorescent, its fluorescent emission was checked in the range of wavelengths where defense phenolic compounds emitted. The fluorescence spectrum of CP in solution was also measured (λ<sub>ex</sub> =365 nm, λ<sub>em</sub> = 380-540 nm). The eliciting activity of proteins was assayed in droplets and expressed as arbitrary fluorescence intensity units in each droplet, where phenolic defence compounds accumulated. Fluorescence values from 10 µL of water were used to eliminate the phenol compounds that could be non-specifically synthesized by leaves as above described. Fluorescence was recorded using a Perkin Elmer spectrofluorimeter 650-10S (Perkin Elmer, Wellesley, MA, USA), using λ<sub>ex</sub> =365 nm, λ<sub>em</sub> = 460 nm and slit 5.

2.5 Leaves treatments, protein extraction and immune-blot analysis

*S. paradoxa* leaves were excised from the plant and floated for 5h on water with gentle shaking. Leaves were infiltrated by means of a hypodermic syringe with 3×10<sup>-4</sup> M CP. Control leaves were infiltrated with distilled water. Leaves were incubated for 15 -60 minutes at room temperature and then frozen in liquid nitrogen. For protein extraction, leaves were grounded to a fine powder in liquid N<sub>2</sub> and added of 400µl of extraction buffer containing 50 mM Tris at pH 7.5, 200 mM NaCl, 1 mM EDTA, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM sodium molybdate, 10% (v/v) glycerol, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride , 1x protease inhibitor cocktail P9599 (Sigma-Aldrich) and 1 mM dithiothreitol (Galletti et al., 2011). After the samples were centrifuged at 14000×g and the clear supernatants were assayed for protein concentration by the Bradford assays method and for MAP kinases phosphorylation. Before performing kinase analysis, the quality of each protein extract (treated and control) was examined by SDS-PAGE (data not shown).

Equal amounts of proteins (about 15 mg) were resolved on 12% polyacrylamide gels and transferred onto a PVDF membrane (Biorad). Primary antibodies against human phospho-p44/42 MAP kinase (Cell Signaling Technologies) were used; horseradish peroxidase-conjugated anti-rabbit as secondary antibody (Cell Signaling Technologies) and the ECL western detection kit (GE healthcare) were used. Membranes were stripped with 50mM Tris-HCl pH 7.0 buffer containing 2% SDS and 0.1M 2-mercaptoethanol for 30 min at 55°C and then incubated with *A. thaliana* MPK3.
and MPK6 antibodies (Sigma-Aldrich), which were used as quantitative references. Signal
detection was performed as above reported.

2.6 Statistics

Statistical analysis was carried out with ANOVA, one-way and two-way (considering
cerato-platanin exposition and metal treatment as main factors), using the statistical program SPSS
13.0 (SPSS Inc., Chicago, IL, USA). A posteriori comparison of individual means was performed
using Tukey post hoc test (with at least p<0.05 as significant level).

3. Results

3.1 Effects of copper and nickel on plant growth

Both the metallicolous populations did not show any metal induced root or shoot biomass
reduction, whereas the sensitive plants exhibited a significantly lower copper and nickel tolerance,
as their root and shoot biomass production was significantly affected by the metal treatment (Tab.
1).

3.2 Copper and nickel accumulation

Regarding copper accumulation, both in roots and in shoots the copper mine population
showed the lowest concentration and the serpentine population the highest (Tab. 2). As for nickel
accumulation, all the populations displayed similar values in roots and in shoots (Tab. 2).

3.3 Fluorescence of cerato-platanin

The fluorescence spectrum of cerato-platanin is shown in Fig. 1 together with the spectrum
of one of the samples of our experiments. No interference of the two spectra was present at 460nm,
that is the emission value generally used to detect the presence of phenolic compounds in such
kinds of samples (El Modafar et al 1995, Du Fall and Solomon 2013). The fluorescence values
registered at such wavelength can be considered representative of the exclusive presence of
phenolic molecules produced after the treatments of our experiments and from now on they will be

3.4 Copper and nickel effect on phytoalexin production

Leaves from the three Silene paradoxa populations were assayed for phenolic compound
production after a 24h treatment with cerato-platanin (Fig. 2).
When leaves were not exposed to CP, in the sensitive and the copper mine populations the levels of fluorescence were not significantly different between control and metal treated plants, whereas in PSS population such levels were significantly higher in metal treated plants as compared to control plants.

