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Responses to environmental stresses in metallicolous and non metallicolous *Silene paradoxa* L. populations

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SUMMARY

Due to the high heavy metal concentrations, metalliferous soils, both of natural and anthropogenic origin, provide a very restrictive and selective environment for plant life. Some plants, named “metallophytes”, have evolved tolerance mechanisms that allow them to cope with toxic heavy metal concentrations.

A good model for comparative studies of metal tolerance is the specie *Silene paradoxa* L. (Caryophyllaceae), an excluder facultative metallophyte that presents populations living in metalliferous and non-metalliferous soils.

This project aims to investigate responses to different environmental stresses, both biotic and abiotic, in metallicolous and non-metallicolous population of this specie.

At the root level, we investigated how Cu affects the morphology and composition of such organ in a non-metallicolous and a Cu-tolerant *Silene paradoxa* population. We found out that in the Cu-tolerant population some of the possible Cu exclusion strategies could be the mucilage and lignine production and the reduction of subapical zone of the root. Passing to the shoot level, we investigated the different effects of Cu excess on photosynthetic parameters in the same two populations. The Cu-tolerant population showed a more efficient photosynthetic activity in respect to the non-metallicolous population and a different nature of photosynthetic limitations, being mostly stomatal, compared to non-metallicolous mostly diffusional and biochemical limitations.

Metalliferous soils are characterized by low macronutrient concentrations and availability, if compared to non-metalliferous ones, so that metallophytes have to adapt even to nutrient scarcity. We compared the ability to use nutrients in a non-metallicolous, a Cu-tolerant and a serpentine Ni-tolerant population exposed to Cu and Ni excess. We found out that metallicolous populations have

evolved mechanisms to adapt to Ca and Mg scarcity in a metal-dependent way, optimizing nutrient utilization.

Responses to biotic stresses in excluder metallophytes are poorly studied. These plants, unlike hyperaccumulators, cannot rely on the accumulation of the metal to defend themselves from pathogens. Therefore, we quantified the responses to pathogen attack in a metallicolous and Cu-tolerant *Silene paradoxa* population exposed to Cu. As an elicitor of defense responses, we used a purified fungal protein with PAMP activity, called cerato-platanin (CP). An over-production of phytoalexins was recorded for the Cu-tolerant population exposed to Cu, suggesting that adaptation to metalliferous soils can affect plant response to biotic stress. Remaining in the same outline and with the same experimental setting, we further investigated whether the pathway leading to induction of defense responses is dependent on ROS (reactive oxygen species) production or not. Our results showed incompatibility between the ordinary ROS-mediated response to fungal attack and the acquired mechanisms of preventing oxidative stress in the Cu-tolerant population. Therefore, the same incompatibility of hyperaccumulators in ROS-mediated biotic stress signals seemed to be exhibited by this excluder metallophyte, but without the advantage of being able to rely on the elemental defense for plant protection from natural enemies.

GLOSSARY

Silene paradoxa L. populations:

CVD	Colle Val D'Elsa (Siena) metal sensitive population
FC	Fenice Capanne (Grosseto) Cu-tolerant mine waste population
PSS	Pieve Santo Stefano (Arezzo) Ni-tolerant serpentine population
TOL	Cu- tolerant mine waste population
NONTOL	metal sensitive population

ABA	abscissic acid
A_N	net photosynthetic rate
BL	biochemical limitation
Chl	chlorophyll
C_i	CO_2 internal concentration
CP	cerato-platanin
d.w. (or D.W.)	dry weight
ETR	electron transfer rate
f.w. (or F.W.)	fresh weight
F_v/F_m	maximum dark-acclimated quantum yield of PSII
Φ_{PSII}	light-acclimated electron flow through PSII
GLVs	green leaf volatiles
g_m	mesophyll conductance
g_s	stomatal conductance
GSH	glutathione
HPLC	high performance liquid cromatography
J_{max}	maximum rate of the electron transport
MAPK	MAP-kinase
MDA	malondialdehyde

Me	metal
MCL	mesophyll limitation
MTs	metallothioneins
m/z	mass-to-charge ratio
ncps	number of counts per second
NUE	nutrient use efficiency
ROS	reactive oxygen species
PAMP	pathogen associated molecular pattern
PCs	phytochelatins
Pheo	pheophytin
PS	photosystem
PTR-MS-ToF	proton transfer reaction- time of flight-mass spectrometry
Q_A, Q_B	quinone A and B
RBOHD	NADPH/respiratory burst oxidase protein D
SL	stomatal limitation
SOD	superoxide dismutase
TL	total photosynthetic limitation
TPU	use of triose- P
$V_{c,\ max}$	maximum carboxylation rate of Rubisco
VOCs	volatile organic compounds

All the elements were indicated with the corresponding chemical symbol of the Mendeleev periodic table.

CHAPTER 1

General introduction

Silene paradoxa growing in a serpentine outcrop in Pieve Santo Stefano (Arezzo)

1.1 Heavy metals: essentiality and toxicity

Heavy metals constitute an ill-defined group of inorganic chemical hazards that include metals and metalloids with an atomic density at least 5 times higher than water. The term "heavy metal" is commonly used to define metals connected with contamination and toxicity, but there is still not a universal agreement on the definition (Duffus, 2002). Metal ions and atoms and most cations are Lewis acids (Sparks, 1995) and most have low positive charge and large size and form covalent bonds with ligands (Roberts et al., 2005).

Among the heavy metals, copper (Cu), chromium (Cr), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), zinc (Zn) and, only for legumes, cobalt (Co) are called essential micronutrients. These elements, in comparison to the macronutrients which are required in thousands of mg kg^{-1}, reach their optimal concentration in relatively small amounts (5-100 μg g^{-1} d.w.) and they are considered essential for the plant because it cannot complete its lifecycle without them, they cannot be replaced and they are involved in important physiological and biochemical functions (Alloway, 1995).

As shown in Fig.1.1A, a concentration range exists for these essential elements and going below or above this regime can result in deficiency and toxicity symptoms, respectively (Roberts et al., 2005).

The remaining heavy metals as arsenic (As), antimony (Sb), cadmium (Cd), lead (Pb), mercury (Hg), are non-essential, they are intrinsically toxic and at least they can be tolerated by plants in low concentrations (Fig. 1.1B).

So it is strictly needed for all organisms that all fluctuations in nutrients availability must be controlled for the homeostasis manteinance (Nelson, 1999; Clemens, 2001).

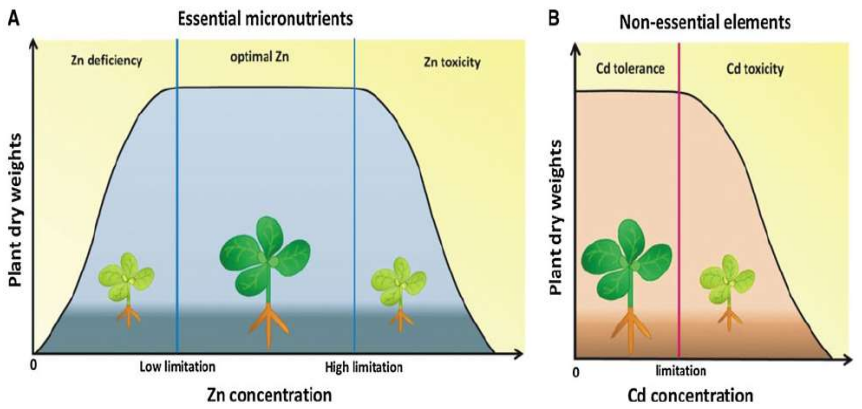

Fig.1.1 Dose-response curves of plants to essential micronutrients (A) and non-essential elements (B) (Lin and Aarts, 2012) shown as plant dry weight against metal concentration.

The main toxicity factor for organisms, including plants, is the participation of heavy metals in chemical reactions that release reactive oxygen species (ROS), like hydroxyl radical, superoxide anion or hydrogen peroxide, causing "oxidative stress", a phenomenon that potentially leads to serious cell damages due to the imbalance between oxidants and antioxidants in favor of the oxidants (Sies, 1997; Kehrer, 2002). The oxidative damage generated by metals with redox activity, as Fe or Cu, is explained by Fenton and Haber-Weiss reactions, catalized by metal ions (Kehrer, 2000), as shown above:

Haber-Weiss reaction: $Me^{n+} + \bullet O_2^- \rightarrow Me^{(n-1)+} + O_2$

Fenton reaction: $Me^{(n-1)+} + H_2O_2 \rightarrow Me^{n+} + OH^- + \bullet OH$

Net reaction: $\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$

Reactive oxygen species, in particular hydroxyl radical (•OH), can cause lipid peroxidation (thus disrupting cell membranes), suppressed photosynthesis, damage to nucleic acids, protein denaturation, interference with signal

transduction, enhanced programmed cell death and induction of senescence (Kehrer, 2000; Lin and Aarts, 2012).

Another toxicity factor is the high affinity of metals to binding nitrogen-, sulfur- and oxygen-containing functional groups that lead to denaturation and inactivation of several biomolecules and enzymes (i.e., Cd substituting Zn, or Mn substituting Mg).

1.2 Metalliferous soils

Heavy metals are naturally occurring in the earth's crust in trace concentration (ppb) with Fe being the most abundant one, making up to the 34% of the Earth's mass. As the term says, metalliferous soils are characterized by a higher content of heavy metals than the non-metalliferous ones. Establishing a metal concentration range for a normal soil compared with a contaminated soil is very difficult as the toxicity does not depend on concentration but on the speciation of the element (Roberts et al., 2005).

In non-metalliferous soils, Zn, Cu, Pb, Ni, Cd and Cr are present in concentration that range between 0.0001 and 0.065%, while Mn and Fe can be present up to 0.002% and 10%, respectively (Ernst, 1974). With the exception of Fe, all heavy metals above a concentration of 0.1% in the soil become toxic to plants and therefore change the plant community structure in contaminated environment (Bothe, 2011).

As soil provides the nutrient-bearing substrate for plant life, its contamination by these elements constitute a high challenge for the plants due to the high toxicity of these contaminants.

Soils can be naturally metal-enriched or contaminated by anthropogenic sources (Siegel, 2002) but separating out the two sources is not often an easy task (Roberts et al., 2005). Without human impact, excessive concentrations of some heavy metals in soils are the result of natural mineralization due to the presence of undisturbed ore bodies near the surface (Ernst, 1989) but also physical and

chemical weathering of bedrocks, volcanic eruptions, forest fires and crustal dust contribute to natural metal spreading in the environment (Fergusson et al., 1990; Nriagu, 1989). Natural sources of pollution are also due to chemical and environmental processes such as metal corrosion and soil erosion, atmospheric deposition, sediment re-suspension and leaching of heavy metals to soils and groundwaters (Nriagu, 1989).

The anthropogenic contamination comes, for the most part, from mining and smelting activities, followed by industrial production (metal processing in refineries, paper productions, power plants, coal burning, spillage of petrochemicals, leather and textile factories, plastic factories), domestic activities (mostly combustion and waste, sewage sludge) and agricultural activities which use metal-containing compounds (fertilizers, fungicides, pesticides). These sources cause the release of metal emissions in atmosphere and the production of solid and liquid wastes containing heavy metals.

Soils are the major sinks for heavy metals released into the environment and, unlike organic contaminants, most metals do not undergo biological or chemical degradation, thus being highly persistent and needing physical remove in order to remediate the soil (Adriano, 2003). Heavy metal in soils can be found in water solution, in solid-liquid interface and in the solid phase. Adsorption is the main process that leads to accumulation of heavy metals in soils and it depends on various parameters such as content of organic matter, content of inorganic colloids, metal speciation, soil type, contact time, soil: solution mass ratio, cation exchange capacity, pH and Eh. In general, water solubility decreases as soil pH increases (Bradl, 2004).

Metal toxicity does not depend only on total content, but above all, on its bioavailability, which depends on physical, chemical and biological soil properties and on the metal speciation. Heavy metals can be immobilized in metal oxides and hydroxides, metal carbonates and phosphates, can remain in liquid phase as soluble ions, complexes or chelates or can be adsorbed by colloid matter

of detrital origin, by the organic matter and by organic and inorganic ligands both of natural (humic and fulvic acids) and anthropogenic (EDTA, polyphosphates) origin (Schlautmann and Morgan, 1994; Siegel, 2002; Bradl, 2004). Studies like the one by Miller et al. (1983) show that the largest fraction of most metals is bound to the organic matter, with Pb having the highest percentage associated with organic matter, and Cd the lowest.

1.3. Plant adaptation to metalliferous soils

Metalliferous soils provide very restrictive habitats for plants, resulting in high selection pressure. As soon as a specie colonizes a metal-enriched soil, it has to adapt its metabolism to the new situation by reaching a new metal homeostasis. One of the basic condition for the colonization of metalliferous soils is the ability of plants to evolve metal resistance (Levitt, 1980) which can be achieved through avoidance (by which a plant is protected externally from the influence of stress) or most likely tolerance (by which a plant survives the effect of internal stress) (Levitt, 1980; Baker, 1987).

Heavy metal tolerance has been developed by plants from totally unrelated taxa (Bothe, 2011). Up to 34 plant families have developed metal tolerant species (Verbruggen et al., 2009). The majority of species are found in Brassicaceae, but also in Caryophyllaceae, Plumbaginaceae, Violaceae, Asteraceae, Poaceae and others (Bothe, 2011).

Plants with ability to tolerate metal toxicity and survive and reproduce in this kind of environments are called metallophytes and can be classified in two big groups: obligate and facultative. The first groups is constituted by species that evolved physiological mechanisms to tolerate exceptional concentrations of heavy metals in soils; they represents typical endemisms and are not found outside this narrow ecological amplitude. Facultative metallophytes (or pseudo-metallophytes) are genotypes or ecotypes of common species with a specific

tolerance to metals and can occur both in metalliferous and non- metalliferous soils (Baker et al., 2010).

The classification of physiological adaptation strategies is provided by Baker and Walker (1990) based on the differences in the internal metal allocation between roots and shoots: excluders, indicators and accumulators (or hyperaccumulators), as shown in Fig.1.2.

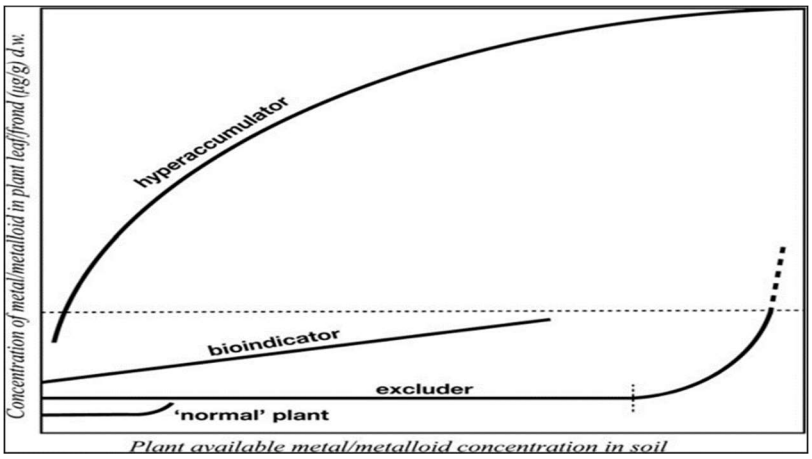

Fig.1.2. Response diagram for metal uptake in plant shoots, adapted from Baker, 1981.

Excluder species actively restrict heavy metal translocation to the shoots within a wide range of metal concentration in soil and retain most of the metals in their roots (Lolkema et al., 1984). Indicator species accumulate metals in the shoots and the concentration of metal in their tissues reflects metal level in the soils. Accumulators increase heavy metal transfer the shoots at higher level than the

substrate. Some species can accumulate only one metal, while others can accumulate more than one metal each. Among this category, the hyperaccumulators show extremely high tolerance to specific elements often present at concentrations in the substrate which would normally exert phytotoxic effects (Peterson, 1982; Baker, 1981). In these plants the element concentration

can exceed 1% in the dried plant material. Brooks (1998) indicates as hyperaccumulators those plants who contain more than 0,1% Ni, Co, Cu, Cr, Pb and more than 1% Mn and Zn in their dried tissues.

1.4. Heavy metal tolerance in plants

Despite the independent evolution of metal tolerance inside various groups of Angiosperms, its physiological regulation is almost identical for all the species involved (Ernst et al., 1992). The plant can defend itself from the toxic effects of the metal basically by limiting its access in organs and tissues and, at the cellular level, by sequestration or compartimentalization of the free metal ions, especially in the vacuole, and protecting the cell from oxidative stress by active ROS scavenging. The removal of surplus metals from the cytosol and their transport across the tonoplast are accelerated in metal-tolerant plants (Verkleij et al., 1998; Assunçao et al., 2001).

Roots are the first organs to be exposed to metal surplus in soil. If the metal concentrations are not homogeneously distributed, roots may grow in search of less contaminated soil patches (Whiting et al., 2000) but that is almost impossible in high and homogeneously contaminated soils such as mine tailings or serpentine outcrops. Another avoidance mechanism is related to symbiosis with mycorrhizal fungi that can enhance the plant metal tolerance by changing metal speciation or by restricting the transfer of the metal into the plant (Dahlberg, 1997).

At the root level, plants can also limits metal uptake by a down-regulation of transporter activities, but such strategy is not so beneficial because the chemical properties or the metal ion size are often too similar to essential micronutrients, causing a possible nutrient deficiency. For example several grass species growing on As-enriched soils down-regulate the high affinity phosphate transporter to lower arsenate uptake (Meharg and Macnair, 1991). The avoidance mechanisms are not so efficient for plants growing on metalliferous soils, thus

they must develope metal tolerance in order to survive in these kind of environments.

In facultative metallophytes, metal tolerance is expressed only by the populations growing on metalliferous soils (Macnair, 1993) whereas the non metallicolous populations can contain tolerant mutants in low frequencies (Gartside e McNeilly, 1974; Wu et al., 1975). Since most metal-tolerances are strongly, if not exclusively metal-specific, it is expected that tolerance should be confined to the metals that are present at toxic concentrations in the soil, because excessive tolerance appears to be unfavorable and negatively selected (McNeilly, 1968). It is accepted that metal specific tolerances have independently evolved several times in different species from local non-tolerant ancestral populations (Pauwels et al., 2005). Several genetics studies have reported metal tolerance being dependent from a few major genes and some minor genes ("modifiers") that enhance the effect of the major gene(s) and increase the metal tolerance (Smith e Macnair, 1998; Schat e Ten Bookum, 1992).

At the cellular level, plants can regulate/limit the entrance of metals by releasing carboxylic acids (malate, citrate, oxaloacetate ecc.) or phytosiderophores that bind heavy metals outside the roots or in the root apoplast, thus preventing metal uptake. Once inside the cell, the metal ion must be detoxified by bonds with several molecules containing –SH groups (free histidines, glutathione (GSH), polyamines, metallothioneins (MTs) and phytochelatins (PCs)), removed from cytosol and compartimentalized in the vacuole. For redox active metals, cells must be also equipped of a strong antioxidant machinery, both enzymatic (SODs, catalases, peroxidases) and non enzymatic (GSH, ascorbate).

Metal-tolerant plants have to face high energetic costs that reflect on a reduced biomass and low growth and reproduction rates, if compared to their non-tolerant ancestors. This diminished biomass is an integration of all costs related to survival on metal-enriched soils, indicating an investment of up to 20% (Ernst,

1983) and also including “ecological” costs for adaptation to other environmental constraints of metal-enriched soils, such as the drought stress and nutrient deficiencies.

1.5 *Silene paradoxa* L.

Silene paradoxa L. (Caryophyllaceae) is an erbaceous perennial specie present in a wide distribution area in Southern Europe ranging from the South-East of France to Greece and from the sea level up to 1300 m of altitude in dry regions. The adult plant reaches a maximum height of 30-40 cm with narrow and long leaves and flowering occurs in the hot season going from May to September. Flowers with nocturnal anthesis attract nocturnal moths for the zoophilic pollination (Pignatti, 1976).

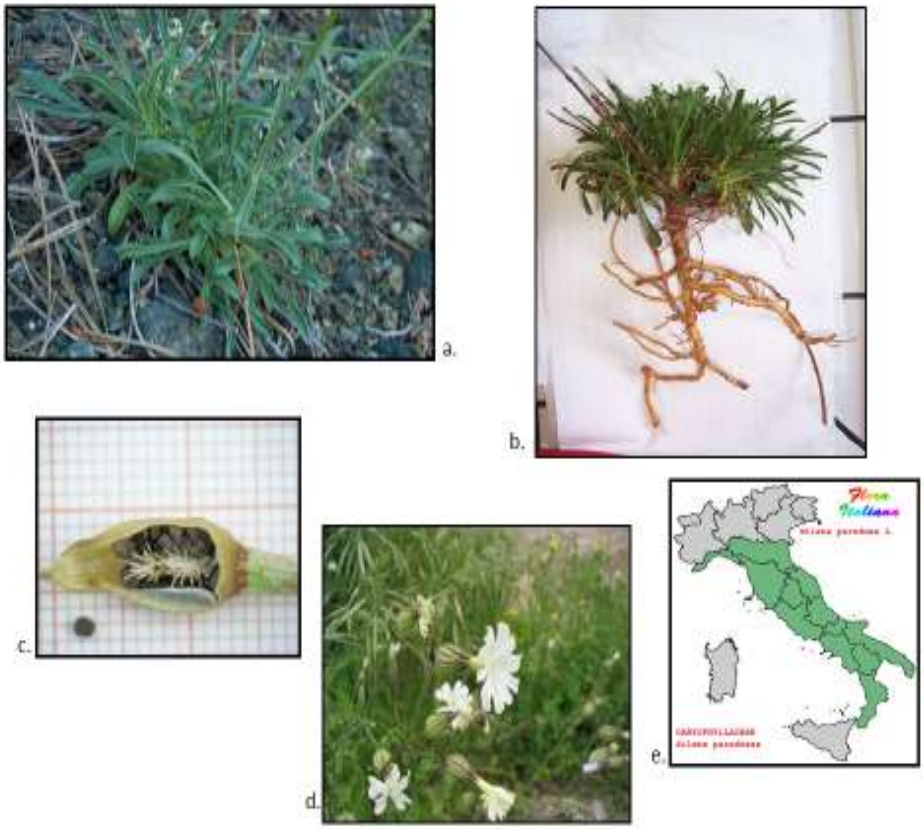

Fig. 1.3. *Silene paradoxa*. a) rosette b) habitus c) seeds inside the calyx d) flowers e) spatial distribution in Italy

Silene paradoxa is an excluder facultative metallophyte as it is commonly located in non-metalliferous dry soils. Anyway, in Italy, several populations are adapted to live in metalliferous soils, as mine deposits or serpentine soils (Chiarucci et al., 1995). This presence of metallicolous and non metallicolous populations makes this specie a suitable model for comparative studies in genetic, ecology and physiology of heavy metal tolerance.

For the present thesis, three Tuscan *Silene paradoxa* populations have been used, two metallicolous and a non-metallicolous one (Fig.1.4).

Fig. 1.4. The three *Silene paradoxa* populations considered in the present work, with the corresponding locations of their sampling sites in Tuscany (Italy).

The Colle Val D'Elsa (Siena) population (CVD) grows on a calcareous non-metalliferous soil.

The Fenice Capanne (Grosseto) population (FC) grows on a polymetallic sulfide deposit that has been mined at least from Etruscan times (7^{th}-6^{th} century B.C., Benvenuti et al., 1999) until 1985. The main exploitation occurred in the Medieval Age and during the 19^{th}-20^{th} centuries. The more abundant metal in the soil is Cu, but also Zn, As, Pb, Fe and Ag are present, though in much lower concentrations (Mascaro et al., 2001). This population has shown to be tolerant to Cu (Gonnelli et al., 2001) but also to other heavy metals, as for example Cd and Zn, suggesting the development of tolerance to these metals in situ (Arnetoli et al., 2008a). Also, this population, if compared to the CVD population, shows to be significantly more tolerant to As, but, interestingly, it uses a mechanism of detoxification not dependent from phytochelatins (Arnetoli et al., 2008b). Colzi et al. (2011) reported a possible exclusion mechanism undergoing Cu tolerance at root level in a decrease of root cell wall pectins and an increase of pectins methylation degree in order to guarantee a low apoplastic Cu accumulation and limit symplastic Cu uptake by the root cells (Colzi et al., 2012).

The Pieve Santo Stefano (Arezzo) population (PSS) grows on a serpentine outcrop, which is a naturally metal-enriched soil. These soils originate from weathering of different ultramafic rocks composed of ferromagnesian silicates, especially serpentinite. These soils are often shallow and rocky, poor in clay minerals and hence not suitable for retaining water; they are generally deficient in plant macronutrients (N, P, K and S), have a Ca/Mg molar ratio less than 1 and reach a high temperature under sunny conditions. They contain toxic metals in high concentrations, such as Ni, Cr and Co. The main toxicity factor in these soils in the high Ni concentration (up to 3600 $\mu g\ g^{-1}$, McGrath, 1995), as Co is present at a lower degree and Cr presents a low bioavailability (Proctor and Woodel, 1975). The abnormal chemical and edaphic composition of serpentine soils promotes the development of tolerant plants called "serpentinophytes" that can

be obligate (endemisms) or facultatives, such as *Silene paradoxa* population that showed to be Ni tolerant (Gonnelli et al., 2001).

From studies on genetic polymorphisms and phylogenetic relationships on these three populations, Mengoni et al. (2001) reported a possible descendence of Cu-tolerant populations from a Ni-tolerant ancestral population coming from serpentine.

1.6 Copper and Nickel

In the present thesis, the experiments were carried out exposing plants to copper and nickel in the culture medium. In order to better understand their role, a brief review on these two metals is presented in the following paragraphs, with a more detailed focus on Cu, since this metal is involved in the majority of the experiments.

1.6.1. Copper: functionality and toxicity

Copper (Cu) is a chemical element which appears in the periodic table among the transition metals at the top of Group 11 above Ag and Au but it constitutes an exception among the transition metals as it possesses a full *d* sub-shell with a single *4s* electron (Cuillel, 2009). In nature, Cu is a relatively unreactive metal, it is very resistant to corrosion and is only slowly oxidized by moist air. Copper exhibits a rich coordination chemistry with complexes known in oxidation states ranging from 0 to IV, although the cupric (II) and the cuprous (I) oxidation states are by far the most common. The cupric oxidation state is more stable than the cuprous because of their redox potentials (Cuillel, 2009), therefore it is predominating.

Copper binds with high affinity to cysteine in proteins and to carboxylic and phenolic groups. In soil solution, up to 98% of Cu is complexed to low molecular weight organic compounds.

The abundance of Cu in the Earth's crust is estimated to be about 70 ppm and that makes it the 8th most abundant element. It is classified as a “trace

element" due to its presence in concentration less than 0,1% in various environmental matrices (Kabata-Pendias, 2001). Small amounts (about 1 ppb) also occur in seawater. It is present as free metal or as ores with oxide minerals such as malachite ($Cu_2CO_3(OH)_2$), cuprite (Cu_2O) and azurite ($Cu_3(CO_3)_2(OH)_2$) and as ores with sulfide minerals such as bornite (Cu_5FeS_4) chalcopyrite ($CuFeS_2$) and chalcocite (Cu_2S) (Enghag, 2004). Copper is widespread in the environment. Natural Cu ores are mainly distributed in North America, Latin America and Central Africa. About 310.000 tons were released into the environment by industries in 2014 (ICSG, 2014) and it is often found near mines, smelters, industrial settings, landfills, and waste disposal sites (ATSDR, 2002). It is known to be a broad spectrum pesticide and it has been used to produce several compounds for plant diseases prevention. The "Bordeaux mixture" ($CuSO_4 \cdot 3Cu(OH)_2 \cdot 3CaSO_4$) was the first fungicide to be intensively used in Europe for the control of grapevine downy mildew caused by *Plasmopara viticola*. Also other copper based compounds such as $3Cu(OH)_2 \cdot CuCl_2$ or $Cu(OH)_2$ are used with satisfying results.

This element was proven to be a micronutrient for plants in 1931 (Schulte and Kelling, 2004). Plants need Cu for their normal growth and development (Yruela, 2005). Due to its multiple oxidation states *in vivo*, Cu acts as a structural element and prosthetic group in regulatory proteins and participates in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism and lignification and hormone signalling (Marschner, 1995; Raven et al., 1999; Yruela, 2005). The redox active property of Cu is used by cuproenzymes involved in redox reactions (Stern, 2010; ATSDR, 2002) and electron transport.

Copper is found in:

- Cu/Zn superoxide dismutase (SOD) that is located in chloroplasts (together with the FeSOD), cytosol and peroxisomes.

- cytochrome c oxidase, located in the mitochondrial membrane, where it participates to electron transport chain of the respiration process.

- other enzymes such as ascorbate oxidase (present in cell walls and cytoplasm), laccases (present in the thylakoid membranes of the chloroplasts for the synthesis of plastoquinone), diamine oxidases (located in the epidermis and xylem of mature tissues where it functions as an H_2O_2 –delivery system for peroxidase activity in the process of lignification and suberization) , polyphenol oxidases (involved in lignin biosynthesis) and molybdopterins (nitrate reductase, sulphite oxidase, xanthine dehydrogenase and aldehyde oxidase).

- plastocyanin, a protein located in the lumen of the thylakoid where it functions as a mobile electron carrier shuttling electrons from cytochrome c to P700 in Photosystem I (Gross, 1993)

- ethylene receptor proteins (ETR), located in the endoplasmic reticulum membrane that act as negative regulators of the ethylene response pathway (Chen et al., 2002).

Most plants present a critical deficiency level of Cu in the range of 1-5 μg/g, an optimal range from 6-12 μg/g and toxicity above 20-30 μg/g DW in vegetative tissues (Marschner, 1995).

Excess Cu in the growing medium can restrict root growth by burning the root tips and thereby causing excess lateral root growth. High levels of Cu can compete with plant uptake of Fe and sometimes Mo or Zn. The new leaves can become initially greener than normal, then exhibit symptoms of Fe deficiency or possibly other micronutrient deficiencies. If not corrected, Cu toxicity can reduce branching and eventually plant decline follows.

At the cellular level, excess Cu interferes with fundamental processes as photosynthesis and respiration. Cu alters chloroplasts structure by lowering pigments content (Baszinksy et al., 1988; Lidon and Henriques, 1991; Ciscato et al., 1997) and thylakoid membrane compositions by inducing lipid peroxidation (Sandmann and Boger, 1980). Cu was found to interfere with electron transport

mainly at the photosystem II (PSII) level (Droppa and Hòrvath, 1990) and the most sensitive sides for Cu toxicity were found to be the Q_B binding site (Mohanty et al., 1989) and the Pheo-Fe-Q_A domain (Yruela et al., 1993, 1996).

It was also reported an increase in the activity of the antioxidant responses both in roots and shoots in a Cu and time dependent trend (dehydroascorbate reductase (DHAR), glutathione reductase (GR), superoxide dismutases (SODs), guiacol peroxidase) (De Vos et al., 1992; Luna et al., 1994; Navari-Izzo et al., 1998) due to Cu induced release of ROS. Also the ascorbate-glutathione cycle was reported to be involved in response to Cu excess (Gupta et al., 1999).

The Cu content of soils ranges from 2 to 100 μg/g with an average value of about 30 μg/g. Most of this is in a unavailable mineral form. The bioavailability of Cu in soils is held mainly by the cupric (II) form and it depends mainly on soil pH and organic matter. Copper bioavailability decreases in organic soils, as organic matter and clay minerals bind copper more than any other micronutrient.Therefore the lowest concentrations are mainly reported on sandy soils which are low in organic matter. Cu uptake decreases as soil pH increases. Increased P, N and Fe availability in soils also decrease Cu uptake by plants.

High Cu in the soil has triggered the evolution of highly specialized plant species, called cuprophytes (Brooks et al.,1982; Baker et al., 2000) which are globally rare and more the 95% of species capable of accumulating foliar Cu and/or Co up to 300 μg g^{-1} d.w. (van der Ent et al., 2013) are concentrated in the Katangan Copperbelt (D.R Congo) (Brooks et al., 1980). Interestingly, all of the putative Cu/Co hyperaccumulators accumulate these metals in a dose-dependent way, in contrast with the fundamental principle of hypeaccumulation (Lange et al., 2016). Examples of African cuprophytes are the excluder *Haumaniastrum katangense* (used as a biogeochemical indicator of Cu ore) (Chipeng et al., 2010) and the hyperaccumulator *Crepidorhopalon perennis* (Faucon et al., 2012). Since

Cu is a natural pesticide, its presence in soils may limit the development of pathogens and therefore metallophytes may have lost their pathogen-resistence mechanisms, being more susceptible to pathogen attacks (Chipeng et al., 2010; Faucon et al., 2012).

1.6.2. Nickel: functionality and toxicity

Nickel (Ni) is a metal that appears in the periodic table of the elements in the 4th period and in the group of Fe and it's the 22nd most abundant element in the Earth's crust where it occurs in igneous rocks as a free metal or together with Fe (Nielson, 1987; Sunderman and Oskarsson, 1991).

This element is a micronutrient required by the majority of plants in very low concentrations (0,05-10 μg g^{-1} d.w.) (Brown et al., 1987). Ni (II) has been identified as a cofactor of many plant metallo-enzymes such as urease (Polacco, 1977), a few SODs, NiFe hydrogenases, methyl coenzyme M reductase, CO dehydrogenase, acetyl coenzyme-A synthase, hydrogenases, RNase-A (Ermler, 1998; Kupper et al., 2007) and glyoxalases (family I) (Fabiano et al., 2015). Its bioavailabilty for plants decreases with the increase of soil pH (Kabata Pendias and Pendias, 1992). Over 50% of Ni absorbed by plants is retained in the roots (Cataldo et al., 1978), 80% of which is present in the vascular cylinder, suggesting a high mobility of this metal in the xylem and phloem (Riesen et al., 2005).

Ni exerts phytotoxic effects when the concentration in the nutrient medium is > 10 μg g^{-1} d.w. for sensitive species, >50 μg g^{-1} d.w. for moderately tolerant species and > 1000 μg g^{-1} d.w. for Ni hyperaccumulators such as *Alyssum* and *Noccaea* species (Chen et al., 2009). Ni causes toxicity through two indirect mechanisms: competition with other essential metal ions (Ca, Cu, Fe, Mg, Mn and Zn) causing inhibition of adsorption and lead to their deficiency in plants (van Assche and Clijsters, 1990; Gabbrielli et al., 1990) and induction of oxidative stress by reducing the activity of many cellular antioxidant enzymes and plant's capability to scavenge ROS (Gajewska and Sklodowska, 2007).

Excess Ni impairs growth and germination and causes leaf chlorosis and necrosis. At the photosynthetic level, it affects light-harvesting complex II and inhibits the electron transport at the level of PSII (Chen et al., 2009).

1.7 Overview of this thesis

The main objectives of this thesis were to evaluate how the adaptation to metalliferous soils influences the response to different environmental stresses, both abiotic (heavy metals and nutrient deficiency) and biotic (fungal pathogens), in metallicolous and non metallicolous *Silene paradoxa* populations.

The exposition of these populations to heavy metals during the growing phase (Cu in all experiments, Ni in two experiments) was the factor that interconnected all the studies reported in the present thesis. More specifically, the three objectives were:

1. Evaluation of the effect of Cu excess at root level (micromorphological changes and cell wall composition- Chapter 2) and at shoot level (changes in photosynthetic activity- Chapter 3) in CVD and FC populations.

2. Analysis of growth, nutrient allocation and nutrient use efficiency in CVD, FC and PSS populations growing in nutrient deficiency conditions, with and without heavy metals (Cu and Ni) in the nutrient medium (Chapter 4)

3. Quantification of defense responses productions in CVD, FC and PSS populations exposed to heavy metals (Cu and Ni) and to a biotic stress (Chapter 5) and evaluation of whether ROS are used as signalling molecules to produce defense responses against pathogens in CVD and FC populations exposed to Cu and to biotic stress (Chapter 6).

CHAPTER 2

Linking root traits to copper exclusion mechanisms in *Silene paradoxa* L. (Caryophyllaceae)

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Abstract

Copper is one of the most important pollutants in mine-contaminated soils. This study tests the response in a sensitive population vs a tolerant one of the model specie *Silene paradoxa* in order to understand the general mechanisms of tolerance at the micromorphological and ultrastructural level. Two populations of *Silene paradoxa* were grown in hydroponics and exposed to different $CuSO_4$ treatments. The roots were investigated with light, fluorescence and transmission electron microscope. Callose and lignin were spectrophotometrically determined. The tolerant population constitutively possessed a higher amount of mucilage and was able to reduce the length of the zone between the apex and the first lignified tracheids. Callose production decreased. It did not show remarkable Cu-induced ultrastructural modifications, apart from the presence of precipitates in the tangential walls. The sensitive population showed huge nucleoli with a spongy periphery in the central cylinder together with the presence of electrondense granules in the mitochondria. Plastids were rarely observed and generally very electrondense and elongated. In the Cu-tolerant population of *S. paradoxa* some of the root traits concurring to generate metal-excluding roots were suggested to be mucilage and lignin production and the reduction of the subapical root zone.

2.1 Introduction

Because of their sessile lifestyle, plants can only slightly escape with their roots from adverse environmental conditions and have the only option of modifying their anatomical structures and their physiological processes to the characteristics of the environment in which they have germinated. Among the extreme habitats that plants are challenged to colonize, metal-enriched soils are world wide spread, due to both their geogenic and anthropogenic origin. Some plants show the surprising ability to adapt to metal loaded soils under the selective pressure exerted by such unfavorable substrates, where fitness takes benefit from the acquired, even though metabolically costly, tolerance mechanisms achieved through a new regulation of element homeostasis at all the plants levels (Baker, 1987; Ernst, 2006). The majority of these plants, called metallophytes, are able to tolerate specific metals in the substrate by physiologically restricting their entry into the root and/or their transport to the shoot (termed "excluders" by Baker, 1981). However, a few species, the so-called metal "hyperaccumulators" (Brooks et al., 1977), have extremely specialized biological mechanisms to hyperaccumulate metals in their shoots at extraordinarily high concentrations.

As roots are the first plant organ to be exposed to excessively high amounts of elements in the soil solution, during the process of adaptation to metalliferous soils readjustments of root growth, development and function can be critical to plant survival. Then, according to the tolerance strategies chosen, roots of metallophytes specialized to reduce metal uptake and translocation or to enhance them, thus generating the excluder or the hyperaccumulator phenotype. Of course, a concomitant evolution of the shoot was necessary, in the latter case with the realization of extremely high levels of metal tolerance at the cellular level.

Most of the studies pertaining to metal induced changes in root development and morphology generally regard metal non-adapted plants and, particularly, cadmium. For example, this element has been found to provoke the formation of

Casparian bands and suberin lamellae closer to the root apex in *A. thaliana* (Schreiber et al. 1999), *Karwinskia humboldtiana* (Zelko and Lux, 2004), and maize (Vaculík et al., 2009), thus reducing the apoplasmic movement of the ion to the xylem and its translocation to the shoot (Lux et al., 2011). Martinka and Lux (2004) found the same Cd-induced response also in different populations of *Silene dioica*, one of the few metallophytes investigated, and demonstrated that plants from a metal contaminated soils developed Casparian bands closer to the root apex than plants from non-contaminated soils. Regarding Cu, generally there are only papers reporting Cu excess induced root malformations and ruptures (Panou-Filotheou and Bosabalidis, 2004) and no adaptative response has been identified yet. When Cu-tolerant plants are taken into account, literature is mainly inexistent and very few reports can be found about Cu-induced morphological changes concurring to Cu-tolerance mechanisms (Llugany et al., 2003) that related abnormal root branching pattern, slough-off of root cap cells and mucilage production, number of border cells, and swelling of the subapical root zone to Cu-tolerance and exclusion mechanisms in metallicolous populations of *Silene armeria*). Also at the ultrastructure level, there are generic reports on Cu-induced plasmolysis, disintegration of cell membranes and non-recognition of any organelles in root cells of Cu-exposed plants (Panou-Filotheou and Bosabalidis, 2004), without any comparative study on metallophyte populations for the individuation of adaptative responses.

Among the plant polymers involved in the formation of root apoplastic barriers against ion movement, callose and lignin are the most studied and in literature there are reports about their quantitative determinations under metal stress. Callose (1,3-β-glucan), synthesized in response to various biotic and abiotic stresses, is known to be impermeable to metal ions, therefore in the apoplast metals can not only be bound by the cell wall compounds, such as pectins, but also be blocked in their migration by the callose layer and then limited in their uptake (Krzesłowska, 2011). Furthermore, callose deposition was found

to protect the cell from cell wall sequestered metal returning into the protoplast in the case of lead (Krzesłowska et al., 2010). As for Al, results are still contrasting and the appearance of callose in Al-exposed plants is not regarded as a tolerance mechanism, but rather as a symptom of its sensitivity (Krzesłowska 2011). Furthermore, callose production can be variably associated with accumulation of Al in the roots. Smith (2011) reported that elevated callose production was negatively correlated with apoplastic Al accumulation and positively correlated with symplastic Al accumulation in poplar genotypes, whereas Horst et al. (1997) did not find any correlation in maize. Heavy metal induced deposition of callose is reported to occur in plasmodesmata as well (Sivaguru et al., 2000) inhibiting cell-to-cell transport of toxic metal ions, but simultaneously also of other molecules (Krzesłowska 2011). Regarding lignin, its deposition under heavy metal stress was demonstrated to be localized in the middle part of the root, probably at the endodermis, as shown upon Cd excess by van de Mortel et al. (2006), or at the xylem cell walls. This response is thought to limit the efflux of metals from the vascular cylinder into the shoot (van de Mortel et al., 2008). In addition, as suggested by Sasaki et al. (1996), lignification could also limit cell growth, reducing cell wall plasticity and cell elongation, and is reported to predominantly happen in sensitive species, such as in the study on Al by Tahara et al. (2005). Exposure to high Cu is known to induce lignin deposition in roots in several plant species (Lequeux et al., 2010).

Even if root morphology is such an important trait related to metal uptake, yet there are very few reports in the literature (Llugany et al., 2003) that compare root traits between metallicolous and non metallicolous populations and investigate how they can affect metal exclusion. Recently, we investigated the role of the cell wall in the phenomenon of naturally- evolved Cu-tolerance studying metallicolous and non-metallicolous populations of *Silene paradoxa* L. (Colzi et al. 2011; 2012). In the Cu-tolerant population the generation of metal-excluding root cell walls with a low metal binding capacity was suggested to be

one of the factors concurring to guarantee a low apoplastic Cu accumulation and probably also to limit symplastic Cu uptake by the root cells. To decrease the amount of metal bound to the root cell wall, in the tolerant population Cu treatments was shown to decrease root cell wall pectin concentration and increase pectin methylation degree (Colzi et al., 2011; 2012).

In this study we shed new light on the link between root traits and the phenomenon of Cu exclusion, comparing a Cu-tolerant and a Cu-sensitive population of *S. paradoxa* and investigating Cu-induced changes in root structure and in callose and lignin deposition.

2.2 Materials and methods

2.2.1 Plant material and experimental conditions

Seeds of *S. paradoxa* L. were collected at the Fenice Capanne (FC) mine waste and at Colle Val D'Elsa (CVD) uncontaminated soil (Arnetoli, 2004; 2008; Pignattelli et al., 2012). Plants were cultivated in the same experimental conditions as in Colzi et al. (2012), where the different effect of Cu treatment on plant growth and element accumulation was already assessed. In such previous studies the mine population resulted clearly more Cu-tolerant and able to limit Cu accumulation in its tissues. Seeds were sown in peat soil and after six weeks seedlings of both populations were transferred to hydroponic culture, in 1-L polyethylene pots (3 plants per pot) containing a modified half-strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.50 mM $MgSO_4$, 20 μM Fe(Na)-EDTA, 1 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.1 μM $CuSO_4$ and 0.1 μM $(NH_4)_6Mo_7O_{24}$ 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 a series of $CuSO_4$ concentrations (0, 5 and 10 μM). Nutrient solutions were renewed weekly and plants were grown in a growth

chamber (24/16 °C day/night; light intensity 75 µE m^{-2} s^{-1}, 12 h d^{-1}; relative humidity 60–65 %).

2.2.2. Preparation for light microscopy

Samples of roots were fixed in formaldehyde—acetic acid – ethanol (FAA). Some samples were cut with a razor blade to observe the distance of the beginning of lignification from the root apex. Phloroglucinol–HCl staining (Jensen 1962) was employed to mark the lignified cells in the root central cylinder. Some samples were dehydrated and embedded in Technovit resin 7100 (Kulzer, Weinheim, German), cut on glass knife and mounted on slides. The samples were stained with toluidine blue (C.I. 52040, Sigma) and PAS-acriflavine (Bruni and Vannini, 1973) to investigate the presence of anionic substances (pectins) and observed with a light Leica microscope DM RB Fluo with UV light, bright field and polarized light.

The distance from the apex of the first lignified tracheid, the number of cells and the mean cell length of such region were manually measured directly observing slides from 12 independent replicates from each sample.