After the exposition to CP, all the samples showed significantly higher values of fluorescence, from a two-fold to a six-fold increase in the different cases. Comparing such data in the case of metal exposed plants, the copper mine population displayed a higher value of fluorescence when grown in the presence of copper and a lower one when grown in the presence of nickel, whereas in the other two populations the fluorescence values obtained where not dependant on the growth conditions.

Comparing the populations among them, the only significant difference scored was between copper treated CP exposed plants in the case of the copper mine population compared to sensitive or the serpentine one (p<0.05).

3.5 Time dependant-production of phytoalexins

In Fig. 3 the fluorescence values from samples exposed for different times is reported. In all the sensitive population samples, the fluorescence intensity increased over time. In samples not exposed to CP the increase was slight and non significantly different between the two different treatments. In both the CP exposed samples the values of fluorescence, not significantly different between themselves, were significantly higher than in CP non exposed samples (p<0.05). In the copper mine population samples not exposed to CP the fluorescence values did not change over time, irrespectively of the presence of copper in the culture medium. In CP exposed samples, an increment of that value occurred and it was significantly higher in the samples from copper-treated plants (p<0.05), except for 6h of exposition. Comparing the populations between themselves, the only significant difference present was between copper treated - CP exposed plants, being the values of the samples from the copper mine population higher than the ones from the sensitive population (p<0.05).

An estimation of the net production of phytoalexins induced by CP, independently from the amount of such molecules induced by the copper treatment, was calculated subtracting the values of fluorescence of the CP non exposed samples from those ones of the relative CP exposed samples. The obtained values are reported in Fig. 4. In all the samples the net phytoalexin production increased over time, but in a population- and treatment-dependent way. When the plants were cultivated in control conditions there were no significant differences between the populations. In respect to those values, in the presence of copper in the culture medium samples from the sensitive
plants showed lower values (p<0.05) and those ones from the copper mine plants showed increasingly higher values that became significantly different at the longer time of exposition (p<0.01). In this case, the values of fluorescence from the copper mine population samples were significantly higher than those ones from the sensitive population samples (p<0.01).

3.6 Copper concentration dependant-production of phytoalexins

Fig. 5 reports the fluorescence values from plants cultivated at different copper concentrations. In CP non exposed plants, the level of phytoalexins did not increased significantly in both the populations. In copper treated CP exposed plants, the values of fluorescence were significantly higher than in non exposed plants (p<0.01), but varied only in the copper mine population, increasing till 5µM CuSO$_4$ concentration (p<0.05). The fluorescence of samples from copper mine plants was significantly higher than that one from sensitive plants (p<0.05).

In sensitive plants the net production of phytoalexins did not vary with copper in the culture medium (Fig. 6). In copper mine plants the net amount of phytoalexins produced increased with increasing copper concentration in the culture medium, except at the higher concentration used. Copper mine plants showed higher values of net phytoalexin production at all the CuSO$_4$ concentrations used as compared to sensitive plants (p<0.05).

3.7 MAP kinase activation

The expression level of two protein kinases was assayed using the anti-human phospho-ERK1/2 antibodies because plant kinases show a sufficient level of homology with the phosphorylation sites of mammalian kinases. ERK1/2 are equivalent to MAPK6 and MAPK3 from Arabidopsis which are involved in the defence response (Galletti et al., 2011).

The level of MAPK phosphorylation was measured at 15 and 60 min, as the kinase cascade activation is one of the first events in plant defence signalling (Pitzschke et al., 2009). Immunoblot analysis of CP-treated leaves showed that either in copper mine and sensitive plants treated with water the activation of kinases was not detectable (Fig 7A, C). On the contrary, the treatment with CP induced an increase in kinases phosphorylation and such increase was more evident in both sensitive and copper mine plants grown in copper containing medium when compared to the same plants grown in control medium (Fig 7B, D). Results also showed that the kinase activation was one of the first signal in defence responses: in all the assayed samples the phosphorylation signal started after 15 min and it was off after 60 min of incubation. Finally, in the copper mine population grown in 5µM copper a basal level of kinase activation was observed also without CP treatment (fig. 7D).
4. Discussion