2.2.3 Preparation for transmission electron microscopy

Samples of roots were collected and fixed for 24 h in 1.25 % glutaraldehyde at 4 ° C in 0.1 M phosphate buffer (pH 6.8), then post-fixed in 1 % OsO_4 in the same buffer for 1 hr. After dehydration in an ethanol series and a propylene oxide step, the samples were embedded in Spurr's epoxy resin (Spurr 1969). Transverse sections approximately 80 nm thick were cut with a diamond knife and a Reichert-Jung ULTRACUT E ultramicrotome (about five to ten sections per thicker transverse section), stained with uranyl acetate and lead citrate, then examined with a Philips EM300 TEM at 80 kV. About five Cryostat sections 20 nm thick (Cryo- Cut American Optical Corporation) were stained with Lugol's

solution and observed with a Leica microscope DM RB Fluo to detect carbohydrates. A few cryostat sections were stained with 0.05% (w/v) toluidine blue in a 0.05M benzoate buffer (pH 4.4) to depict general orientation. TEM analysis was done with a PHILIPS CM 12 TEM.

2.2.4. Callose and lignin determination

Callose extraction and quantification was performed according to Smith et al. (2011), using curdlan (Sigma) as standard and expressing callose concentration as μg curdlan equivalent (CE) g-1 FW. Regarding lignin determination, the followed method was the one of Brinkmann et al. (2002) using commercial lignin (Aldrich) as reference standard. Lignin concentration was expressed as mg/g DW. Measurements were performed on an UV/Vis spectrophotometer (Lamba 35, Perkin-Elmer).

Measurements were performed on 12 plants per population per Cu concentration.

2.3 Results

2.3.1 Light microscope observations

In control conditions, in sections at less than 1 mm from the root apex, the amount of toluidine blue positive material outside the root cap and rhizodermis (mucilage) was more abundant in the FC plants (Fig. 2.1a) with respect to the CVD plants (Fig. 2.1b). Apart from that, regarding the general organization of root apex, the two populations did not show striking differences: at 1–2 mm from the apex the central cylinder began to differentiate with respect to the cortical cylinder, with larger cells forming the latter in the FC population (Fig. 2.1c). Root hairs were present in both populations and some cells of the central cylinder appeared thickened and lignified (Fig. 2.1d). At 4 mm from the root apex, endodermis Casparian bands appeared formed in both FC and CVD plants (Fig. 2.1e and f). In the FC samples the walls of the cortical cylinder cells appeared

more toluidine blue positive (Fig. 2.1e), than those of the CVD samples (Fig.2.1f), probably also for the wall thickness, about 4–5 μm with respect to 1–2 μ of wall thickness in the CVD. In FC plants grown at 5 μM $CuSO_4$ at less than 1 mm from the apex (Fig. 22a), the cell cytoplasm of the cortical cylinder cells was quite toluidine blue positive. In the CVD plants the cortical cells presented a weak positive reaction to toluidine blue and large vacuoles (Fig. 22b). In the FC population, at 2 mm from the apex, some lignified cells at the level of the cortical cylinder was already present (Fig. 2.2c). In the CVD population no lignification was detectable at the same level and position (Fig. 2.2d). As for plants grown at 10 μM CuSO4, at the level of the root apex (less than 1 mm from the root cap) in the FC population (Fig. 2.3a), the presence of mucilage appeared higher with respect to the CVD population (Fig. 2.3b). At 4 mm from the apex the tracheids appeared already formed also in the center of the central cylinder in the FC plants (Fig. 2.3c), while in the CVD plants they were limited to the periphery of the central cylinder (Fig. 2.3d). To better distinguish the walls with a prevalent pectin concentration from those that were already, at least partially, lignified, we used a PAS-acriflavine staining. The tracheids appeared to be formed and lignified already at 1–2 mm from the apex (Fig. 2.3e) in the FC population at 10 μM, while in the CVD some tracheids appeared to form in the central cylinder only at 4 mm from the apex (Fig.2.3f).

2.3.2 Beginning of tracheid formation in the root

The distance from the root apex at which the procambium cells began their lignification to tracheids is reported in Table 1. In the CVD population lignification started at about 0.7 mm from the apex in control conditions and that value significantly increased in the presence of Cu in the culture medium. In the FC population such distance from the apex was not different from that of the CVD population in control conditions, but it decreased as the Cu concentrations in the culture medium increased, significantly for the highest treatment. In the CVD population the number of cells in such a region significantly increased with increasing copper concentration in the culture

medium (Table 2.1), while in the FC on it followed a slight decrease, significant only at the highest Cu concentration used. Comparing the populations, in control condition the FC population showed a significantly higher number of cells of the subapical region and a significantly lower one at the highest Cu concentration used in respect to the CVD population. Regarding the mean length of those cells (Table 2.1), CVD plants showed a Cu-induced significant increase in such parameter, while in FC plants it did not change. At the highest Cu concentration used, the CVD population

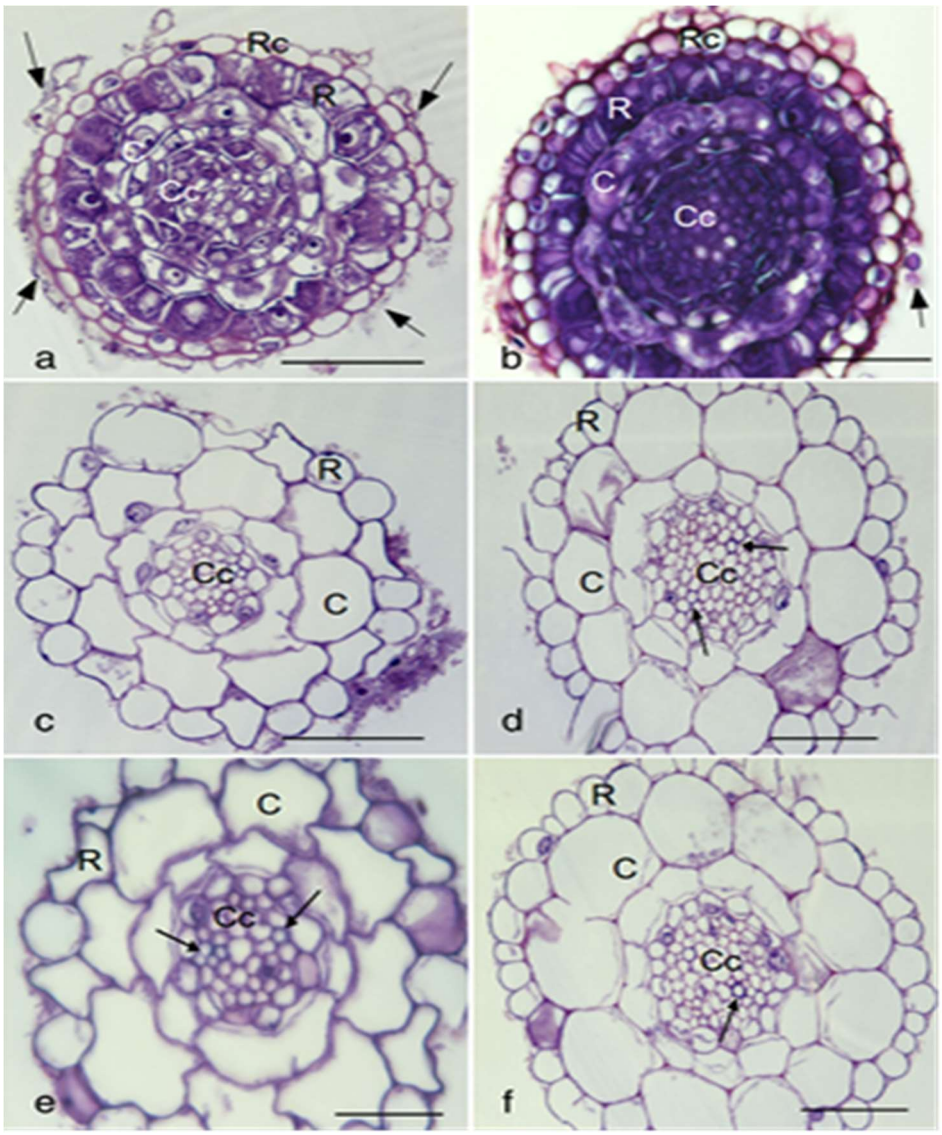

Fig. 2.1 Light microscope. a and b: root, cross section, control, close (less than 1 mm) to the root apex. a - FC: Abundant mucilage is present outside the root cap (arrows). Bar=50 µm. b - CVD: A low amount of mucilage is present outside the root cap (arrow). Bar=50 µm. c and d: root, cross section, control, 2 mm from the root apex. c - FC: he central cylinder (arrows) begins to differentiate. Bar=50 µm. d – CVD: Some cells (arrows) of the central cylinder appear thickened and lignified (arrows). Bar=50 µm. e and f: root, cross section, control, 4 mm from the root apex. e - FC: the walls appear quite toluidine positive. Endodermis Casparian bands are visible. Two cells in the central cylinder are lignifying their walls (arrows). Bar=50 µm. f - CVD: endodermis Casparian bands formed. A cell in the central cylinder is lignifying its wall (arrow). Bar=50 µm. Key to labelling: C cortex, Cc central cylinder, D dictyosome, M mitochondrion, N nucleus, P plastid, R rhizodermis, Rc root cap, V vacuole, W wall

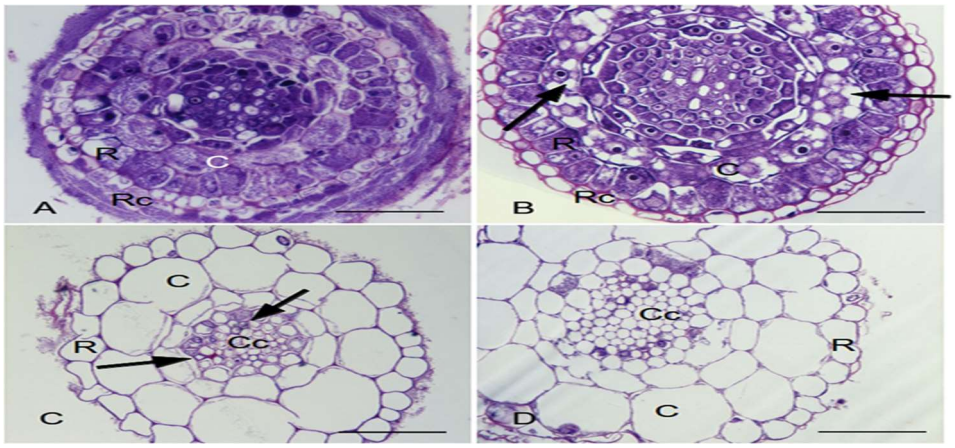

Fig 2.2 Light microscope, a and b: root, cross section, 5 µM Cu. Less than 1 mm from the root apex. a – FC: Bar=50 µm. b - CVD: the first cortical layer underneath the rhizodermis contains large vacuoles (arrows). Bar=50 µm. c and d: root, cross section, 5 µM Cu. 2 mm from the root apex. c - FC; some lignified cell is already detectable. Bar=50 µm. d- CVD: the lignified cells are not detectable. Bar=50 µm

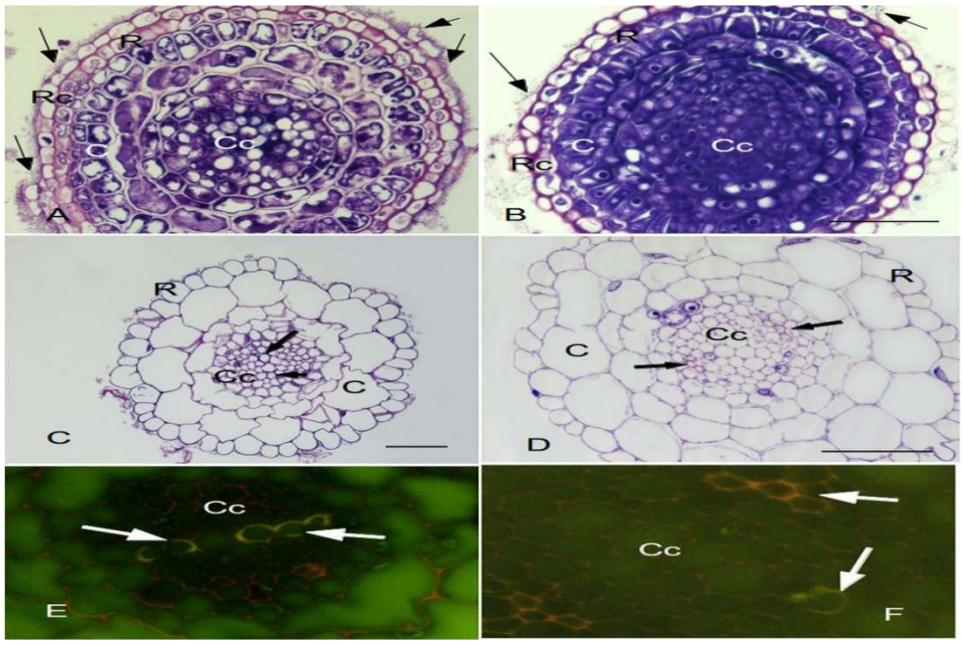

Fig. 2.3 Light microscope. a and b: root, less than 1 mm from the apex, cross section, 10 µM Cu. a - FC: the production of mucilage is quite abundant (arrows). Bar=50 µM. B - CVD: the production of mucilage is lower (arrow) than in the FC. Bar=50 µM. c and d: root, 4 mm from the apex, cross section, 10 µM Cu. c - FC: some tracheids (arrows) are formed also towards the center of central cylinder. Bar=50 µm. d: some tracheids (arrows) are formed at the periphery of central cylinder Bar=50 µm. e and f: root, 1–2 mm from the root apex, cross section, 10 µM Cu. e – FC: acriflavin-PAS staining. The tracheids are differentiated (arrows). Bar=25 µm. f - CVD: acriflavin-PAS staining. Some tracheids are formed at the periphery of the central cylinder (arrow). Bar=25 µm

showed a significantly higher mean length of the cells in the region between the apex and the first lignified tracheid in respect to the FC population.

2.3.3 Callose and lignin production

Callose and lignin concentrations after copper exposure are shown in Table 2.2. Results indicated that Cu treatments gave an inverse trend for the two populations. In CVD plants a significant increase was observed, significantly at the highest Cu concentration, while in the FC population a significant decrease of callose concentrations was found at both the Cu concentrations in respect to control. Significant differences were found between the two populations in callose concentrations, as they were higher in FC population in respect to CVD one in control conditions and lower at the highest concentration used. As for lignin concentration, a significant difference was observed in CVD population exposed to 10 μM Cu, where it was significantly lower as compared to the control and to 5 μM Cu. In the FC population the opposite situation was found and the lignin concentration was higher at that concentration in respect to the others. Comparing the two populations, the lignin concentrations were lower in FC plants than in CVD plants in control conditions and at 5 μM Cu and higher at 10 μM Cu.

2.3.4. Transmission electron microscope (TEM) observations

In control plants, in both the CVD and FC apexes, at about 1–2 mm from the root cap, the parenchyma cells underneath the rhizodermis had quite sinuous radial wall, cytoplasm in peripheral position and open plasmodesmata (Fig. 2.4a for the FC population, CVD, with similar results, not shown). The more internal cortical cylinder cells showed a thick wall, 2–4 large vacuoles containing electrondense material, mitochondria with quite dilated cristae and electron dense plastids (Fig. 2.4b). The cells of the more internal central cylinder appeared more

elongated, with much thinner walls than the cortical layer, less cytoplasm occupied by vacuoles, a large nucleus with a huge nucleolus

Table 2.1 Distance from the apex of the first lignified tracheid, number of cells and mean cell length of such region in two populations of *Silene paradoxa* after exposure to increasing $CuSO_4$ concentrations. Values are means of 12 replicates ± standard error, significant differences (at least $P<0.05$) between the means appear with different letters, capital for the inter-population and small for the intra-population ones

	CVD			FC		
	Control	5 μM $CuSO_4$	10 μM $CuSO_4$	Control	5 μM $CuSO_4$	10 μM $CuSO_4$
Distance mm	0.68±0.11 aA	1.93±0.19 bB	3.51±0.60 cB	0.91±0.05 bA	0.82±0.06 abA	0.74±0.04 aA
Number of cells	4.2±0.3 aA	9.5±1.2 bA	13.5±1.4 cB	7.5±0.7 aB	6.7±0.9 abB	5.8±0.4 bA
Mean cell dimension mm	0.16±0.03 aA	0.24±0.08 abA	0.27±0.04 bB	0.13±0.03 aA	0.13±0.04 aA	0.14±0.04 aA

Table 2.2 Callose and lignin concentration in two populations of *Silene paradoxa* after exposure to increasing $CuSO_4$ concentrations. Values are means of 12 replicates ± standard error, significant differences (at least $P<0.05$) between the means appear with different letters, capital for the inter-population and small for the intra-population ones

	CVD			FC		
	Control	5 μM $CuSO_4$	10 μM $CuSO_4$	Control	5 μM $CuSO_4$	10 μM $CuSO_4$
Callose μg g^{-1} FW	0.26±0.03 aA	0.33±0.06 abA	0.40±0.06 bB	0.48±0.05 cB	0.35±0.02 bA	0.12±0.01 aA
Lignin mg g^{-1} DW	14.6±0.8 bB	14.4±0.6 bB	9.9±0.7 aA	10.2±0.6 aA	10.8±0.6 aA	14.2±0.8 bB

mitochondria had almost the same appearance as those of the cortical layer, while the plastids appeared less electrondense (Fig.2.4c). At about 1 to 2 mm from the apex, some of the cells of the central cylinder appear lignified (Fig. 2.4d). In CVD plants grown at 5 μM $CuSO_4$, the cells showed features intermediate between the control and 10 μM $CuSO_4$ (data not shown). In this latter case (10 μM $CuSO_4$), the walls of the rhizodermal cells appeared to be thickened with a translucent material (Fig. 2.5a). In the same cells the vacuoles appeared large and containing some electrondense material, while apparently the plastids were spherical in shape and containing electrontransparent globules that were observed also in the cytoplasm (Fig.2.5b). The plasma membrane of the parenchyma cells underneath the rhizodermis appeared to be very irregular in shape showing frequent invaginations (Fig.2.5a). The most striking feature of the parenchyma cells of the cortex and of the central cylinder (respectively Fig. 2.5c and d) was the huge nucleolus within the nucleus. Such nucleoli had a peripheral spongy crown surrounding a more homogenously electrondense central disk. Some mitochondria had a quite electrontrasparent matrix, which contained few cristae and electrondense granules, while other mitochondria were more electrontransparent with a rarefied area in the middle of the matrix (Fig. 2.6a). The plastids were rare, very electrondense and with a twisted shape (Fig. 2.6b). In all the treated samples from the FC population, the most evident feature was the accumulation of granular electrondense material, mainly in the internal tangential wall of the rhizodermal cells (Fig. 2.6c). The parenchyma underlying the rhizodermis had an aspect similar to the control, with mitochondria showing dilated cristae (Fig. 2.6d). In the central cylinder the cells were more elongated and had large vacuoles containing decomposing organelles. Some mitochondria were present and apparently similar to the control, while plastids were very rare (Fig.2.6e). Some cells of the central cylinder were beginning the lignification process (Fig.2.6f).

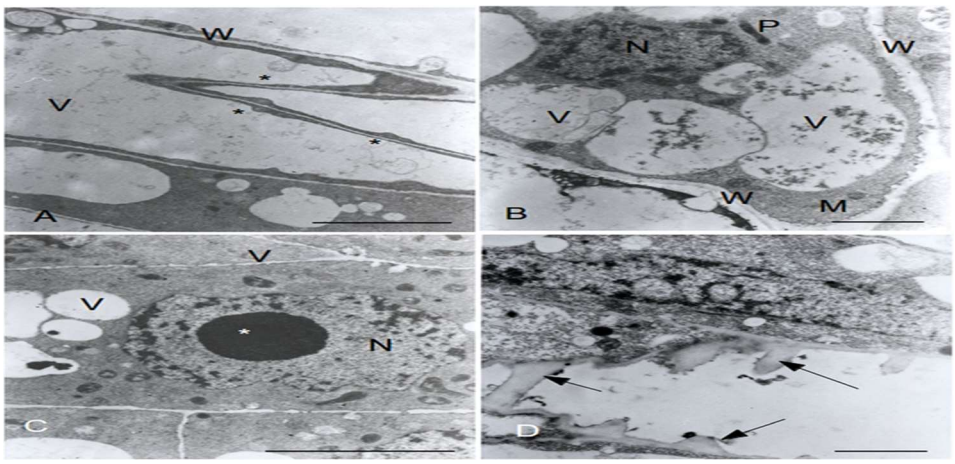

Fig.2.4 TEM. FC root under control treatment. Longitudinal sections. 1–2 mm from the root apex. **a** - The parenchyma cells underneath the rhizodermis show sinuous radial walls (asterisks) and cytoplasm in peripheral position. Bar=5 µm. **b** - The more internal cortical cylinder cells show thick walls, 2–4 large vacuoles containing electrondense material, mitochondria with quite dilated cristae and electron dense plastids. Bar=2 µm. **c** - The cells of the more internal central cylinder appear elongated, with thin walls, small vacuoles and a large nucleus with a huge nucleolus (asterisk). Mitochondria have almost the same appearance as those of the cortical layer, while the plastids appear less electrondense. Bar=2 µm. **d** - Lignification (arrows) of tracheids in the central cylinder. Bar=2 µm

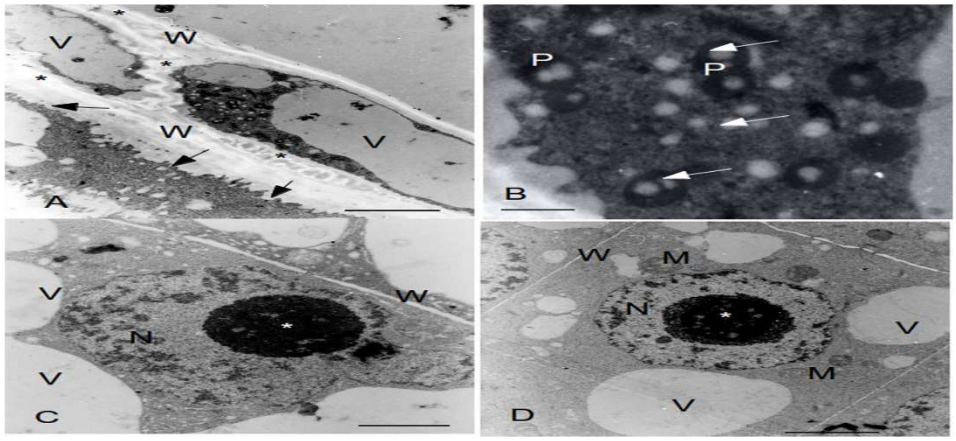

Fig.2.5 TEM. CVD root, 10 µM copper. Longitudinal sections. 1– 2 mm from the root apex. **a** - The walls of the root cap cells appear thickened with a translucent material (asterisks). Vacuoles appear large and containing some electrondense material, while the plastids are spherical and contain electron transparent globules. The plasmamembrane of the parenchyma cells underneath the rhizodermis appears very irregular (arrows). Bar=3 µm. **b** – Detail of Fig. 5a: electrontransparent globules (arrows) can be observed in the plastids and in the cytoplasm. Bar=0.5 µm. **c** – Parenchyma cells of the cortex. A huge nucleolus (asterisk) is visible within the nucleus. The nucleolus exhibits a peripheral spongy crown surrounding a more homogenously electrondense central disk. Bar=2 µm. **d** - Parenchyma cells of the central cylinder. The nucleolus (asterisk) is similar to that of the cortical layer. Some mitochondria are of medium electrondensity and have few cristae. Bar=2 µm

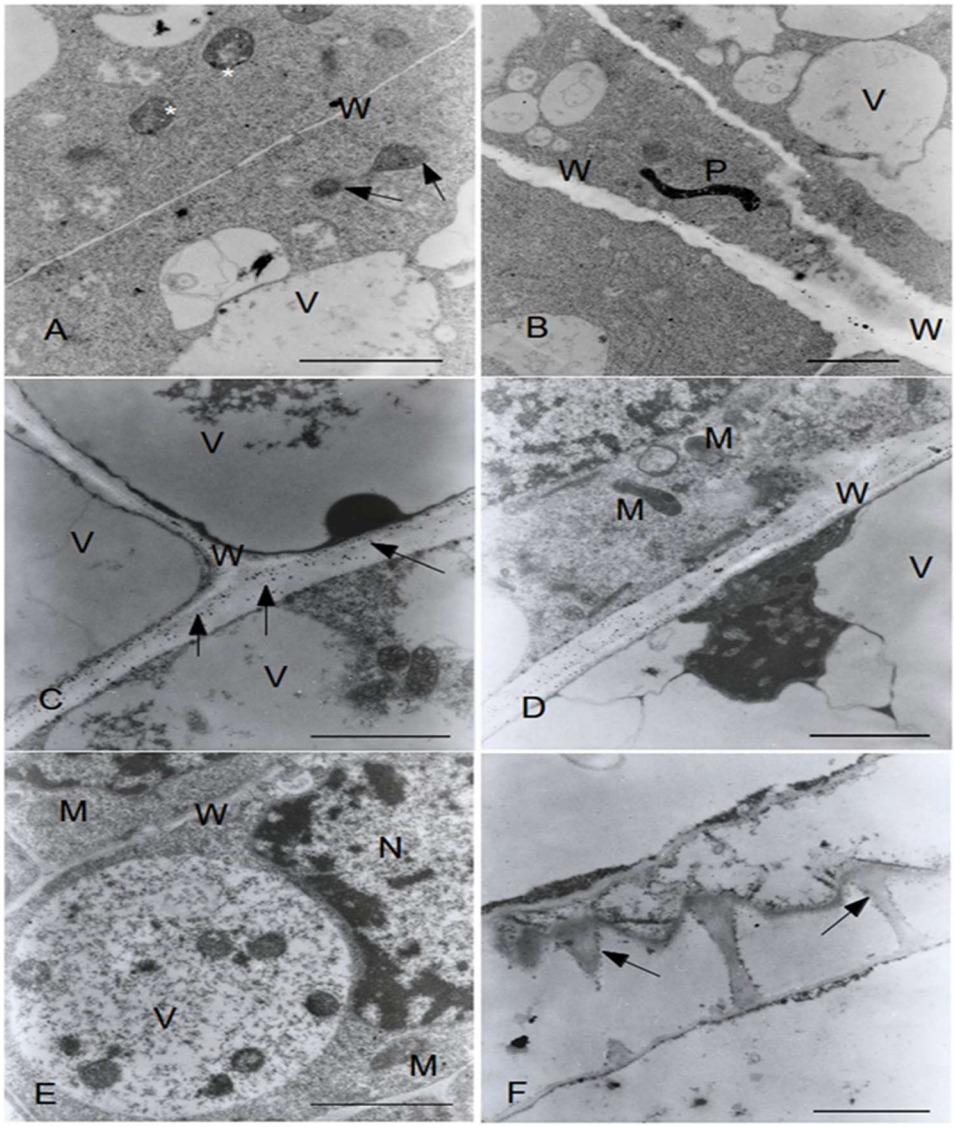

Fig.2.6 TEM. Longitudinal sections, root, 10 µM Cu. 1– 2 mm from the root apex. **a** – CVD: some mitochondria (arrows) have a quite electrontrasparent aspect, with few cristae and electrondense granules in the matrix, while other appear very electrontransparent showing a rarefied matrix (white asterisks). Bar=2 µm. **b** – CVD: the plastids are rare, very electrondense and with a twisted shape. Bar=2 µm. **c** – FC: the accumulation of granular electrondense material (arrows), in the internal tangential wall of the rhizodermal cells is shown. Bar=2 µm. **d** – FC: the parenchyma underlying the rhizodermis had an aspect similar to the control, with mitochondria showing dilated cristae. Bar=5 µm. **e** – FC: central cylinder parenchyma. The cells are elongated and show large vacuoles containing decomposing organelles. Some mitochondria are present and apparently similar to the control. Plastids are rare. Bar=2 µm. **f** – FC: central cylinder parenchyma. Some cells show lignifications (arrows). Bar=5 µm

2.4 Discussion

2.4.1. Copper induced effects on root morphology

In the tolerant population no remarkable changes were found in the root morphology of Cu-treated plants in respect to control plants, most probably because of both its lower Cu accumulation and/or the presence of more efficient tolerant mechanisms at the cellular level, such as enhanced antioxidant defenses of the root apex itself, as demonstrated for a Cu-tolerant non-excluding maize cultivar (Madejon et al., 2009) . On the other hand, the macroscopical effects of Cu on the sensitive population roots were generic malformations, swellings and ruptures, as already reported by several authors for non metal adapted plants (Panou-Filotheou and Bosabalidis, 2004; Sheldon and Menzies, 2005) and in one report on metallicolous plants (Llugany et al., 2003). The increased production of lateral roots in the Cu- exposed sensitive population was observed also in Arabidopsis by Lequeux et al. (2010).

2.4.2. Involvement of root apex toluidine blue positive material outside the root cap and rhizodermis in Cu exclusion mechanisms.

In comparison to the sensitive population, the tolerant one seemed not only to constitutively possess a higher amount of toluidine blue positive material outside the root cap and the rhizodermis, identifiable as mucilage, at the root apex, but also to be able to increase its level in presence of Cu. Actually, mucilage, mainly exuded from the outer layers of the root cap and largely consisting of negatively charged polysaccharides (Iijima et al., 2008), can be involved in metal immobilization mechanisms in plants (Hawes et al., 1998; Geng et al., 2012; Cai et al., 2011) due to its selective absorption and storage of ions, such as Al^{3+}, Pb^{2+}, Cd^{2+} and Na^{+} (Archambault et al., 1996; Ghanem et al., 2010; Morel et al., 1986). A definite role of mucilage in protecting the roots from

metal excess has been shown only for Al, studying differently tolerant cultivars of *Glycine max* (Cai et al., 2013) and in *Vigna unguiculata* (Horst et al.,1982). Therefore, in the metallicolous population of *S. paradoxa* the presence of abundant mucilage already in control conditions could represent a population-specific adaptation to the differential nutrient availability levels in the population natural environment, aimed at restricting metal accumulation in the root apex. In addition, such mechanism showed also a Cu inducible nature, thus probably concurring to the multiple metal imposed exclusion mechanisms that generate the lower Cu accumulation showed by the tolerant population in such growth conditions (Colzi et al., 2012). Consequently, our data indicated the mucilage response to heavy metal treatment, until now evaluated only in selected cultivars of crop plants and mainly for Al, as one of the mechanisms actually accounting for naturally-evolved plant tolerance to Cu.

Moreover, the tolerant population presented some toluidine blue positive material at the level of the walls of all the cells of the cortical cylinder at 4 mm from the apex, while the sensitive one showed normal walls at that level, indicating a different wall composition between the two populations. Intriguingly, a Cu-induced opposite trend for the changes in another kind of metal-binding root polysaccharides is worth to be noted: Cu treatment induced a decrease in root cell wall pectin concentration in the tolerant population and an increase in the sensitive one (Colzi et al., 2011; 2012), exactly the contrary of what happened in the above-mentioned case of external mucilage, substance present only at the level of the root cap. Pectic polysaccharides are heterogeneous polymers constituting part of both the external mucilage and cell wall matrix (Willats et al., 2001). Here the different localization of this compounds (respectively outside the root cap and rhizodermis and as a component of the root cell walls) could be probably related, in both cases, to its capability of linking cations, but with a different functioning. The discriminating factor for such

contrasting trends could probably be the very localization of the polysaccharides. Actually, the amount of metal bound to pectins can affect the ionic composition of the apparent free space and then could influence the amount of Cu available for plasma membrane transport. Cu is known to be, together with Pb, the metal most capable to be bound by pectins (Dronnet et al., 1996). As for mucilage, being this polysaccharide prevalently localized in a structure without any sort of actual connection with the root, it cannot affect the concentration of the ions in any of the root compartment, free space included. Therefore, the presence of mucilage could represent an out-of-root ion immobilizing mechanism, that wisely the tolerant population seemed to exploit for reducing Cu accumulation in the root apex and consequently in the whole root itself.

2.4.2 Can differentiation of the xylem and deposition of lignin and callose be involved in copper tolerance mechanisms?

As for a possible Cu-induced changes in the xylem development, in presence of the metal vessel, lignification started further away from the root apex in the sensitive population, while the opposite phenomenon was present in the tolerant population. In literature there is information on such metal imposed effect only on Cd and with contrasting results (Lux et al., 2011), whereas this topic would require more attention, because metal uptake and xylem loading are for the most part carried out exactly by young subapical regions of actively growing roots. Even if the number of cells present between the apex and the first lignified tracheid was slightly lower in treated plants than in untreated ones, while the mean length was the same, the phenomenon of the reduction of the subapical region length could difficultly be interpretable as an indirect and passive effect of a Cu-imposed decrease in the mitotic activity of the apex itself, as the tolerant population did not show any impaired growth in such conditions (Colzi et al., 2012), thus implying an early differentiation as the more probable option.

Therefore, also a metal- imposed length reduction of such zone of the root apex seemed to be involved in the several exclusion mechanisms operated by this naturally-evolved metal tolerant *S. paradoxa* population. On the other hand, in the sensitive population, even though in such experimental conditions a Cu-induced decrease in root length was present (Colzi et al., 2012), the Cu-mediated expansion of the subapical region was coupled to an increase in both the cell number and the cell mean length. Therefore, the ion could have actually provoked a delay in the differentiation of the xylem, thus concurring to the higher copper accumulation displayed (Colzi et al., 2012).

At the level of the whole root, the above mentioned result was coupled to a Cu-induced decrease in lignin concentration in the sensitive population and a Cu-induced increase in the tolerant one. The induction of lignin could be a mechanism for the generation of root apoplasmic barriers, as this polymer, together with suberin, forms the impregnation material of Casparian bands developed in the endodermal cell walls (Lux et al., 2011). In fact, while for hyperaccumulators lignin deposition may represent a mechanisms to prevent excess efflux of metals from the vascular cylinder (van de Mortel et al., 2006), in non-accumulating plants such phenomenon may have a different function, representing a specific detoxification mechanism that limits the entry of toxic metals in the roots, as hypothesized for Cd (van de Mortel et al., 2008; Lux et al., 2011). Our results on *S. paradoxa* could suggest an intriguing hypothesis on the discriminating factor for metal induced lignification and the generation of the excluder or the hyperaccumulating phenotype, that could be the length of the subapical region. In presence of the metal, for reducing such length, and thus limiting the area of the root that presents the highest rate of metal uptake, some excluder plants could accelerate the lignifications of the vessels, thus unavoidably resulting also in a general lignification of the root. Even if such lignification can favor the translocation of the metals limiting their efflux, the advantage of the above-mentioned decrease could represent the primary determinant reason for

such a response. On the other hand, any reduction of the subapical region has never been reported in hyperaccumulators, that seem to just directly exploit root lignification for its capacity of limiting metal efflux to the cortical parenchyma.

Regarding variation in root lignification in Cu-exposed plants, Cu-unaffected lignin accumulation was found for metallicolous populations of *S. vulgaris*, probably due to the short time of exposition (Kováčik et al., 2010), whereas there are many similar examples of Cu-imposed increase in lignin concentration in non metallicolous plants (Lequeux et al. 2010), but always without any information about the distance of the differentiation zone from the apex. In fact, even if it is well-known that Cu induces alterations in root lignin metabolism (Chen et al., 2002), the structural changes in root anatomy resulting from the response of plants to excess Cu are scarcely documented. In this context, our results represent the first report that couples the Cu-induced lignin production to the differentiation of the xylem and relates that to Cu tolerance mechanisms in a metallophyte. On the other hand, probably the sensitive population was unable to enhance its lignification degree, and actually it intriguingly decreased, thus resulting also in an increase of the length of the subapical region. In such conditions, Cu could reach more easily the central cylinder and then harmfully move towards the aerial parts.

Interestingly, the callose response was triggered in the sensitive population, whereas in the tolerant one the production of this polysaccharide was diminished. In the sensitive population the translucent material present in 10 μM $CuSO_4$ treated plants at the rhizodermis wall level could be probably represented just by callose deposition. The general capability of callose deposition in the wall to make the apoplast semipermeable is considered a mean to control water and solute flow (Pirselova and Matuskova, 2013). This peculiar property is linked to the fact that callose linear chains form triple helixes that can exclude water in some points via interchain hydrogen bonds, also called ‘hydrophobic bonds’ (Gidley and Nishinari, 2009). At the root level, metals can be blocked in their

migration by the callose layers (Krzesłowska, 2011) impermeable to many metal ions (Hall, 2002; Patra et al., 2004). Anyway, there are cases where the protective role of callose appearance under heavy metal stress is still elusive (Krzesłowska, 2011), so that its barrier role against ion penetration appears less obvious than previously believed, whereas most significant appears its distribution (Samardakiewicz et al., 2012). Specifically in the case of Cu, a metal-induced production of callose was found in bean roots (Bouazizi et al., 2010), in *Brassica juncea* (in this case at a higher level in respect to the more tolerant species *B. napus* as reported by Feigl et al., 2013), whereas it did not appear in Cu-exposed poplar (Qin et al., 2007). Therefore, in *S. paradoxa* the tolerant population seemed to shunt its metabolic resources in favor of lignin deposition at the expense of the callose one, maybe because of a supposed greater protecting effect of the former and/or because of its more strategic localization in comparison to the latter. This wise response, able to limit Cu accumulation, was not present in the sensitive population that even chose the opposite behaviour, exactly as it was demonstrated in the case of Cu-induced pectin production (Colzi et al., 2012).

Beyond the generation of the differences in copper accumulation in such growth conditions between the two populations observed by Colzi et al. (2012), the above mentioned differences in the Cu-induced lignification and callose deposition, and their derived alteration of ion uptake and translocation, may be one of the causes of the already reported changes (Pignattelli et al., 2013) in the root and shoot ionome of Cu- treated *S. paradoxa* populations.

2.4.3. Copper-induced ultrastructural changes and copper-induced root cell wall precipitates

The most striking ultrastructural feature of the sensitive population in presence of Cu, that is the presence of huge nucleoli with a spongy periphery, may be referred to the need of an increased translation of proteins linked to the stress. A change in nucleoli morphology under stress in mammals was already observed

by many authors (summarized by Boulon et al., 2010). Boulon et al. (2010) considered the nucleolus as a hub in the stress response in the mammalian cell. The effect of stress on nucleoli in plants is less known, but some studies linked excess of Zn to the reduction of the nucleolus volume, while Cd would elicit the increase in nucleoli number in the root (Balsberg Påhlsson, 1989). The presence also of dilated RER, connected to the vacuole, would enforce this hypothesis. Another feature of the sensitive population was the presence of electrondense granules in the mitochondria and in the autophagic vacuoles, this latter probably derived by phagocytized granule-containing mitochondria. Also the plastids appeared to be affected by Cu in the sensitive population, being rare and in some cases very electrondense and elongated. Excess Cu can affect the chloroplasts grana and cause a modification of the lipid-protein ratio of thylakoid membranes (Maksymiec, 1998) while Panou-Filotheou et al. (2001) observed a reduction in volume and number of chloroplast and an increase in plastoglobules. These causes may produce the observed plastid features in *S. paradoxa*. Similar morphology in root plastids was already observed by Panou-Filotheou et al. (2001) in oregano where these organelles appeared more swollen with respect to our observations.

The thickened and translucid walls of the sensitive plants exposed to Cu had the morphology of callose containing walls. No plasmolysis was observed in *S. paradoxa* root as instead observed in *Theobroma cacao* (Souza et al. 2014).

The higher degree of methylation of pectins and their lower concentration in the tolerant population may be related to one of its most striking ultrastructural feature, which was the precipitation of abundant electron dense granules in the epidermal internal tangential walls of the tolerant population roots. Precipitates in the root cells wall in presence of Pb was already observed by Krzesłowska (2011), even if those precipitates showed a very different ultrastructure compared to our observations. Our observations would be the first of this kind induced by copper.

2.5 Conclusions

Our results demonstrated that the Cu-induced production of mucilage at the root tip level can concur to the tolerance mechanisms in the Cu-tolerant population of *Silene paradoxa*. Furthermore, in such population the presence of the metal was able also to increase root lignification and to promote an early differentiation of the vessels. All these Cu-imposed features could contribute to the exclusion mechanisms evolved by this species to limit Cu uptake and translocation. The observation of Cu-induced precipitates in the root walls of such population was the first observed in presence of this metal.

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CHAPTER 3

Photosynthesizing on metal excess: Copper differently induced changes in various photosynthetic parameters in copper tolerant and sensitive *Silene paradoxa* L. populations

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Abstract

This work investigated Cu-induced changes in photosynthetic activity in contrasting populations of *Silene paradoxa* L. A metallicolous Cu-tolerant population and a non-metallicolous sensitive population were grown in hydroponics and exposed to different $CuSO_4$ treatments for different times. Copper accumulation, MDA concentrations, and several photosynthetic parameters were measured to assess different effects of Cu exposure on plants from the two populations. A more efficient ability to photosynthesize in the presence of Cu excess was showed by the Cu-tolerant population with respect to the sensitive one. Interestingly, Cu-imposed limitations were present not only at a different degree, but also of different nature in the two populations. In the tolerant population, the most limiting factor to photosynthesis seemed to be Cu-imposed stomatal closure, whereas Cu-mediated biochemical limitation was scarce and Cu-mediated reduction in mesophyll conductance almost non-existent. In the sensitive population, Cu largely affected all the measured parameters, so that its photosynthetic activity experienced any kind of limitation, diffusional and especially biochemical. The lower Cu concentrations accumulated in the tolerant plant could be one of the factors concurring to the reported differences in photosynthetic activity, but also a higher capacity of internal detoxification and compartmentalization of the metal could not be excluded.

3.1 Introduction

A number of plant species occupy a very broad niche with respect to soil heavy metal concentrations. In many of these species, this is due to the evolution of heavy metal-tolerant populations, with a number of particular traits enabling them to cope with the surplus of essential and metabolically non-essential elements (Baker, 1987; Ernst, 2006). Such "metallophytes" can deal with excess metal in very different ways, depending on the species. The majority of these plants are able to tolerate high metal concentration in the substrate by physiologically restricting their entry into the root and/or their transport to the shoot (termed "excluders" by Baker, 1981), whereas only a few species, the so-called metal "hyperaccumulators" (Brooks et al., 1997), can accumulate metals in their shoots at extraordinarily high concentrations. Metallophytes are not only excellent biological systems to study plant adaptation to extreme environments, but also the optimal choice for the development of environmental technologies for the remediation of metal-contaminated soils (Whiting et al., 2004).

The exclusion mechanism acts to restrict uptake and accumulation of metals by preventing their entry into the cytoplasm, either by an altered ion binding capacity of the cell wall and/or by a different regulation of the metal influx and efflux from the cytosol (Tong et al., 2004). Moreover, chelation of heavy metals with organic acids (e.g. citric, malic, oxalic acids) outside the plasma membrane can reduce their uptake (Ryan et al., 1995; Zeng et al., 1998). As a consequence, most tolerant excluder plants often accumulate less metal in tissues when compared with non- tolerant individuals of the same species (De Vos et al., 1992; Ouzounidou et al., 1995; Van Hoff et al., 2001; Llugany et al., 2003). In addition to the exclusion of metals, these plants may also show mechanisms of internal tolerance to effectively face toxic metal concentrations in the cytoplasm (Baker and Walker, 1989). At the cellular level, tolerance mechanisms can protect metabolically active sites through the synthesis of metal-binding ligands to sequester excess of metals in the citosol (Hall, 2002; Verkleij et al., 2009) and/or

through a more efficient cellular compartmentation of the metal surplus, especially into the vacuole. In any case, the picture of metal tolerance mechanisms is far from being complete and the whole plant physiology of metallophytes has not been sufficiently studied. For example, the effect of metals on the photosynthesis of such plants remains almost completely unexplored and whether metallophytes have evolved tolerance mechanisms at the level of photosynthetic processes remains unknown. Actually, considerable information is available on the inhibitory effect of metal excess on photosynthesis in non-metallophytes, even if a complete analysis of the different photosynthetic aspects is rare to be found. In general, photosynthetic reactions are known to represent the most important sites of inhibition by many heavy metals and photosystem II (PSII) has long been identified as the main target of metal toxicity (Clijsters and Van Assche, 1985). In this context, Cu is one of the most studied metals, due to its widespread use in agricultural practices and its importance as an environmental pollutant. In non-metallophytes, Cu negative influence on pigment concentration and photosynthesis biophysics is largely documented (Maksymiec, 1997; Barón et al., 1995; Küpper et al., 2002). On the other hand, as for the limitations to photosynthesis, different kinds of results have been reported, depending on the species and the experimental conditions used. Anyway, reports about Cu-imposed limitations to photosynthesis independent from variations in leaf CO_2 concentration are actually the more frequent. For example, in *Cucumis sativus*, Cu treatment induced a stomatal closure that did not account for the inhibition of photosynthesis, and also affected more the dark phase of photosynthesis rather than the light phase (Vinit-Dunand et al., 2002). Non-stomatal limitations were found also in *Limoniastrum monopetalum* (Cambrollé et al., 2013) and in *Atriplex halimus* (Mateos-Naranjo et al., 2013) where the Cu-imposed reduced photosynthetic carbon assimilation was mainly generated by the adverse effect of the metal on the photochemical apparatus. In contrast,

Phyllostachys auresulcata and *Pleioblastus chino* showed also a Cu- imposed decrease in the levels of internal leaf CO_2 concentrations (Jiang et al., 2013).