4.1 Growth conditions and differential phytoalexin production

The metal concentrations used in the experiments proved to be adequate to the proposed aim, both in terms of metal imposed effect on plant growth and metal accumulation in the plant organs. Actually, metal treatment provoked an impaired development of the non-metallicolous population (Tab. 1). This effect was significant, but slight (on average around 30% growth reduction in roots and about 45% in shoots), thus suggesting the plants to be still metabolically efficient to produce any other stress-imposed response. At such concentrations, the development of the metallicolous plants was unaffected (Tab. 1), even if their features of copper and nickel tolerance were reported to be different. The serpentine population (PSS) was shown to be nickel tolerant but not copper tolerant, whereas the mine population was shown to be copper tolerant and nickel co-tolerant (Gonnelli et al., 2001). Probably, the copper concentration used was too low to reveal the sensitivity of the serpentine population to this metal in respect to the mine population (FC), but increasing it to that point could provoke an effect on the sensitive population (CVD) so deleterious to render the experimental design not suitable to the proposed aim. As far as metal accumulation is concerned (Tab. 2), metal concentrations were similar among the populations in most of the cases. In fact, the only significant difference was the low copper concentration shown by the Cu mine population and generated by its well known behavior as a copper excluder (Gonnelli et al., 2001, Colzi et al., 2011, 2012).

Cerato-platanin was used as biotic stimulus to detect the ability of metallicolous and nonmetallicolous populations of Silene paradoxa to activate the defense response measured as the ability to induce phenolic compound accumulation on leaves (El Modafar et al 1995; Großkinsky et al 2012; Du Fall and Solomon 2013). The emission fluorescent spectra obtained (Fig.1) and the values obtained upon treatment of leaves with water indicated that the assayed fluorescence was due to the activation of defense responses and not to non-specific responses or to the cerato-platanin itself.

After cerato-platanin exposition, all the samples showed significantly higher values of fluorescence, indicating that this well characterized protein fungal PAMP was able to induce the synthesis of phytoalexins in the non-host plant Silene paradoxa (Fig. 2). The presence of copper or nickel in the culture medium did not affect the production of such molecules after the exposition to cerato-platanin, except in one case. Interestingly, when the mine tolerant population was grown in presence of copper, it showed a level of phytoalexin production higher than in control conditions and higher than the other two populations. Intriguingly, the presence of nickel did not induce a
similar response. Therefore, at least for the populations studied, a metal specificity for such particular behavior could be suggested and it is interesting to note that it was for a metal well known as a fungicide.

The serpentine population uninterestingly showed fluorescence values in the range of the reference population. Therefore, the previous particular behavior seemed to be dependant also on the kind of metalliferous environment the plants came from. For that reason, this population was excluded from the further experiments on the response of *S. paradoxa* to PAMP exposition present in this study. Nevertheless, only the serpentine population showed a metal-induced production of phytoalexins in cerato-platanin not exposed plants. This mechanism could represent a possible metal-induced response exclusive of that population, thus deserving further investigation.

The over-production of phytoalexins of the copper tolerant population grown in the presence of such metal suggested that, in specific cases, the adaptation to metalliferous environments can effectively affect plant response to biotic stress. Some considerations on the role in plant defense mechanisms of such large phytoalexin production can be intriguingly drawn and would deserve to be verified in other copper tolerant populations. Considering that copper-rich soils may have lower pathogen loads compared to normal soil, thus relaxing the selection for resistance mechanisms, the over-production of phytoalexins could reflect a greater susceptibility of such population to a pathogen attack. Curiously, that behavior was different when the Cu metallicolous population was grown in control conditions and similar to that on one of the sensitive population. The mine population seemed to be able to “sense” the presence of copper in its environment. Probably, at normal copper level, the mine population synthesized its constitutive defenses against natural enemies at a normal rate. In effect, this population never shows problems of germination and establishment on uncontaminated soil (pers. obs.) and neither a fungicide nor elevated copper concentrations are required to optimize its growth, as for example in the case of *Haumaniastrum katangense* (Chipeng et al., 2010). This condition could result in a response to fungal attack similar to the sensitive population. On the other hand, copper in the culture medium could simulate a well known environment for that population, with a low pressure of natural enemies. Such environment could not require a high investment of resources in basal defenses against, at least some kinds, of pathogens. Therefore, in case of attack the population would be constrained to a higher production of inducible defenses.

### 4.2 Characterization of copper effect on phytoalexin production

The different behavior that the copper tolerant population had shown when grown in the presence of that metal was confirmed by time-dependence experiments (Fig. 3). The cerato-platanin
imposed production increased with the exposition time in a similar way for the sensitive plants, irreversibly of the presence of copper in the culture medium, and for the non treated metallicolous plants. All the values of the previous samples were lower than the copper treated cerato-platanin exposed metallicolous samples. This behavior was even more evident when the net production of phytoalexins was calculated to eliminate the effect of the phytoalexin production due to the metal treatment only. The result of this calculation further confirmed the larger activation of defenses, in the presence of copper, in the tolerant population compared with the sensitive one (Fig. 4).