In the case of metallophytes, there is some interesting information about the effect of Cd on photosynthesis in Zn/Cd hyperaccumulating plants, even if the primary mechanism of Cd- induced inhibition of photosynthesis in such species is still a matter of controversy (Ying et al., 2010; Zhou and Qiu, 2005; Küpper et al., 2007; Tang et al., 2013). In the case of metal excluder plants, it is not known if the photosynthetic machinery has evolved in response to natural selection by high levels of metals in the soil, if all the aspects of their photosynthesis are equally affected by metals and what processes are most sensitive in non-tolerant populations compared to tolerant ones. To our best knowledge, some information on this topic can be retrieved only for Cu, with just four reports dealing uniquely with metal-induced effects on pigment concentration and biophysics of photosynthesis. In *Haumaniastrum katangense* (Peng et al., 2013) a loss of pigments at high external Cu was detected only in the sensitive populations, but the effect of Cu stress on photosynthesis biophysics was not clearly different between sensitive and tolerant populations. In *Elsholtzia splendens* (Peng et al., 2013) Cu toxicity negatively affected pigment concentration and the photosynthetic parameters considered, but the study was conducted only on a Cu-tolerant population without a non-tolerant one for reference. Always studying only a Cu-tolerant population, a similar situation was described for the invasive amphibious water plant *Crassula helmsii* (Küpper et al., 2009), whereas in *Erica andevalensis* a Cu-mediated increase in pigment concentration was ascribed to decreased cell size (Rossini Oliva et al., 2010). A comprehensive study of the whole photosynthetic response to heavy metals in metallophytes, in comparison to non-tolerant populations, has yet to be performed, and the present work was conducted to fill this gap of knowledge.

Silene paradoxa L. (Caryophyllaceae) is a good study model to conduct comparative studies of response to heavy metals, as it is a species generally found in non-contaminated dry areas and occasionally evolving metal-tolerant populations on metalliferous soils (Chiarucci et al., 1995). In previous investigations, it was found that the S. paradoxa population growing in the polymetallic mine of Fenice Capanne (Tuscany, Italy) was tolerant to some metals present in the mine waste, such as Cu, As, Cd, and Zn, as compared to a *S. paradoxa* population growing in a non-contaminated soil (Gonnelli et al., 2001; Arnetoli et al.,2008; Mengoni et al., 2003). This species is by now considered as an excluder type and some of its physiological mechanisms concurring to Cu tolerance have been successfully elucidated, such as reduced accumulation, higher antioxidative ability and increased expression of a metallothionein gene (Gonnelli et al., 2001; Mengoni et al., 2003; Colzi et al., 2011; Colzi et al., 2012; Pignattelli et al., 2013). We here test if the response of photosynthesis machinery is different between a metal-tolerant and a non-tolerant population of a metal excluder. Increased metal tolerance at the photosynthesis level is not necessarily expected, since metal excluders are able to restrict metal transfer to the shoot. For the first time, to our knowledge, limitation analysis was applied to the exploration of the Cu-induced changes in photosynthesis of natural plant populations, thus identifying the different relevance of diffusional and biochemical limitations during the acquisition of metal tolerance. In addition, the present paper is also the first report that, in such plant systems, took into consideration the effect of Cu excess on both biochemical and biophysical photosynthetic factors at the same time.

3.2 Materials and methods

3.2.1. Plant material and growth conditions

Seeds of *S. paradoxa* L. were collected at the Fenice Capanne mine waste (tolerant population, TOL) and at Colle Val D'Elsa uncontaminated soil (non-tolerant population, NONTOL) (Arnetoli, 2004). Seeds were sown in peat soil; 6-week-old seedlings were transferred to hydroponic culture, in 1-L polyethylene pots containing a modified half-strength Hoagland's solution (Hoagland and Arnon, 1950) in milliQ-water (Millipore, Billerica, MA, USA) buffered with 2 mM 2-morpholinoethanesulphonic acid (MESA), pH 5.5, adjusted with KOH. Nutrient solutions were changed weekly and plants were grown in a growth chamber as described in Pignattelli et al. (2013). After 3 weeks of pre-culture, plants were exposed to a series of $CuSO_4$ concentrations (1, 10, 20 µM), in a background solution of the same composition as the pre-culture solution, for 1, 7, 14, and 21 days.

3.2.2. Measurement of copper concentration

Copper concentrations were determined in roots and shoots by acid digestion of oven-dried material as in Colzi et al. (2012). Prior to analysis, root material was carefully washed with a solution of 10 mM $Pb(NO_3)_2$ for 30 min to remove metals adhering to the root cell wall. Copper was determined on an atomic absorption spectrophotometer (Perkin-Elmer, Analyst 200).

3.2.3 Determination of MDA concentration

Lipid peroxidation was evaluated as the amount of malondialdehyde (MDA, end product of lipid peroxidation), measured by the thiobarbituric acid reaction (Buege and Aust, 1978). The method described in Gonnelli et al. (2001) was followed: briefly, about 1 g of fresh sample was homogenized with TCA and

supernatant used for the determination after centrifugation. After reaction with a solution of TBA, the absorbance of the supernatant was recorded at 532 nm and then at 600 nm for the subtraction of the non-specific absorption and an extinction coefficient of 155 mM^{-1} cm^{-1} was used.

3.2.4 Photosynthesis limitation analysis and chlorophyll fluorescence measurements

Leaf gas exchange and chlorophyll fluorescence parameters were determined simultaneously with chlorophyll fluorescence measurements using the portable photosynthesis system Li-6400 XT (LiCor Inc., Lincoln, NE, USA). Leaf gas exchange measurements were taken on the youngest fully expanded leaves at the following time points: 0 (before the treatments), 1, 7, 14, and 21 days of treatments. Measurements of net photosynthetic rate (A_N) and stomatal conductance (g_s) were determined with reference CO_2 of 400 µmol mol^{-1}, ambient relative humidity, flow rate of 500 µmol s^{-1}, chamber temperature at 20 ◦C and 300 µmol m^{-2} s^{-1} of photosynthetically active radiation. At the end of the treatments, for more in-depth analyses of the photosynthetic limitations in each ecotype, CO_2 response curves (A_N/C_i) were determined for all treatments with the following external CO_2 concentrations: 400, 50, 100, 150, 250, 350, 500, 700, 900, 1200, and 400 µmol mol^{-1}. A_N/C_i curves were analyzed using the curve fitting utility described in Sharkey et al. (2007) and the following key biochemical parameters were determined: V_{cmax} (maximum rate of carboxylation), J_{max} (light-saturated rate of electron trans- port), and TPU (rate of triose-P utilization for sucrose and starch synthesis). Subsequently mesophyll conductance (g_m) was estimated from combined gas exchange and chlorophyll fluorescence measurements (Harley et al., 1992; see Sagardoy et al., 2010 for more detailed information) as $A_N/(C_i - (T^*(ETR_{flu} + 8(A_N + R_d))/ETR_{flu} - 4(A_N + R_d)))$. In this equation, T * was taken after Bernacchi et al. (2002), and dark respiration was

used as a proxy for R_d (Pinelli and Loreto, 2003). ETR_{flu} is the electron transport rate calculated from fluorescence measurements, R_d is the mitochondrial respiration in the light, and T * is the CO_2 compensation concentration in the absence of mitochondrial respiration. Relative photosynthetic limitations were then partitioned into different functional components related to stomatal (SL) and mesophyll conductance (MCL) and leaf biochemical characteristics (BL), following the method of Wilson et al. (2000) modified by Grassi and Magnani (2005) that requires measuring A_N, g_s, g_m, V_{cmax}, and the comparison with a hypothetical reference state when all parameters are at their maximum, i.e., in control plants (Sagardoy et al., 2010; see Harley et al. (1992) for further information and formulae).

3.2.5. Pigment concentration

Leaf pigment concentrations were determined as described in Rich et al. (2005). Briefly cold 100% methanol was added to ground freeze-dried leaf tissues and subsequently samples were incubated in darkness at 4 ◦C for 30 min. Following the incubation, samples were centrifuged at 9300 g for 10 min at 4 ◦C, and then the supernatants were removed and their absorbance determined at 470, 665.2, and 652.4 nm, using a Tecan Infinite 200 Spectrophotometer (Männedorf, Switzerland). Chlorophyll a (Chla) and b (Chlb) and total carotenoids concentration were obtained using the equations from Wellburn (1994).

3.2.6. Stomatal density

Stomatal density was assessed with a Zeiss Axio Observer Z1 fluorescence microscope (Carl Zeiss, Thornwood, NY). Leaves were collected at the end of the experiment and five areas of 0.361 mm^2 of the same leaf were observed and the stomata counted. Measurements were performed on three different leaves of

the same plant. Stomatal density and pore size were measured manually using the Adobe Photoshop 7.0 software.

3.2.7. Statistics

One-way and two-way ANOVA, considering population and metal treatment as main factors, were used for the statistical analysis of the data (statistical program SPSS 13.0, SPSSInc., Chicago, IL, USA). Tukey post hoc test was used for a posteriori comparison of individual means (with at least $p < 0.05$ as significant level).

All the measurements were evaluated on 12 plants per population per concentration.

3.3 Results

3.3.1 Copper accumulation and MDA concentrations

Copper concentrations in roots and shoots of both the populations increased with increasing metal concentrations in the nutrient solution for all the $CuSO_4$ concentrations used (Fig.3.1). Copper concentrations in shoots were much lower than those in roots in both populations. Statistically significant differences in root and shoot Cu concentrations were found between the two populations at all the metal concentrations used, except for the lowest one, being metal accumulation lower in the TOL population in respect to the NONTOL one (at least $p < 0.05$).

Plantlets from both the populations showed an increase in MDA concentration in roots and shoots after copper treatment (Fig.3.2). In the NONTOL population, with respect to the TOL one, such increase started to be significant, as compared to control plants, from lower times of exposure and lower $CuSO_4$ concentrations. In both populations, levels of MDA in leaves were greater than those in roots. Comparing the populations between themselves, when

the effect of the metal was significant, also significant differences in root and shoot MDA concentrations were generally found, showing the NONTOL plants higher values than the TOL ones (at least $p < 0.05$).

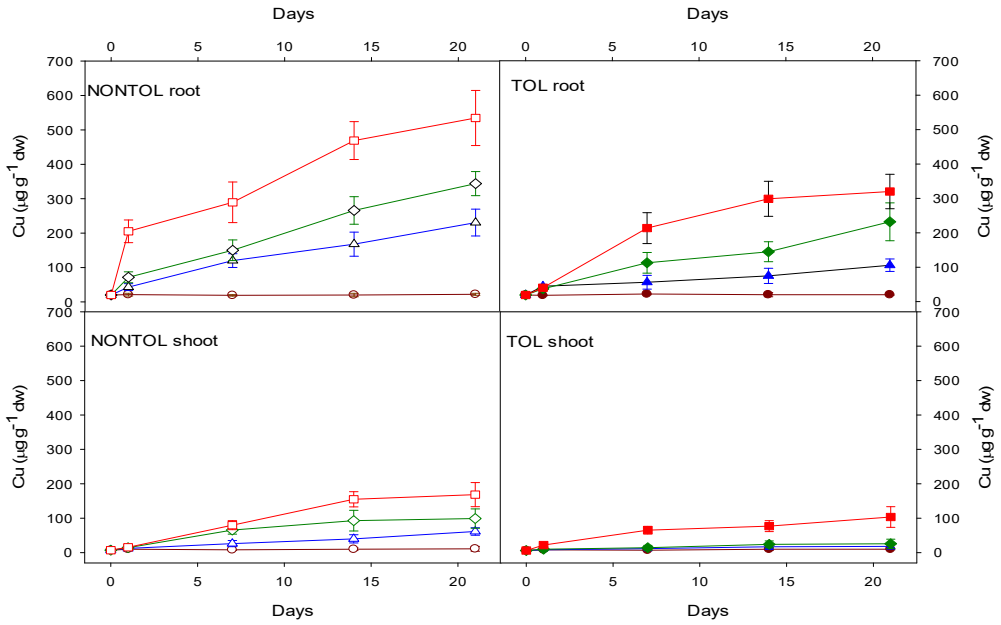

Fig.3.1. Root and shoot Cu accumulation of two *S. paradoxa* populations after exposure to increasing $CuSO_4$ concentrations for 21 days (NONTOL: Colle Val D'Elsa, sensitive population, white symbols; TOL: Fenice Capanne, Cu-tolerant population, black symbols; circle, brown: control conditions, triangle,blue: 10 µM $CuSO_4$, diamond,green: 20 µM $CuSO_4$, square,red: 40 µM $CuSO_4$). Values are means of 12 replicates ± standard deviation.

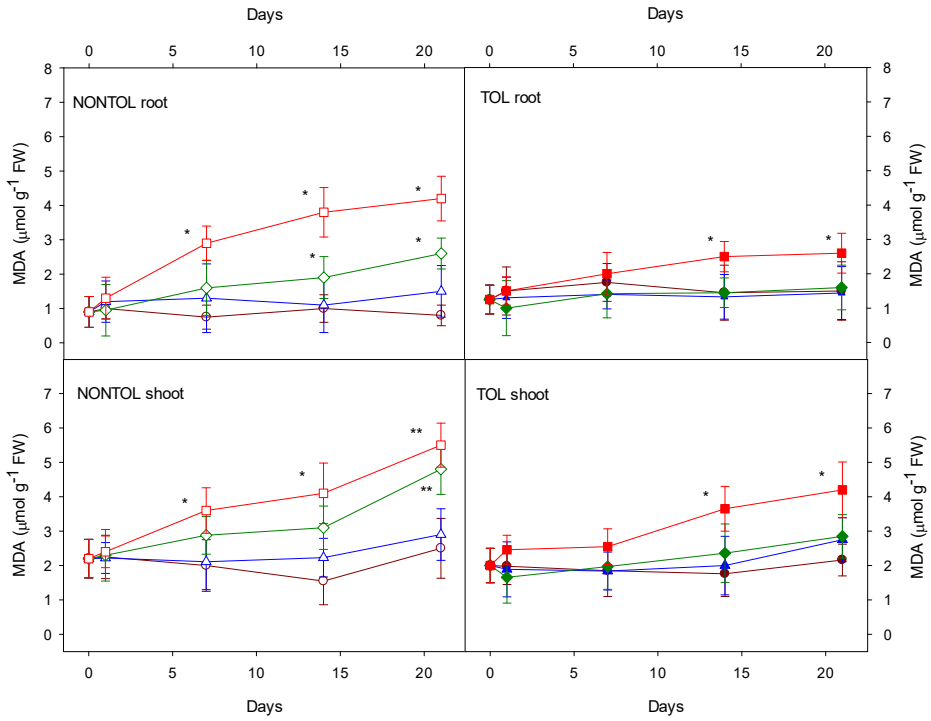

Fig.3.2. Root and shoot MDA concentrations of two *S. paradoxa* populations after exposure to increasing $CuSO_4$ concentrations for 21 days (NONTOL: Colle Val D'Elsa, sensitive population, white symbols; TOL: Fenice Capanne, Cu-tolerant population, black symbols; circle,brown: control conditions, triangle,blue: 10 µM $CuSO_4$, diamond,green: 20 µM $CuSO_4$, square,red: 40 µM $CuSO_4$). Values are means of 12 replicates ± standard deviation. Intra-population significant differences between the means appear with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.3.2. Gas exchange parameters

Two-way ANOVA displayed significant effects of populations and Cu treatment for most of the parameters studied, whereas the interaction population*treatment was showed to be always significant (Table 3.1).

In the youngest fully expanded leaves of the NONTOL population a significant decrease of the net photosynthetic rate (A_N) was present already after

1 day of exposure at the highest Cu concentration used. The other significant differences in A_N in respected to the control conditions were after 7 days, at the same concentration, after 14 days, at the two highest concentrations used, and after 21 days, at all the Cu concentrations used (Fig. 3.3a). In the TOL population the effect of metal exposure on A_N was present only at the highest Cu concentration used, starting from 7 days of treatment (Fig. 3.4b). In control conditions, NONTOL population showed higher, even if not significantly, values of AN in comparison to the TOL population.

Regarding the stomatal conductance (g_s), all the Cu concentrations used produced a significant decrease in this value in the NONTOL population (Fig.3.3c) at any time, excluded after 1 day of treatment when the effect was significant only at 40 µM CuSO4. In the TOL population (Fig.3.3d), Cu-induced changes in g_s were significant only at the highest $CuSO_4$ concentration from 14 days of treatment onwards. Interestingly, in control conditions, the two populations showed very different values of g_s, being the one of the TOL population about the half of that of the NONTOL population ($p < 0.05$).

In both the populations, the internal concentration of CO_2 (C_i, Fig.3.3e and f) decreased in the presence of Cu only at the highest concentration used from 7 days of exposure onwards. The only difference was that in the NONTOL population C_i significantly increased with respect to the control at the end of the exposure time at 20 µM $CuSO_4$. In control conditions, C_i showed the same value in both the populations.

As the more significant differences among the different treatments were present at 21 days of Cu exposure, at that time also the key biochemical factors (maximum carboxylation rate of Rubisco ($V_{c,max}$), maximum rate of the electron transport (J_{max}) and use of triose-P (TPU), calculated from the A_N/C_i curves) and the mesophyll conductance (g_m) were measured, as reported in Table 3.2. The values of $V_{c,max}$, J_{max}, and TPU were significantly lower only at 20 and 40 µM $CuSO_4$ in comparison to the control conditions in NONTOL and, to a lower extent

and especially in the case of V_{cmax}, in TOL population. Regarding g_m, in plants from the NONTOL population a Cu-induced wide decrease in g_m was present, significantly already at the lowest Cu concentration used, whereas plants from the TOL population showed a slight decrease in such parameter, significantly only at the highest Cu treatment. In control conditions, the TOL population showed an extremely low g_m value in comparison to the NONTOL population.

Before the treatment, stomatal density and pore size were measured in both the populations. Plants showed the same stomatal density for the leaf lower side (42 ± 2 and 40 ±3 stomata per 0.361 mm^2 leaf area, in NONTOL and TOL population, respectively) and a significantly different one for the leaf upper side (39 ± 3 and 24 ± 6 stomata per 0.361 mm^2 leaf area, in NONTOL and TOL population respectively, $p < 0.05$). Pore size was the same in both the populations, with values around 37 ± 1 μm for both the leaf lower and the upper sides.

3.3.3. Limitation to photosynthesis

To compare the Cu different effect on photosynthesis between the populations after 21 days of treatment, the limitations to photosynthesis were calculated. The photosynthesis limitation parameters, expressed as percentage of the control condition, in *S. paradoxa* plants grown in nutrient solution with a range of Cu concentrations are reported in Fig.3.4 and the relative results of two-way ANOVA in Table 3.3.

Regarding the NONTOL population, plants supplied with 10 μM $CuSO_4$ experienced a slight degree of total limitation to photosynthesis (TL), whereas, at the highest rates of Cu supply, TL increased substantially, showing values of about 90%. In these latter cases, the limitation was for a remarkable part determined by biochemical limitation (BL) with respect to the diffusional limitation (DL, defined as the sum of stomatal limitation (SL) and mesophyll lim itation (MCL)). Anyhow, at 20 μM $CuSO_4$ MCL was preponderant on SL, whereas at 40 μM $CuSO_4$ their contribution was similar.

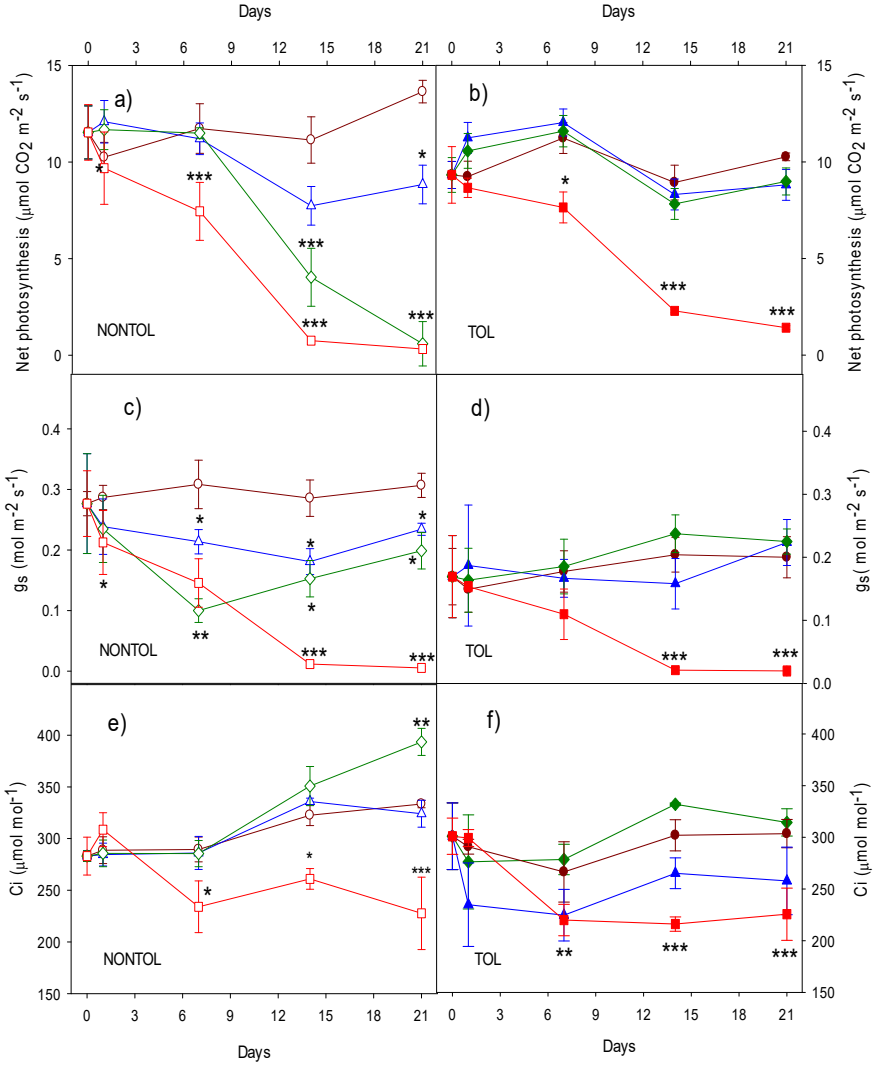

Fig 3.3. Net photosynthetic rate (A_N), stomatal conductance (g_s), and CO_2 internal concentration (C_i) in leaves of two *S. paradoxa* populations grown in nutrient solution with a range of Cu concentrations for 1, 7, 14, and 21 days. (NONTOL: Colle Val D'Elsa, sensitive population, white symbols; TOL: Fenice Capanne, Cu-tolerant population, black symbols; circle, brown: control conditions, triangle,blue: 10 µM $CuSO_4$, diamond,green: 20 µM $CuSO_4$, square,red: 40 µM $CuSO_4$). Values are means ± standard deviation of 12 replicates. Intra-population significant differences between the means appear with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table 3.1 Two-way ANOVA results for the variation in gas exchange parameters in the two *S. paradoxa* populations treated with different $CuSO_4$ concentrations for 21 days.

Parameter	Source of variation					
	Population (*P*)		Treatment (*T*)		Interaction (*P***T*)	
	F	*P*	*F*	*P*	*F*	*P*
P_n	32.62	***	165.50	***	34.31	***
g_s	2.29	ns	73.45	***	5.34	**
C_i	78.10	***	75.30	***	9.46	***
V_{cmax}	23.75	***	61.00	***	19.39	***
J_{max}	21.03	***	54.70	***	16.72	***
TPU	18.06	***	100.20	***	18.06	***
g_m	0.61	ns	18.38	***	6.53	**

Table 3.2. Photosynthetic biochemical parameters and mesophyll conductance in leaves of two *Silene paradoxa* populations grown in nutrient solution with a range of Cu concentrations for 21 days. Values are means ± standard deviation of 12 replicates. Significant differences between the means appear with different letters when intra-population and with asterisks when inter-population (*p < 0.05).

Parameter	NONTOL				TOL			
	Control	10 µM $CuSO_4$	20 µM $CuSO_4$	40µM$CuSO_4$	Control	10 µM $CuSO_4$	20 µM $CuSO_4$	40µM$CuSO_4$
V_{cmax} (µmol CO_2 m^{-2} s^{-1})	70 ± 1 c	73 ± 3 c	14 ± 3 a*	30 ± 4 b*	68 ± 2 b	63 ± 5 b	49 ± 2 a*	54 ± 5 ab*
J_{max} (µmol CO_2 m^{-2} s^{-1})	110 ± 3 c	112 ± 7 c	26 ± 4 a*	49 ± 6 b*	103 ± 1 b	104 ± 9 b	83 ± 4 a*	77 ± 5 a*
TPU (µmol Pi m^{-2} s^{-1})	8.5 ± 0.2 c	8.9 ± 0.5 c	2.4 ± 0.2 a*	5.0 ± 0.3 b	8.0 ± 0.1 c	7.9 ± 0.4 c	6.5 ± 0.2 b*	5.8 ± 0.1 a
g_m (mmol CO_2 m^{-2} s^{-1})	183 ± 47 c*	66 ± 14 b	5 ± 1 a*	2 ± 0 a	83 ± 8 b*	63 ± 9 b	62 ± 9 b*	8 ± 1 a

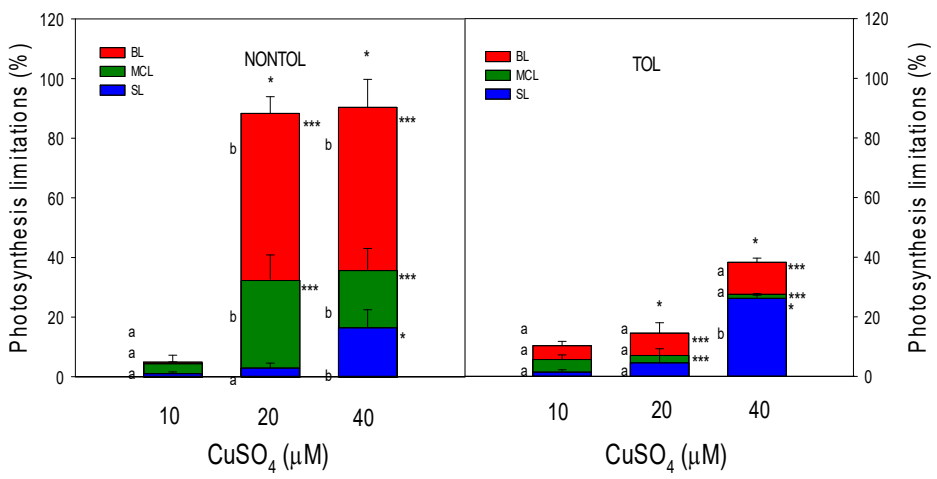

Fig.3.4 Photosynthesis limitation parameters (%) in the two *S. paradoxa* populations grown in nutrient solution with a range of Cu concentrations for 21 days (SL: stomatal limitation, MCL: mesophyll limitation, BL: biochemical limitation). Significant differences between the means appear with different letters when intra-population and with asterisks when inter-population (*p < 0.05, **p < 0.01, ***p < 0.001).

Table 3.3 Two-way ANOVA results for the variation in photosynthetic limitations in the two *S. paradoxa* populations treated with different $CuSO_4$ concentrations for 21 days.

Limitation	Source of variation					
	Population (*P*)		Treatment (*T*)		Interaction (*P***T*)	
	F	*P*	*F*	*P*	*F*	*P*
Total	338.30	***	257.00	***	124.50	***
SL	2.25	ns	28.77	***	1.52	ns
MCL	26.74	***	5.35	*	7.82	**
BL	46.53	***	25.57	***	18.31	***

In the TOL population, TL increased following the increase of Cu concentrations in the culture medium till reaching values of about 40% at the highest Cu treatment. At 20 and 40 μM $CuSO_4$, concentrations at which TL was appreciable, the contribution of BL was quite constant, whereas the one of SL tended to increase. Values of MCL were instead negligible. Comparing the populations between themselves, TL was higher in the NONTOL population in respect to the TOL one, excluded for the lowest Cu concentration used that generated similar values. At 20 and 40 μM $CuSO_4$, also the diverse contributions were different, being BL and MCL always higher in the NONTOL population with respect to the TOL one, whereas SL showed a similar value at 20 μM $CuSO_4$ and a lower one at 40 μM $CuSO_4$. ANOVA gave significant results for all the parameters studied, except in the case of SL where the effect of populations and the interaction population*treatment were not significant (Table 3.3).

3.3.4. Chlorophyll fluorescence parameters, distribution of excitation energy absorbed in PSII antennae and pigment concentration

Copper treatment induced significant changes in parameters of photosynthesis biophysics, differently in the two *S. paradoxa* populations, as shown in Table 3.4.

In the NONTOL population the maximal dark-acclimated quantum yield of PSII photochemistry (F_v/F_m) significantly decreased at the two highest Cu concentrations used, whereas in the TOL population the decrease was present only in plants exposed to 40 μM $CuSO_4$. At the two highest Cu concentrations used, values of electron transfer rate (ETR) significantly decreased in both the populations, particularly to a greater and significant extent in the NONTOL population. The same condition was displayed by data about the light-acclimated electron flow through PSII (Φ_{PSII}). ANOVA results were always significant, except for F_v/F_m, that showed a significant effect only in the case of treatment.

Table 3.5 reports the concentration of Chla, Chlb, and total carotenoids in the two populations of *S. paradoxa* exposed to increasing Cu concentrations for 21 days. Total Chl concentration decreased in the presence of the metal in the culture medium, to a greater extent in NONTOL plants, even if in a non-significant way. In both the populations a Cu-mediated decrease in Chla was present, significantly at the two highest concentrations used, but at a lower extent for the TOL population. Chlb concentration followed the same trend, in this case significantly from 10 µM $CuSO_4$ in the tolerant populations, even if without any significant difference between the two populations. In both the populations, at the two highest concentrations used, Cu treatments induced a decrease also in total carotenoid concentration, at a higher extent in the NONTOL population at 20 µM $CuSO_4$, in a similar way for both the populations at 40 µM $CuSO_4$. As for pigment concentration, ANOVA showed significant results only for the treatment effect.

3.4. Discussion

3.4.1. Growth conditions, Cu accumulation and Cu-imposed lipid peroxidation

As expected, due to the already assessed behavior of *S. paradoxa* as a Cu excluder (Gonnelli et al., 2001; Colzi et al. ,2011; 2012), metal accumulation was lower in the tolerant population with respect to the sensitive one. The same trend was displayed by Cu-induced lipid peroxidation, as reported in Gonnelli et al. (2001) and related to the population difference in Cu tolerance. In any case, the fundamentally important result for the present study was that, for most of the concentrations used and times applied, the plants could be considered still

Table 3.4. Chlorophyll fluorescence parameters and two-way ANOVA results of total photon energy absorbed by photosystem II (PSII) antennae of leaves of the two *S. paradoxa* populations grown in nutrient solution with a range of Cu concentrations for 21 days. Values are means ± standard deviation of 12 replicates. Significant differences between the means appear with different letters when intra-population and with asterisks when inter-population (*p < 0.05).

Parameter	NONTOL				TOL			
	Control	10 μM $CuSO_4$	20 μM $CuSO_4$	40 μM $CuSO_4$	Control	10 μM $CuSO_4$	20 μM $CuSO_4$	40μM $CuSO_4$
F_v/F_m	0.84 ± 0.01 b	0.85 ± 0.01 b	0.79 ± 0.01 a	0.76 ± 0.02 a	0.83 ± 0.02 b	0.85 ± 0.02 b	0.84 ± 0.02 b	0.78 ± 0.01 a
ETR (μmol e^- m^{-2} s^{-1})	77 ± 2 c	80 ± 1 c	27 ± 3 b*	57 ± 9 a*	85 ± 1 b	80 ± 2 b	68 ± 1 a*	71 ± 2 a*
δ_{PSII}	0.59 ± 0.02 c	0.62 ± 0.01 c	0.20 ± 0.03 a*	0.48 ± 0.06 b	0.65 ± 0.01 b	0.61 ± 0.02 b	0.52 ± 0.01 a*	0.54 ± 0.01 a

Parameter	Source of variation					
	Population (P)		Treatment (T)		Interaction (P*T)	
	F	*P*	*F*	*P*	*F*	*P*
F_v/F_m	1.80	ns	9.67	***	1.40	ns
ETR	37.80	***	38.05	***	12.05	***
δ_{PSII}	32.44	***	41.70	***	14.85	***

Table 3.5 Chlorophyll and carotenoid concentration in leaves of the two *S. paradoxa* populations grown in nutrient solution with a range of Cu concentrations for 21 days and two-way ANOVA results. Values are means ± standard deviation of 12 replicates. Significant differences between the means appear with different letters when intra-population and with asterisks when inter-population (*p < 0.05).

Parameter	NONTOL				TOL			
	Control	10 μM $CuSO_4$	20 μM $CuSO_4$	40μM $CuSO_4$	Control	10μM $CuSO_4$	20 μM $CuSO_4$	40μM $CuSO_4$
Total chlorophyll (μg m g^{-1} d.w.)	1279 ± 176 c	927 ± 94 c	722 ± 45 b	409 ± 33 a	1248 ± 61 c	1025 ± 68 c	856 ± 58 b	532 ± 58 a
Chlorophyll a (μg m g^{-1} d.w.)	738 ± 65 b	550 ± 48 b	282 ± 14 a*	186 ± 22 a	636 ± 73 c	636 ± 53 c	476 ± 5 b*	222 ± 20 a
Chlorophyll b (μg m g^{-1} d.w.)	489 ± 88 b	431 ± 31 b	439 ± 14 b	222 ± 12 a	510 ± 22 b	389 ± 18 a	366 ± 40 a	310 ± 22 a
Carotenoid (μg m g^{-1} d.w.)	92 ± 14 b	96 ± 21 b	22 ± 22 a*	24 ± 7 a	96 ± 21 c	110 ± 9 c	63 ± 19 b*	17 ± 1 a

Parameter	Source of variation					
	Population (P)		Treatment (T)		Interaction (P*T)	
	F	*P*	*F*	*P*	*F*	*P*
Total chlorophyll	1.81	ns	30.54	***	0.40	ns
Chlorophyll a	1.97	ns	32.44	***	2.60	ns
Chlorophyll b	0.00	ns	12.44	***	1.71	ns
Carotenoids	1.32	ns	12.39	***	0.82	n

metabolically efficient to produce Cu-imposed responses. In fact, the metal-mediated damage to the plants, evaluated by lipid peroxidation, was significant only for part of concentrations and times considered, but most importantly its increase was not dramatic, as the maximum shoot MDA value was only the double of the controls. Certainly, the lower Cu concentrations accumulated in the tolerant plants could be one of the factors concurring to the above- mentioned differences in lipid peroxidation levels, but also a higher capacity of metal detoxification and compartmentalization could not be excluded. Similarly, the Cu effects on photosynthesis, discussed as follows, could also have derived, at least in part, from such a scenario.

3.4.2. Effects of Cu on gas exchange and photosynthetic biochemical parameters

Our results demonstrated that, in terms of Cu-mediated changes in gas exchange and photosynthetic biochemical parameters, the response of the photosynthesis machinery was different between a metal-tolerant and a non-tolerant population of a metal excluder. In the sensitive population, Cu excess induced a decrease in g_s almost at any time and metal concentration used, whereas the A_N Cu-imposed inhibition followed a less pronounced and more delayed diminishing trend. For Cu concentrations lower than 40 μM $CuSO_4$, A_N and g_s decrease did not result in C_i reduction, suggesting that the A_N limitation was not due to g_s, probably because the lower CO_2 influx was of the same entity of its lower assimilation. At 20 μM $CuSO_4$ and at the exposure time end, the opposite occurred and C_i increased, most probably because the A_N Cu-induced decrease was extremely remarkable. At 40 μM $CuSO_4$, with respect to control conditions and almost for any time, C_i was significantly lower, thus becoming the stomatal closure the most important determinant for shaping such reduction; this occurred despite the evident declines in A_N, which depended on both stomatal and non-stomatal limitations (see also Flexas et al., 2002; Perez-Martin et al., 2009). Although in non-metallicolous plants Cu-induced stomata independent

limitation in A_N is more frequently reported (see for example Cambrollé et al., 2012; 2013), Cu has also been found to decrease C_i (Jiang et al., 2013); this clearly demonstrates that some conditions can generate also a Cu-imposed stomatal limitation to photosynthesis, as in our case at 40 μM $CuSO_4$. The tolerant population showed a Cu effect on the considered parameters that shifted in a time- and concentration-dependent manner, with a A_N and g_s decrease only at the highest Cu concentration and only after 7 days of treatment. Although limitation on A_N, when present, appeared before significant g_s reductions, the C_i decrease suggested that, already after 7 days of treatment, limited CO_2 diffusion, associated with small stomatal closure, was behind the reduction in CO_2 assimilation rates, as found in case of drought stress (Medrano et al., 2002), meanwhile later declines in g_s became more evident.

In control conditions, the tolerant population showed significantly lower g_s values with respect to the sensitive one. Probably, as a result of the adaptation to the Fenice Capanne extreme environment, subjected also to water deficit (Mascaro et al., 2001), the tolerant population constitutively operated a reduction in its transpiration rate, useful to limit water loss together with metal translocation to the shoots. Confirming this hypothesis, a significant difference was found in plant stomatal density, with the tolerant population constitutively showing a lower number of stomata on the leaf upper side. The reduced g_s in the tolerant population did not result in a lower C_i probably due to a lower, although not significantly different, A_N value. The lower biomass production always reported in the tolerant population in control conditions (see for example Colzi et al., 2011;2012) could therefore be associated with this lower A_N together with a possible shunt of the photosynthetic products toward the realization of the constitutive tolerance mechanisms. Thus, such A_N and g_s reduction in the tolerant population could be considered an indication of the evolutionary cost of metal tolerance in terms of plant photosynthetic performances.

Regarding V_{cmax}, J_{max}, and TPU values, Cu excess determined their decrease, as found for example in Cu-treated poplar species (Borghi et al., 2008) as a result of Cu-imposed stomatal closure and Cu direct impairment of the photosynthetic machinery. In any case, the two populations displayed different susceptibility to Cu also in terms of its effect on these photosynthetic parameters, the tolerant population showing lower Cu-imposed decrease. Interestingly, in the tolerant population A_N was stable till 20 µM $CuSO_4$, even if at that concentration V_{cmax}, J_{max}, and TPU had already declined, whereas in the sensitive population A_N was already affected at 10 µM $CuSO_4$, even if such parameters significantly decreased only at 20 µM $CuSO_4$. Probably, one of the reasons of the opposite response in the two populations could be the different stomatal behavior. In the tolerant population at 20 µM $CuSO_4$ stomata were still open so that lower CO_2 availability was not added to the present detrimental effect on the photosynthetic machinery and A_N was preserved. In the sensitive population at 10 µM $CuSO_4$ the Cu-mediated stomatal closure was already sufficient to decrease A_N, even if an impairment of the photosynthetic machinery was had yet to occur.

In both the populations, Cu treatments decreased g_m values, especially in sensitive plants. Probably, Cu differentially imposed on the populations morphological alternations and stunned development in leaves, that could have interfered with g_m, thus further decreasing, together with the Cu-mediated stomatal closure, the CO_2 diffusion and restricting the photosynthetic rate indirectly. Interestingly, in control conditions g_m of the tolerant population was about half of that of the sensitive one, suggesting again its constitutive adaptation to limit leaf water loss as above-mentioned.

3.4.3. Unraveling the differences in Cu-imposed effects through photosynthesis limitation analysis

Photosynthesis limitation analysis allowed a direct comparison of the Cu effect on the two populations. To the best of our knowledge, this is the first time that such analysis is applied to Cu-induced effects on photosynthesis. To date there is similar information only on a Cd-treated population of the Zn/Cd hyperaccumulator *Picris divaricata* (Tang et al., 2013). TL Cu-dependant increase was two-fold higher in the sensitive population in respect to the tolerant one and the difference was not only quantitative, as already showed by the parameters analyzed separately, but also, and most importantly, qualitative. Our analysis showed that the two populations were limited, under Cu excess, in a different way for all the parameters studied, except one. Compared to the sensitive population, in the tolerant one the effect of Cu on BL was lower, on MCL minimal and most of the limitation was due to SL, with the latter being the only limitation that did not show significant results for the inter- action population*treatment. Therefore, in the tolerant population the primary component of the Cu-induced inhibition of photosynthesis was the decrease in leaf CO_2 diffusion, even if the lower Cu-imposed stomatal closure, in respect to the sensitive population, could have advocated a different conclusion. Such result could suggest that, even if metal excluders can restrict shoot metal transfer, the evolution of metal adaptation may have involved also increased metal tolerance at the photosynthesis level. Specifically, in the tolerant population the whole photosynthetic machinery appeared less negatively affected by the metal, with the stomatal closure emerging as the most limiting factor, even if it was less negatively metal-affected as well.

3.4.4. A possible cause of the population different response: Cu effects on photosynthetic biophysical parameters and pigment concentration

Data about F_v/F_m, ETR, and Φ_{PSII} showed that such parameters underwent a lower decrease in presence of Cu in the metallicolous population with respect to the

non-metallicolous one, thus revealing, even under such photosynthetic aspect, its higher tolerance to Cu, significantly in the case of the latter two parameters. In any case, Cu-mediated enhancement of photoinhibition is a well- known Cu-imposed effect (see for example Yruela et al., 1996; Ciscato et al., 1997), probably resulting from the denaturation of antenna protein complexes or the direct inhibition of the PSII reaction center by the Cu insertion into pheophytin (Küpper et al., 2002) and the subsequent impairment of PSII electron donation (Yruela et al., 1996; Küpper et al., 2002).

Copper exposition affected pigment concentrations in both the populations, probably as a result of the direct metal toxicity or the formation of Cu–Chl complexes (Küpper et al., 2002). Even if a significant result for the interaction population*treatment was not found for such parameters, the tolerant population showed a lower decrease in pigment concentration, with significant differences for some of the $CuSO_4$ concentrations used, thus probably displaying another one of the reasons of its higher capacity of photosynthesizing on Cu excess. Beyond the different level of Cu accumulated in the leaves and the probable presence of efficient detoxification mechanisms, in this case the lower decrease of Chl concentration showed by the tolerant population could be explained also by the already assessed different interference of this metal with Fe leaf concentration (Pignattelli et al., 2013), being the Cu-induced decrease in Fe accumulation more important in the sensitive population. Copper also decreased carotenoid concentrations, probably due to the denaturation of the light harvesting complexes after Cu–Chl formation, as suggested by Küpper et al. (2002). Also in this case, the lower decrease shown by the tolerant population in the concentration of such pigments could have contributed to the maintenance of a higher photosynthetic ability in the presence of Cu excess.

Overall, our results demonstrate that, under anomalous Cu concentrations, *S. paradoxa* metallicolous and non-metallicolous populations are characterized by diverse photosynthetic responses, not only at a different degree, but also of different

nature. Some of the differences are present also under control conditions and probably largely reflect divergent evolutionary adaptation to soil factors other than Cu itself. In any case, beyond including in the research a higher number of populations, further investigations on Cu-induced effects on different parameters, such as for example ABA signaling and Cu accumulation in stomata cells, are needed for a comprehensive understanding of the observed effects of Cu exposure in the different populations.

Acknowledgements

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CHAPTER 4

Adaptation to metalliferous soils: relationship between metal stress and nutrient use efficiency in *Silene paradoxa* L. populations

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Abstract

Plants adapted to live in metal-enriched soils have to face the stress due both to high metal concentrations and to nutrient deficiency. In this work, we investigated the responses to two secondary macronutrients (Ca^{2+} and Mg^{2+}) deficiency stress by two metallicolous (Cu-tolerant and Ni-tolerant) and a non-metallicolous *Silene paradoxa* populations to evaluate the interaction between heavy metal stress and nutrient starvation. Plants were grown in hydroponics and exposed to metals (Cu and Ni) and nutrient deficiency, separately and in combination. Results showed that the two metallicolous populations were less sensitive to the nutrient deficiency compared to the non-metallicolous one, thus demonstrating that, despite the scarcity of nutrients in the soil, these populations are able to optimize the nutrient accumulation and allocation in their organs to maintain an adequate growth. When exposed to combinations of the two stresses, the metallicolous populations showed to be more efficient in nutrient utilization according to the sites of origin and to the specific metal-tolerances possessed. Moreover, metal accumulation showed to be increased in nutrient deficiency conditions, with the Cu-tolerant population being the most efficient to limit metal accumulation in its organs. The NUE (nutrient use efficiency) values demonstrated that the two metallicolous populations are indeed more efficient than the non-metallicolous one in facing the nutrient deficiency stress, depending on the presence and on the nature of the metal to which they were exposed.

4.1 Introduction

Soil is the main source of macro and micronutrients for plant life. Among soils, the metalliferous ones, both from natural and anthropogenic origin, contain a higher concentration of toxic heavy metals generally combined to a lower concentration of essential macronutrients, if compared to non-metalliferous soils. One of the basic conditions for plants to live in such restrictive environments is their ability to evolve metal tolerance (Levitt, 1980; Baker, 1987). These "metallophytes", depending on the species, can tolerate high metal concentration by limiting their root uptake and/or their translocation to the shoot ("excluders") (Baker, 1981) or they can actively accumulate metals in the shoot at extremely high concentrations ("hyperaccumulators") (van der Ent et al., 2013). These species constitute an excellent model to study the adaptation to harsh and highly stressful environments, often characterized by the simultaneous presence of different kinds of abiotic stresses. So far, in the study of metallophyte physiology there is no information about the relationship between adaptation to heavy metal excess and to the nutrient deficiency, characteristic of metalliferous soils.