In copper concentration dependent experiments the differences between the different populations and treatments were maintained and shown to be dependent on the metal level for the first two concentrations used (Fig. 5 and 6). Till 5 µM CuSO₄, the previously hypothesized “metal sensing” of the mine population seemed to increase its effect following the copper concentration, the more the metal was present in the environment, the more phytoalexins needed to be produced under fungal attack. At the highest copper concentration used, the level of phytoalexin production surprisingly fell to control level. Plant growth and copper accumulation were monitored also in this kind of experiment, giving expected results (a higher copper imposed effect on plant growth for the sensitive population, along with a higher copper accumulation in roots and shoots, data not shown) coherent with the already published papers on the presently studied system (see for example Colzi et al., 2012).

4.3 MAPKs, a possible involvement?

Multiple studies have shown that MAPK cascades play important roles in plant responses to biotic and abiotic stresses, such as pathogen infection, wounding, low temperature, drought, hyper- and hypo-osmolarity, high salinity, mechanical stress, metals and ROS (Mishra et al 2006, Pitzschke et al 2009, Tena et al. 2011, Hamel et al. 2012). The results obtained from the phosphorylation level of MAPKs in leaves of Silene paradoxa from non-contaminated and copper mine soil, either grown in the presence/absence of 5µM CuSO₄, showed that CP activated MAPKs in both the populations and that such activation was larger when plants were grown in the presence of copper (Fig. 7). Therefore, the experiments confirmed the ability of cerato-platanin to activate MAPKs in non-host plants (Lombardi et al 2013) and suggested a positive interaction between biotic and abiotic stimuli in inducing an upstream signal of defence at the copper concentration here used.

Biotic and abiotic stress signal transduction results from a complex arrangement of interacting factor that may positively or negatively interact (Pedras et al 2008; Aktinson and Urwin 2012). In this contest, our results can suggest that the interaction between a biotic stimulus, here
represented by the non-catalytic fungal protein cerato-platanin, and an abiotic stress, such as copper excess, can affect the defensive machinery system of a particular metallicolous population of *Silene paradoxa*, as confirmed by MAPks activation as well as phytoalexins production. In fact, each stress elicits a complex cellular and molecular response system implemented by the plant in order to prevent damage and ensure survival as recently reported (Schenke et al 2011, Aktinson and Urwin 2012, Opdenakker et al 2012). Therefore, our results are in agreement with the scanty data present in literature and provide new information about the induction of defenses induced by a fungal PAMP in metal adapted plants.

5. Conclusions

The intensity of response to biotic stress, in terms of phytoalexin production after PAMP exposition, shown by the excluder pseudo-metallophyte *Silene paradoxa* seemed to be dependent both on the kind of metal adapted population and the presence of specific metals in the culture medium. Actually, an over-production of phytoalexins was recorded for the copper mine population grown in the presence of such metal. Therefore, the adaptation to metalliferous environments can effectively affect plant response to biotic stress, but this interesting hypothesis cannot be generalized to all the types of metalliferous environments.

Nevertheless, the present paper showed for the first time that a biotic stimulus, such as the fungal PAMP cerato-platanin, can have a different influence on the induction of defenses in copper tolerant plants grown in the presence of copper. That effect probably was a consequence of a stronger activation of defensive mechanisms that start by the activation of MAPKs and conclude with the secretion of fluorescent phenolic compounds on the lower surface of the leaves.

References


Table 1. Biomass production (mg per plant) of the three *Silene paradoxa* populations treated with 5 µM CuSO₄ or 5 µM NiSO₄ for eight weeks. Values are means ± standard deviation of twelve replicates. Significant differences between the means appear with different letters (*p<0.05, **p<0.01). CVD = Colle Val D’Elsa, noncontaminated soil; FC = Fenice Capanne, copper mine soil; PSS = Pieve Santo Stefano, serpentine soil.