Serpentine soils are naturally metal-enriched soils that originated from the weathering of different ultramafic rocks (Brooks, 1987) composed of ferromagnesian silicates, especially serpentinite (Whittaker, 1954). This kind of soil is characterized by low Ca concentration and extremely high Mg concentration, deficiency in primary macronutrients, such as N, P, K, and high contamination of Ni (up to 3600 $\mu g\ g^{-1}$, McGrath, 1995), Cr and Co (Proctor, 1971; Brooks, 1987).

Anthropogenically contaminated soils derive mainly from mining and smelting activities and present great soil damage and modifications in the vegetation structure (Sheoran et al., 2010). In addition to a possible low nutrient total concentration in such soils, the high amount of contaminants leads to disturbance in microbial communities, thus reducing nutrient bioavailability (Steinhauser et al., 2009), while the lowering of pH and loss of organic carbon lead to an increase in biovailability of

toxic metals (Barceló and Poschenrieder, 2003). In general, low soil pH decreases Ca and Mg solubility (Sheoran et al., 2008), whereas Mn and Al result more soluble reducing Mg uptake (Gransee and Fuhrs, 2013; Chen and Ma, 2013).

Therefore, the above-mentioned biophysiochemical soil properties can affect macronutrients bioavailability in soil, influencing the release of the elements from the solid phase to the liquid phase, the mobility of the elements in the soil solution and the uptake of the nutrients by plant roots (see for example Comerford, 2005). Another limiting factor of nutrient bioavailability can be the presence of high heavy metal concentrations in the soil. In fact, heavy metals are well-known to be strong competitors of other cations and anions at plant uptake sites (Shaw, 1990; Siedlecka, 1995), thus causing potential limited nutrient uptake. For example, $Ca^{2+}$and Mg^{2+} are known to compete with metal cations such as Cu^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+} , Fe^{2+} , Ni^{2+}, in the uptake and translocation by a wide range of plants (Kochian, 1991; Küpper et al., 1996). On the other hand, a beneficial interaction between Ca and Mg and Cu in condition of nutrient sufficiency or excess was widely reported in several species (Chen et al., 2013; Österås and Greger, 2006; Min et al., 2013; Kopittke et al., 2011; Lock et al., 2007; Gabbrielli and Pandolfini, 1984).

Macronutrients are required by the plants in concentrations up to thousands of µg g^{1} d.w. (Alloway, 2013); the primary ones (N, P and K) are the most limiting factors to plant life, while the secondary ones (Ca, Mg and S) are required in lower, but equally fundamental, concentrations. In particular, calcium is required in minimum concentration of 5 µg g^{-1} d.w.; it plays an important role in the cell wall and membranes structure, as an intracellular signal in the cytosol (Marschner, 1995) and as a regulator of stomata opening (Allen et al., 2001). Once taken up, Ca is translocated to the shoots via xylem through transpiration (White, 1998; 2001). In deficiency condition, it cannot be mobilized from older tissues (Clarkson, 1984), thus causing deficiency symptoms especially in young leaves. Magnesium is required at least in 2 µg g^{-1} d.w. (Marschner, 1995); it has key roles in the

photosynthetic machinery and in the structure of several proteins (Shaul, 2002). Being mobile in plant tissues, Mg deficiency symptoms appear firstly in old leaves.

A quantitative measure of plant efficiency in using nutrients for biomass production is represented by the NUE (Nutrient Use Efficiency) index. This index, in nutrient-deprived conditions, can vary depending on plant species and the severity of the deficiency (Bridgham et al., 1994).

To our knowledge, there are only few and contrasting reports about the possible interaction between heavy metal stress and nutrient deficiency conditions. In several species of microalgae, under nutrient deficiency conditions, heavy metals can decrease the nutrient utilization (Miazek et al., 2015). In *Pisum sativum,* Fe^{2+} deficiency induces the expression of Fe transporters and facilitates the transport of other divalent cations such as Cd^{2+} or Zn^{2+} (Cohen et al., 1998). When considering metal adapted species, the information becomes almost negligible. Ionomes of a few hyperaccumulator metallophytes in sufficiency conditions are reported in Singh et al. (2015). A report about the interaction between metals and nutrients in an excluder metallophyte, even though in nutrient sufficiency conditions, is the one by Pignattelli et al. (2012), where a sensitive and a Cu-tolerant population of *Silene paradoxa* showed different behavior in the allocation of some elements (such as Ca and Mg) under Cu exposition. Specifically, Cu excess affected the ionome profile in both the populations, but at a higher extent in the sensitive one, thus suggesting population-specific adaptation to the different nutrient availability in the site of origin (Pignattelli et al., 2012).

Silene paradoxa L. is an excluder facultative metallophyte that generally lives in non-metalliferous soils and occasionally in metalliferous soils (Chiarucci, 2003), thus representing a good model for comparative investigations on metal tolerance.
This study tested the hypothesis whether adaptation to metalliferous soils can modify the plant ability of nutrient utilization and its interaction with the metal accumulation. To this aim, three contrasting *Silene paradoxa* populations, one from

a non-contaminated calcareous soil (sensitive population), one from a Cu mine deposit (Cu-tolerant population) and one from a serpentine outcrop (Ni-tolerant population), were grown in Ca and Mg deficiency and exposed to Cu and Ni excess in the nutrient solution, separately and in combination. We compared root and shoot growth, nutrient and metal accumulation and NUE indexes of the three populations, hypothesizing different responses due to the different sites of origin.

4.2 Materials and methods

4.2.1 Plant material and experimental conditions

Seeds of *S. paradoxa* and adult plants were collected from populations living in a non-contaminated soil (Colle Val D'Elsa, Siena, CVD), in a Cu mine waste (Fenice Capanne, Grosseto, FC) and in serpentine outcrop (Pieve Santo Stefano, Arezzo, PSS): for description of populations and sample sites see for example Chiarucci et al. (1995) and Gonnelli et al. (2001). Seeds were sown in peat soil; 6-weeks-old plantlets of the three populations were transferred to hydroponic culture in 1-L polyethylene pots (three plants per pot) containing a modified half-strength Hoagland solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.50 mM $MgSO_4$, 20 µM Fe(Na)-EDTA, 1 µM KCl, 25 µM**,** H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.1 µM $CuSO_4$ and 0.1 µM $(NH_4)_6Mo_7O_{24}$ 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). For exposing plants to Ca and Mg deficiency, the solution was deprived of 9/10 of the compounds containing the above-mentioned cations. The counter-ions were provided by adding alternative compounds. In Ca deficient solution, $Ca(NO_3)_2$ was reduced to 0,2 mM and the counter-ion was provided by adding 3,6 mM $NaNO_3$; in Mg deficient solution, $MgSO_4$ was reduced to 0,05 mM and the counter-ion was provided by 0,45 mM K_2SO_4 (Hewitt, 1966). The nutrient solutions were supplied with 5 µM $CuSO_4$ and

5 μM $NiSO_4$. Hydroponic solutions were renewed once a week and for four weeks the plants were grown in a growth chamber (for growth conditions see Pignattelli et al., 2012). After the growth period, root samples were desorbed with ice-cold (4°C) $Pb(NO_3)_2$ (10 mM) for 30 min. Plants were then divided into roots and shoots and the dry weight of the organs was measured after oven-drying at 70° C for 1 day. Each measurement was performed on twelve replicates.

Plants collected in the fields were carefully washed with milli-Q water and roots where desorbed with the same method used for plants growing in hydroponics. Finally, they were oven-dried at 70°C for 7 days for further digestion.

4.2.2 Soil material

Soils samples were randomly collected at a depth of 0-15 cm at the three sampling sites over the entire spatial distribution of each population. Samples were oven-dried at 50°C for 7 days. After sieving to 2 mm, soil samples were homogenized and digested in *aqua regia* (3:1 v/v $HCl:HNO_3$) on a hot plate (T<50°C). Nutrient (Ca and Mg) concentrations were estimated by atomic absorption spectrometry (Analyst 200, Perkin Elmer) in six replicates. Each replicate was measured in three times.

4.2.3. Determination of nutrient and metal concentration in plants

Nutrient (Ca and Mg) and metal (Cu and Ni) concentrations in plants were determined by digesting oven-dried plant material in a 5:2 (v/v) mixture of HNO_3 (Romil, 69%) and $HClO_4$ (Applichem, 70%) in 25 ml beakers at 120–200 °C and adjusting the final volume to 10 ml with milliQ-water. Elements were determined by atomic absorption spectrometry (Analyst 200, Perkin Elmer) in six replicates. Each replicate was measured in three times. The ratio between mean dry weight and mean nutrient concentration was used as NUE index (Baligar et al., 2001).

4.2.4 Statistical analysis

Statistical analysis was carried out with ANOVA, one-way and two-way (considering nutrient deficiency 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).

4.3 Results

4.3.1 Element concentration in soil samples and plants collected in the field

Figures 4.1 and 4.2 report the concentration of Ca and Mg in the studied soils and plants. Calcium was present in significantly higher concentration in CVD non-metalliferous soil compared to the two metalliferous soils (Fig 4.1a). The CVD and PSS populations showed a higher Ca concentration in the shoots (Fig 4.1c) while FC population allocated this element preferentially in the roots (Fig.4.1b). Regarding Mg, the highest concentration was found in the PSS soil, while the lowest was found in FC soil (Fig.4.2a). The roots from CVD plants presented a significantly lower Mg concentration compared to the other populations (Fig.4.2b), while, as PSS populations, Mg concentration was higher in the shoots (Fig. 4.2c). FC population presented a significantly lower Mg concentration in shoots and a slightly higher concentration in roots, in respect to the other two populations.

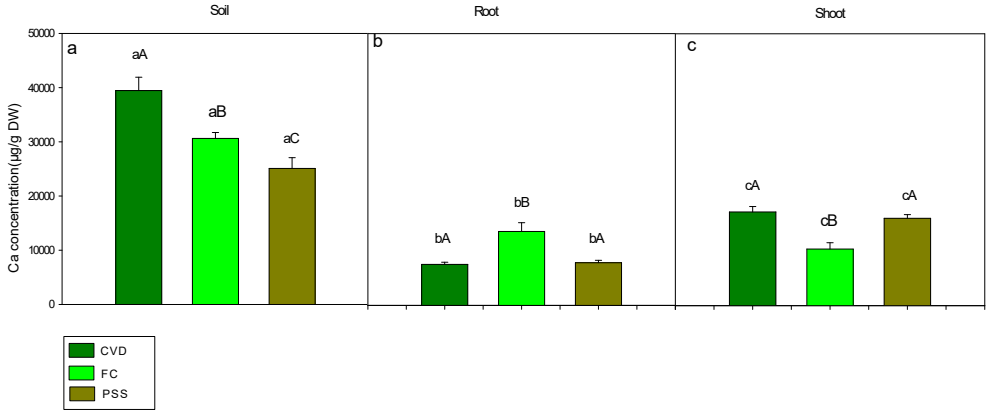


Fig. 4.1 Ca concentration (µg g^{-1} d.w.) in: a-soil; b- roots- c-shoots. Values are means of six replicates ± standard deviations. Significant differences between the means appear with different letters, small for intra-population and capital for inter-population comparisons (at least p<0,05).

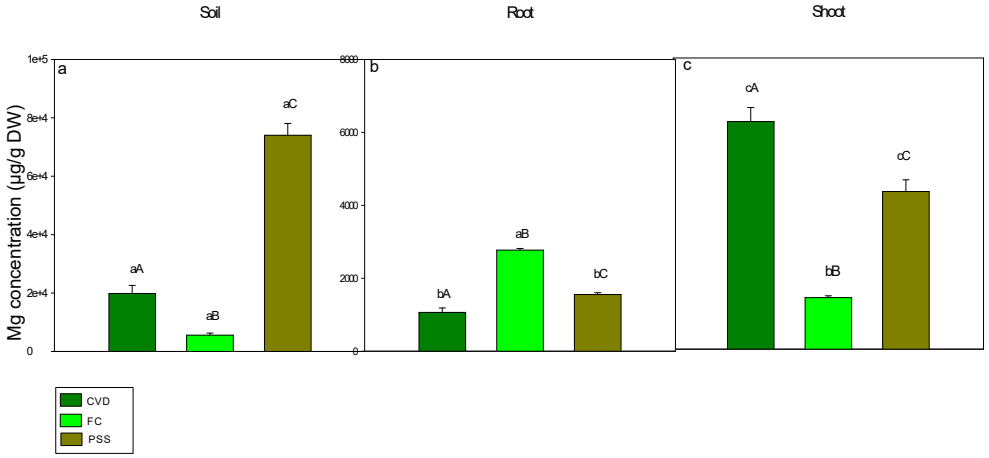
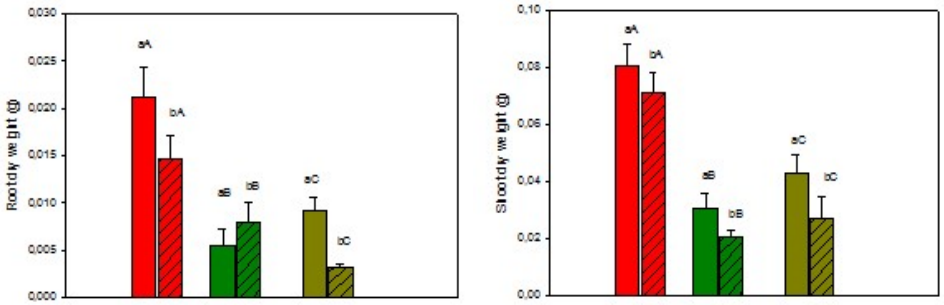


Fig.4.2. Mg concentration (µg g^{-1} d.w.) in: a-soil; b-roots; c-shoots. Values are means of six replicates ± standard deviations. Significant differences between the means appear with different letters, small for intra-population and capital for inter-population comparisons (at least p<0,05).

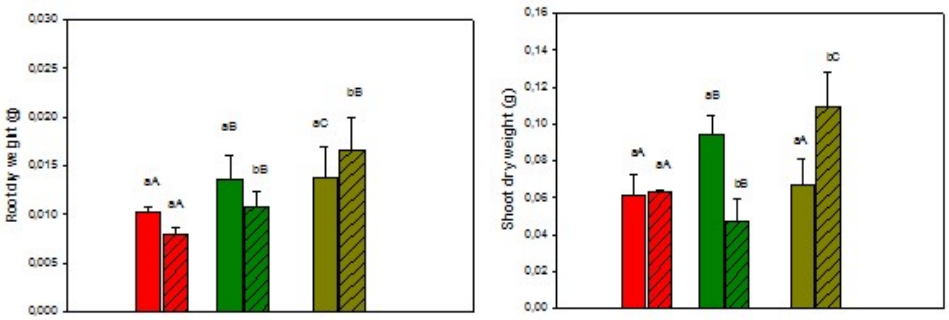
4.3.2. Effects of nutrient deficiency and metal treatment on plant growth

In Ca deficiency conditions (Fig.4.3), only CVD population showed a significant decrease in growth, both at root and shoot level (p<0,01), compared to sufficiency conditions.

CVD (non-metallicolous population)



FC (Mine deposit population)



PSS (serpentine outcrop population)

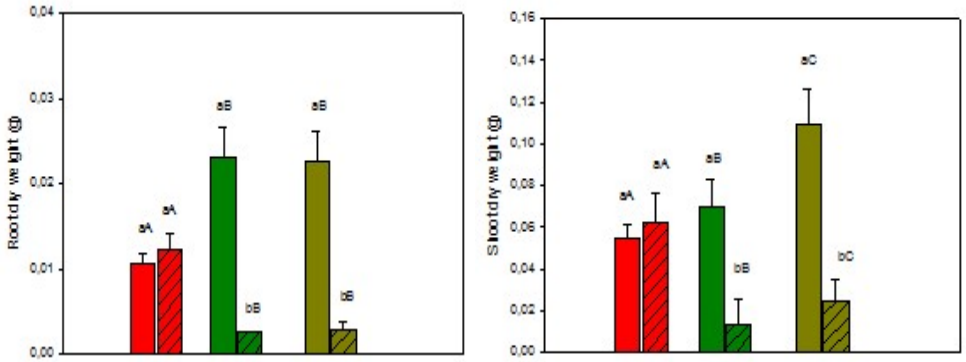
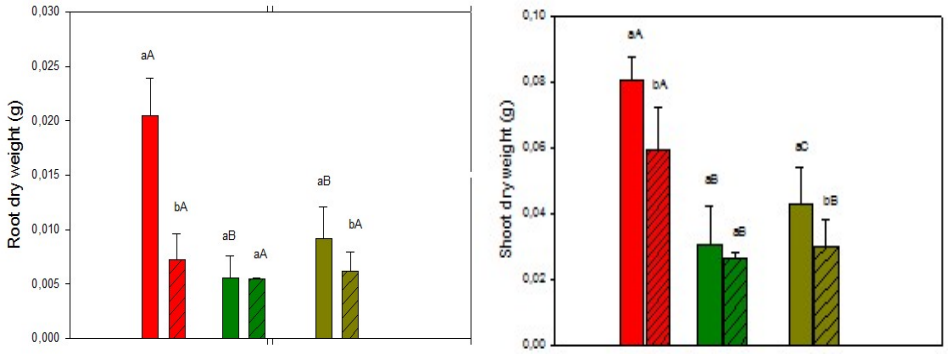
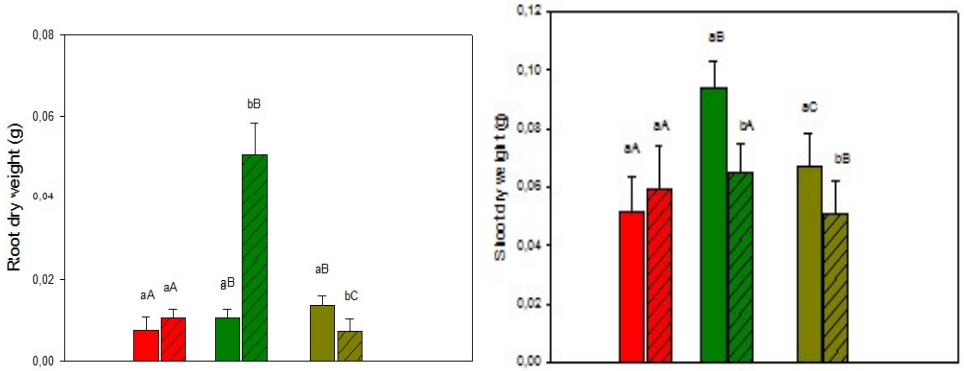


Fig.4.3. Root (on the left) and shoot (on the right) growth (dry weight, g) (means ± standard deviations) of the three *S.paradoxa* populations growing in Ca deficiency (striped) in control (red) $CuSO_4$ (green) and $NiSO_4$ (yellow) treatments. Significant differences between the means are indicated with different letters, small for metal intra-population and capital for metal inter-population comparisons (at least $p<0,05$).

CVD (non-metallicolous population)



FC (Mine deposit population)



PSS (serpentine outcrop population)

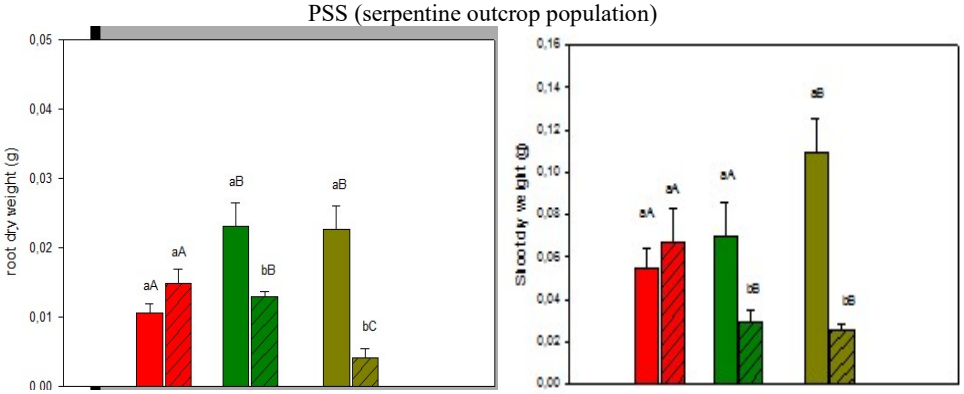


Fig.4.4. Root (on the left) and shoot (on the right) growth (dry weight, g) (means ± standard deviations) of the three *S.paradoxa* populations growing in Mg deficiency (striped) in control (red) $CuSO_4$ (green) and $NiSO_4$ (yellow) treatments. Significant differences between the means are indicated with different letters, small for metal intra-population and capital for metal inter-population comparisons (at least $p<0,05$).

When exposed to $CuSO_4$, CVD plants showed a significant decrease in both root and shoot growth ($p<0,001$), whereas FC and PSS populations were not affected. When Ca deficiency is added to Cu exposition, CVD population increases root growth and decreases shoot growth ($p<0,05$), whereas FC population and, at an extremely high extent, PSS population, reduce both root and shoot growth ($p<0,001$). When exposed to Ni, CVD population shows a reduction in root and shoot growth, compared to non-metal treated plants ($p<0,01$), while in FC population, the growth is increased significantly only at root level ($p<0,05$) and both at root and especially at shoot level in PSS population ($p<0,001$). When Ca deficiency is added to Ni exposition, root and shoot growth is reduced in CVD population ($p<0,01$) and, at extremely high extent, in PSS population ($p<0,001$), whereas, FC population increases root and shoot growth ($p<0,001$).

In Mg deficiency conditions (Fig.4.4.), only CVD population showed a significant decrease in root and shoot growth ($p<0,01$). Treatment with Cu, compared to control conditions, reduced root and shoot growth only in CVD population as compared to the other ones ($p<0,01$). Combining Mg deficiency to Cu treatment, in FC population root growth increased at extremely high extent ($p<0,001$), compared to sufficiency conditions, and shoot growth decreased, whereas in PSS plants both root and shoot growth was reduced ($p<0,001$). Adding Mg deficiency to Ni treatment, root and shoot growth decreased in all the three populations, and at a higher extent in the PSS population ($p<0,001$).