<table>
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<th>Population</th>
<th>Root</th>
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<th></th>
<th>Shoot</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CuSO₄</td>
<td>NiSO₄</td>
<td>Control</td>
<td>CuSO₄</td>
<td>NiSO₄</td>
</tr>
<tr>
<td>CVD</td>
<td>35.7± 2.1a</td>
<td>25.4± 3.3b*</td>
<td>29.0± 2.3b*</td>
<td>169.7± 17.2a</td>
<td>105.8± 23.0b*</td>
<td>79.8± 14.7b**</td>
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<tr>
<td>FC</td>
<td>25.0± 5.4</td>
<td>44.3± 22.3</td>
<td>29.6± 15.0</td>
<td>164.8± 19.4</td>
<td>192.4± 84.0</td>
<td>139.0± 55.9</td>
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<tr>
<td>PSS</td>
<td>26.9± 4.6</td>
<td>42.5± 17.7</td>
<td>24.4± 12.6</td>
<td>170.0± 66.3</td>
<td>184.1± 57.1</td>
<td>150.8± 34.0</td>
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</table>
Table 2. Metal accumulation (µg g⁻¹ dry weight) in the three *Silene paradoxa* populations treated with 5 µM CuSO₄ or 5 µM NiSO₄ for eight weeks. Values are means ± standard deviation of six replicates. Significant differences between the means appear with different letters (*p<0.05, **p<0.01). CVD = Colle Val D’Elsa, noncontaminated soil; FC = Fenice Capanne, copper mine soil; PSS = Pieve Santo Stefano, serpentine soil.

<table>
<thead>
<tr>
<th>Population</th>
<th>Root</th>
<th>Shoot</th>
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<tr>
<td></td>
<td>CuSO₄</td>
<td>NiSO₄</td>
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<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
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<td>105.9±18.7b*</td>
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<tr>
<td>FC</td>
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<tr>
<td>PSS</td>
<td>7.4±3.2</td>
<td>185.4±11.6c**</td>
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**Figure captions**

**Figure 1:** Fluorescence emission spectrum of 18nmol of cerato-platanin (CP) before (dashed line) and after 48h of incubation on lower surface of *S. paradoxa* leaf (solid line).

**Figure 2:** Fluorescence emission at 460nm of 3 nmol of cerato-platanin (CP) after 24h of incubation on lower surface of *S. paradoxa* leaves from the three populations (CVD = Colle Val D’Elsa, noncontaminated soil; FC = Fenice Capanne, copper mine soil; PSS = Pieve Santo Stefano, serpentine soil). Incubation with 10µL of water was used as reference. Values are means of three replicates ± standard deviations. Significant differences between the means appear with different letters, small for metal intra-treatment and capital for metal inter-treatment comparisons (at least p<0.05).

**Figure 3:** Time dependent phytoalexins production upon cerato-platanin (CP) treatment. Fluorescence emission at 460nm in Colle Val D’Elsa (CVD, noncontaminated soil) (A) and Fenice Capanne (FC, copper mine soil) (B) populations grown in control and 5 µM CuSO₄ solution after incubation with 3nmol of CP for 6, 24 and 48h. Incubation with water was used as reference. Values are means of three replicates ± standard deviations.

**Figure 4:** Net production of phytoalexins after incubation with 3 nmol cerato-platanin (CP) for 6, 24 and 48h in Colle Val D’Elsa (CVD, noncontaminated soil) and Fenice Capanne (FC, copper mine soil) populations grown in control and 5 µM CuSO₄ solution. The net production of phytoalexins was calculated by subtracting the production of phytoalexins induced by water from phytoalexins production induced by cerato-platanin at various times. Values are means of three replicates ± standard deviations. Significant differences between the means appear with different letters, small for metal intra-treatment and capital for metal inter-treatment comparisons (at least p<0.05).

**Figure 5:** Concentration-dependent phytoalexins production in *S. paradoxa* Colle Val D’Elsa (CVD, noncontaminated soil) and Fenice Capanne (FC, copper mine soil) populations after 48h of incubation with 3nmol of cerato-platanin (CP). Incubation with water was used as reference. Values are means of three replicates ± standard deviations.

**Figure 6:** Net production of phytoalexins after 48h of incubation with 3 nmol of cerato-platanin (CP) in Colle Val D’Elsa (CVD, noncontaminated soil) and Fenice Capanne (FC, copper mine soil) populations grown at different copper concentrations. The net production of phytoalexins was
calculated by subtracting the production of phytoalexins induced by water from phytoalexins production induced by cerato-platanin at various copper concentrations. Values are means of three replicates ± standard deviations. Significant differences between the means appear with different letters, small for metal intra-treatment and capital for metal inter-treatment comparisons (at least $p<0.05$).

Figure 7: MAPKs activation in *Silene paradoxa* leaves treated with 3 nmol of cerato-platanin (CP). Phosphorylation of MAPK in Colle Val D'Elsa (CVD, noncontaminated soil) (A) and Fenice Capanne (FC, copper mine soil) (C) leaves grown in water (left panel) and respectively (B and D) in copper medium (right panel) visualized with anti ERK1/2 antibodies. In each figure the lower panel represents the results obtained with anti MAPK3 and MAPK6 antibodies; the band intensities were used to normalize the results. The figure and the normalization results are representative of three independent experiments performed in duplicate.