4.3.3. Nutrient (Ca and Mg) accumulation

Calcium accumulation values are shown in Table 4.1. In absence of metal treatment, Ca deficiency, compared to sufficiency conditions, significantly decreased Ca accumulation in CVD and PSS shoots and in FC roots, while it

			Control	$CuSO_4$	$NiSO_4$
CVD	Roots	Sufficient	6290± 2591 aA	4774 ± 832 aA	159 ± 63 aB
		Deficient	7489 ± 858 aA	17366 ± 4891 bB	4280 ± 754 bC
	Shoots	Sufficient	15514 ± 2817 aA	1094 ± 132 aB	1301 ± 548 aB
		Deficient	7909 ± 573 bA	519 ± 75 bB	397 ± 81 bC
FC	Roots	Sufficient	9731 ± 231 aA	4094 ± 625 aB	69± 11 aC
		Deficient	6393 ± 475 bA	303 ± 45 bB	773± 48 bC
	Shoots	Sufficient	11109 ± 751 aA	1012 ± 874 aB	753 ± 270 aC
		Deficient	12901 ± 845 bA	744± 152 bB	236 ± 86 bC
PSS	Roots	Sufficient	4220 ± 651 aA	245 ± 31 aB	72± 37 aC
		Deficient	3949 ± 945 aA	368± 51 bB	476± 62 bC
	Shoots	Sufficient	16050± 903 aA	1629 ± 851 aB	869 ± 247 aC
		Deficient	13018 ± 845 bA	1738± 154 aB	492 ± 258 bC

Table 4.1 Ca accumulation (µg g^{-1} d.w.) in the three *S.paradoxa* populations (means of six replicates ± standard deviations) in sufficiency and Ca deficiency conditions, treated with $CuSO_4$ and $NiSO_4$. Significant differences between the means appear with different letters, small for metal intra-population and capital for metal inter-population comparisons (at least $p<0,05$). Significant variations in values due to Ca deficiency appear with different colors: green for increases and red for decreases.

			Control	$CuSO_4$	$NiSO_4$
CVD	Roots	Sufficient	6129 ± 441 aA	4259 ± 493 aB	1495± 240 aC
		Deficient	13089 ± 1587 bA	5609 ± 906 aB	3044 ± 160 bC
	Shoots	Sufficient	13299 ± 2374 aA	1506 ± 453 aB	856 ± 537 aC
		Deficient	12513 ± 2809 aA	1565±275 aB	571± 65 bC
FC	Roots	Sufficient	5888 ± 421 aA	3149 ± 1311 aB	1002± 124 aC
		Deficient	6875 ± 352 bA	1508 ± 312 bB	767± 94 bC
	Shoots	Sufficient	3239± 321 aA	489 ± 133 aB	784 ± 510 aC
		Deficient	3348 ± 402 aA	904± 286 bB	635 ± 33 bC
PSS	Roots	Sufficient	3781 ± 924aA	1183 ± 118 aB	631 ± 35 aC
		Deficient	2875 ± 532 aA	2745 ± 321 bA	2769 ± 542 bA
	Shoots	Sufficient	2826± 205 aA	760 ± 340 aB	494 ± 221 aC
		Deficient	2556 ± 57 aA	1339 ± 225 bB	577 ± 124 bC

Table 4.2 Mg accumulation (µg g^{-1} d.w.) in the three *S.paradoxa* populations (means of six replicates ± standard deviations) in sufficiency and Mg deficiency conditions, treated with $CuSO_4$ and $NiSO_4$. Significant differences between the means appear with different letters, small for metal intra-population and capital for metal inter-population comparisons (at least $p<0,05$). Significant variations in values due to Ca deficiency appear with different colors: green for increases and red for decreases.

increased in the shoots ($p<0,05$). After Cu treatment and in Ca deficiency conditions, CVD accumulated Ca in the roots up to almost five-fold increase ($p<0,001$), in respect to sufficiency conditions. The same increase in root accumulation was shown by PSS population, but at a lower extent ($p<0,05$). After Ni treatment, all the three populations showed increased Ca accumulation in the roots and a simultaneous decrease in its accumulation in the shoots in Ca deficiency stress, compared to sufficiency conditions ($p<0,01$).

Magnesium accumulation values are shown in Table 4.2. In non-metal treated plants, Mg deficiency led to a significant increase of Mg accumulation in the roots of CVD and FC populations ($p<0,05$), with no corresponding decrease in the shoots. After Cu treatment, CVD population was not affected by Mg deficiency in Mg accumulation, whereas PSS population was significantly affected showing an increase in Mg accumulation in both roots and shoots ($p<0,01$). FC population, treated with Cu showed decreasing Mg concentration in roots and increasing ones in shoots, when in Mg deficiency compared to sufficiency conditions ($p<0,01$). When treated with Ni, CVD population, when in Mg deficiency condition, increased Mg concentration in the roots and decreased it in the shoots ($p<0,05$), compared to sufficiency conditions; FC population showed a decrease of Mg concentration in both the organs, especially at root level ($p<0,001$) whereas PSS population showed an increase in both the organs and at a significantly higher extent in the roots ($p<0,001$).

4.3.4. Metal (Cu and Ni) accumulation

Copper accumulation values are shown in Fig. 4.5. In sufficiency conditions, the Cu concentration in roots and shoots was the lowest in FC population and the highest in PSS population. Roots of the three populations showed a significantly higher increase of Cu accumulation in Ca deficiency compared to Mg deficiency conditions and to sufficiency conditions ($p<0,001$). Mg deficiency led to an increase in root Cu accumulation, at a higher level, in CVD and PSS populations, and at a

lower extent in FC population. A similar trend was shown in the shoots of the three populations, with the difference on PSS population decreasing shoot Cu concentration in Mg deficiency condition, compared to sufficiency one. Comparing the three populations in the same conditions, CVD population showed a significantly higher Cu accumulation in roots when in Mg deficiency ($p<0,05$), whereas FC population showed the lowest root Cu accumulation when in Ca deficiency condition ($p<0,001$).

Nickel accumulation values are shown in Fig. 4.6. In sufficiency conditions, the three populations showed no significant differences between them in Ni concentration both in roots and shoots. Interestingly, roots of the three populations showed an increase in Ni accumulation at a higher level when in Mg deficiency, in respect to Ca deficiency and to sufficiency conditions ($p<0,001$). In shoots, Ni accumulation was higher in Ca deficiency for all the three populations, if compared to Mg deficiency and sufficiency conditions ($p<0,001$). Comparing the three populations in the same conditions, in Mg deficiency, FC population showed the lowest values of root Ni concentration, whereas, in Ca deficiency, CVD population showed the highest value. At shoot level, in Mg deficiency, CVD showed the highest value of Ni concentration, whereas in Ca deficiency, FC showed the lowest value.
FC population showed lower values of metal accumulation in all the conditions and for both the metals, compared to the other two populations.

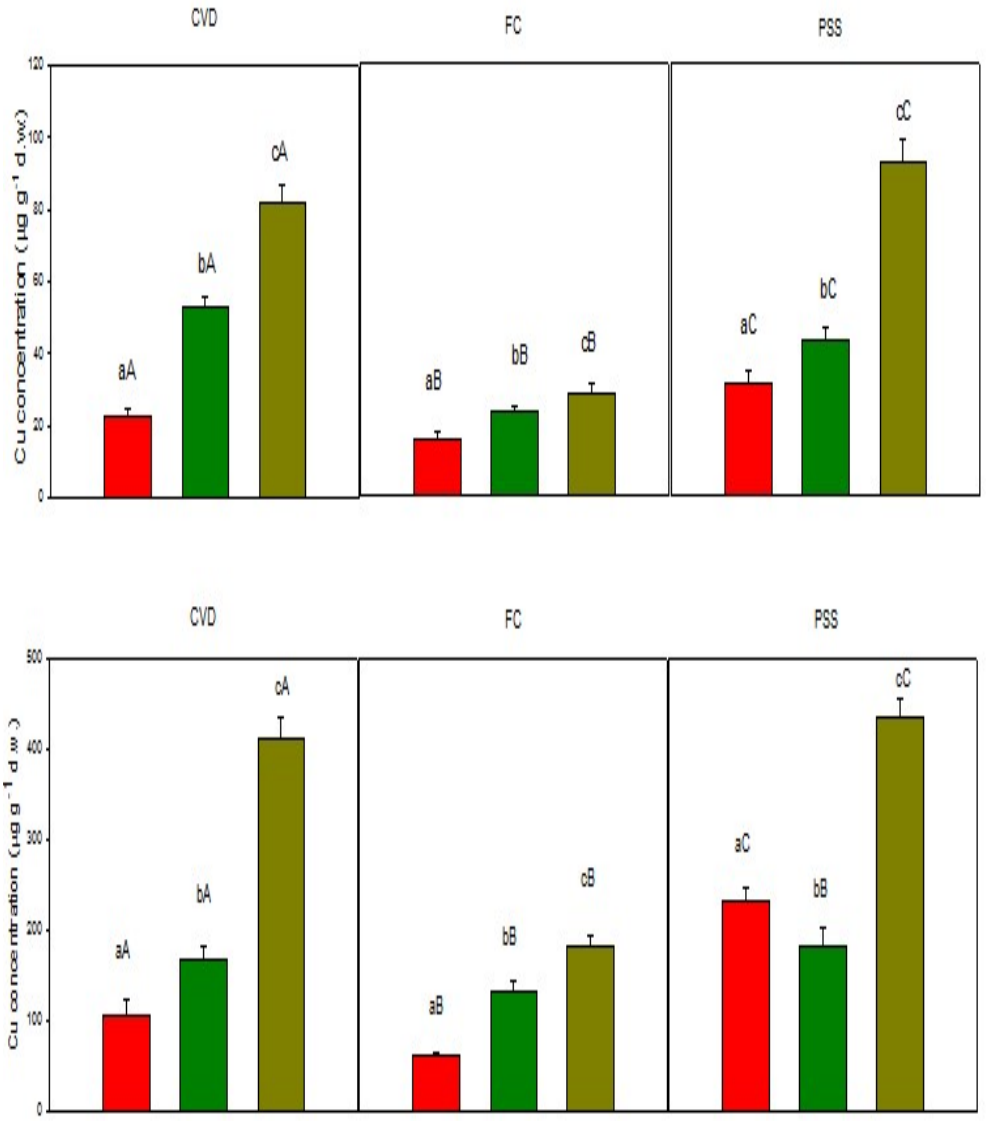


Fig. 4.5. Cu concentration (µg g^{-1} d.w.) in: roots (upper graph); shoots (lower graph) of the three *S.paradoxa* populations in sufficiency conditions (red), Mg (green) and Ca (yellow) deficiency conditions (means of six replicates ± standard deviations). Significant differences between the means are indicated with different letters, small for intra-population and capital for inter-population comparisons (at least p<0,05)

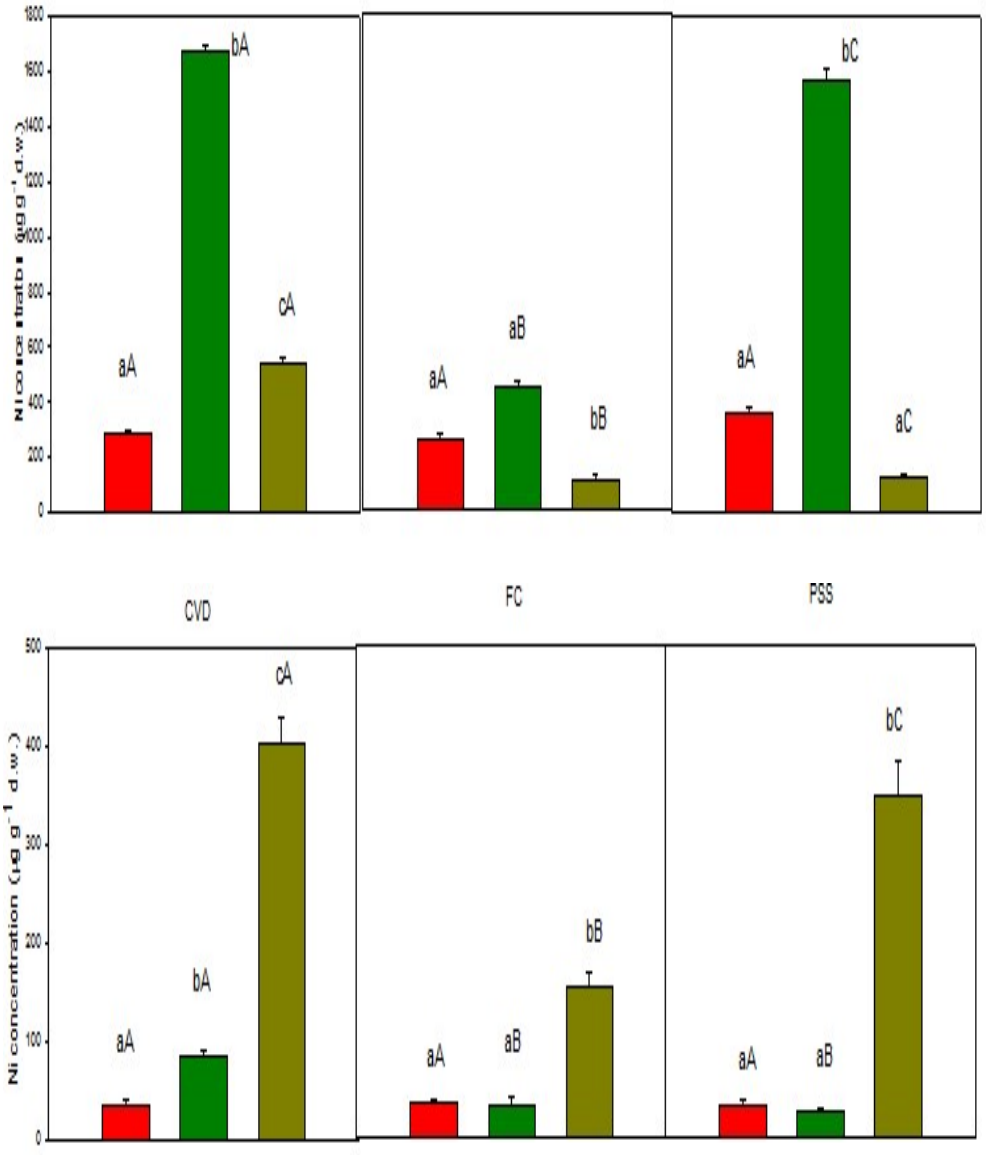


Fig. 4.6. Ni concentration (µg g^{-1} d.w.) in roots (upper graph) and shoots (lower graph) of the three *S.paradoxa* populations in sufficiency conditions (red), Mg (green) and Ca (yellow) deficiency conditions (means of six replicates ± standard deviations). Significant differences between the means are indicated with different letters, small for intra-population and capital for inter-population comparisons (at least p<0,05).

4.3.5. N.U.E. (nutrient use efficiency) for Ca and Mg

Values of NUE index for Ca are reported in Table 4.4. In absence of metal treatment, CVD population showed a reduction in Ca use efficiency in roots and an increase in shoots in Ca deficiency conditions in respect to sufficiency one. The metallicolous populations, FC and PSS, showed an increase in Ca use efficiency at root level. After Cu exposure, NUE values of CVD population remained unaltered both at root and shoot level, whereas FC and PSS population significantly increased NUE values in Ca deficiency conditions, compared to sufficiency conditions, in both the organs. When treated with Ni, CVD and PSS populations showed an increase in Ca NUE for both root and shoots, whereas FC population significantly decreased NUE values for Ca in both organs, when in Ca deficiency condition compared to sufficiency one. Comparing metal treatments among them, Cu- and Ni- treated plants show a significantly higher Ca use efficiency, in respect to non-treated plants, with the exception of CVD roots.

			Control	$CuSO_4$	$NiSO_4$
CVD	Roots	Sufficient	$3.37e^{-6}$	$1{,}41e^{-6}$	$1{,}72e^{-6}$
		Deficient	$1.96e^{-6}$	$3e^{-7}$	$2{,}26e^{-6}$
	Shoots	Sufficient	$5.18\ e^{-6}$	$3{,}07e^{-5}$	$6{,}19e^{-5}$
		Deficient	$9.04\ e^{-6}$	$5.94e^{-5}$	$7{,}24e^{-5}$
FC	Roots	Sufficient	$7.71e^{-7}$	$2{,}57\ e^{-6}$	$5{,}89e^{-4}$
		Deficient	$1.25e^{-6}$	$3{,}83\ e^{-5}$	$1{,}44e^{-4}$
	Shoots	Sufficient	$5.55\ e^{-6}$	$1{,}32e^{-4}$	$9{,}34e\text{-}^{4}$
		Deficient	$4.87\ e^{-6}$	$8e^{-5}$	$5{,}29e^{-4}$
PSS	Roots	Sufficient	$0.253e^{-6}$	$1{,}43e^{-5}$	$9{,}34e^{-4}$
		Deficient	$3.11e^{-6}$	$1{,}77e^{-5}$	$9{,}92e^{-7}$
	Shoots	Sufficient	$3.40\ e^{-6}$	$5{,}76e^{-5}$	$1{,}48e^{-4}$
		Deficient	$4.80\ e^{-6}$	$1{,}97e^{-5}$	$7{,}03e^{-5}$

Table 4.4 N.U.E. index (g dry matter/ µg g^{-1} Ca concentration) referred to Ca (Ca use efficiency) in the three *S.paradoxa* populations in sufficiency and Ca deficiency conditions, treated with $CuSO_4$ and $NiSO_4$. Significant variations in values due to Ca deficiency appear with different colors: green for increases and red for decreases (at least $p<0{,}05$).

Values of NUE related to Mg are reported in Table 4.5 In absence of metal treatment, Mg use efficiency is not significantly affected by Mg deficiency conditions in all the three populations at shoot level, whereas at root level it is evident a reduction in Mg use efficiency for CVD population and an increase for FC and PSS populations. The same trend in CVD population was shown also upon Cu and Ni treatments. In the FC population exposed to Cu, Mg use efficiency was reduced at both root and shoot level by Mg deficiency, whereas in PSS population it was reduced in the roots and increased in the shoots. Treatment with Ni affected all the three populations, exclusively at root level, reducing Mg use efficiency in Mg deficiency conditions. Comparing metal treatments among them, Cu- and Ni- treated plants show a significantly higher Mg use efficiency, in respect to non-treated plants, with the exception of CVD roots.

			Control	$CuSO_4$	$NiSO_4$
CVD	Roots	Sufficient	3.46 e^{-6}	2,17 e^{-6}	1,85 e^{-5}
		Deficient	0.55 e^{-6}	9,89 e^{-7}	4,03 e^{-6}
	Shoots	Sufficient	6.05 e^{-6}	2,39 e^{-5}	1,13 e^{-4}
		Deficient	4.76 e^{-6}	1,72 e^{-5}	1,59 e^{-4}
FC	Roots	Sufficient	1.27 e^{-6}	5,40 e^{-6}	4,10 e^{-5}
		Deficient	1.54 e^{-6}	2,12 e^{-5}	2,86 e^{-5}
	Shoots	Sufficient	19.0 e^{-6}	2,07 e^{-4}	1,67 e^{-4}
		Deficient	19.7 e^{-6}	7,55 e^{-5}	1,20 e^{-4}
PSS	Roots	Sufficient	2.83 e^{-6}	2,97 e^{-5}	1,07 e^{-4}
		Deficient	5.14 e^{-6}	4,92 e^{-6}	4,50 e^{-6}
	Shoots	Sufficient	19.3 e^{-6}	1,31 e^{-6}	3,07 e^{-4}
		Deficient	26.2 e^{-6}	2,34 e^{-5}	1,32 e^{-4}

Table 4.5 N.U.E. index (g dry matter/ µg g^{-1} Mg concentration) referred to Mg (Mg use efficiency) in the three *S.paradoxa* populations in sufficiency and Mg deficiency conditions, treated with $CuSO_4$ and $NiSO_4$. Significant variations in values due to Mg deficiency appear with different colors: green for increases and red for decreases (at least $p<0,05$).

4.4 Discussion

4.4.1 Nutrient concentrations in soil and plants collected in the field

As expected, in the soil samples, the highest Ca concentration was found in the CVD non-metallicolous calcareous soil and the lowest in the PSS serpentine outcrop, whereas the Mg concentration showed a total inverse trend, being the lowest concentration in CVD soil and the highest in PSS serpentine soil. Regarding plants collected in the fields, FC and PSS metallicolous populations showed a high capacity to accumulate Ca, at the same level of CVD population, despite being their soils of origin much poorer in Ca than the CVD soil. Interestingly, PSS population coming from a site of origin extremely poor in Ca, shows no significant difference in Ca accumulation patterns with CVD population. Despite the extremely high and potentially toxic Mg concentration in the PSS serpentine soil, the corresponding plants collected in the field did not present an excessive concentration of the nutrient in their organs compared to the other two populations, whose soils of origin do not contain toxic Mg concentrations. The PSS serpentine population seems to actively limit Mg entrance and allocation in the roots. A possible Mg exclusion strategy was reported also for several other serpentinophyte species, both obligate and facultative (Shallari et al., 1997; Proctor, 1999; Dudić et al., 2007). The preferential allocation of Ca and Mg in the roots of FC population rather than the shoots could be aimed to an increase in root growth. In fact, it is known that, in nutrient deficiency conditions, plants tend to allocate biomass in the underground organs which are closer to the source of deficient nutrient and are appointed for its uptake (Brouwer, 1962; Bazzaz, 1997).

Therefore, a high difference between macronutrients concentrations was showed in soils, but not in plants, suggesting that the studied model is adequate

to investigate on possible differences in the ability of nutrient utilization in plants adapted to metalliferous and nutrient deficient soils.

4.4.2. Effect of nutrient deficiencies and metal treatments on plant growth and nutrient allocation

In absence of metal treatment, Ca deficiency hampered root and shoot growth only in the non-metallicolous population (CVD), probably because this population comes from a Ca-enriched soil of origin and it is not adapted to a shortage in Ca supply. In this population, Ca deficiency leads to a preferential Ca allocation in the roots rather than in the shoots. This phenomenon follows the model of the "functional equilibrium", according to which the plant responds to a decrease in underground resources with an increase in underground allocation (see Poorter and Nagel, 2000). The metallicolous populations were not affected by Ca deficiency in their growth, suggesting an adaptation mechanism to poor nutrient supply of the soils of origin. Metal treatments hampered growth of non-metallicolous population but not of the two metallicolous populations, as already reported in Martellini et al. (2014). In the Cu-treated non-metallicolous population, the Ca deficiency-induced root growth could be related to an increase in Ca root allocation for the reasons above-mentioned (Bazzaz, 1997; Poorter and Nagel, 2000), while in Ni-treated plants, the same increase in Ca root allocation is not corresponding to an increase in root growth. Thus, the non-metallicolous population varies its growth and Ca allocation according to the metal to which it is exposed. In the Cu-tolerant population (FC), Ca deficiency hampered root and especially shoot growth and decreased Ca concentration in Cu-treated plants. This latter result could be probably due to Cu exclusion mechanisms (widely demonstrated in Gonnelli et al., 2001; Colzi et al., 2011 and 2012) that could prevent the uptake of other competing divalent cations such as Ca. In fact, alterations in plant ionome can be caused directly by variations of nutrient content in soils or by the impairment of ion transporter and/or indirectly by changes in

cell wall structure (Salt et al., 2008). Treatments with Ni led to a Ca deficiency-dependent increase in root and shoot growth and a preferential allocation of Ca in the roots. FC population, besides being Cu-tolerant, was demonstrated to be also Ni-tolerant, but at a lower extent compared to Cu (Gonnelli et al., 2001), thus the lower restriction of Ca accumulation compared to Cu-treated plants. The PSS serpentine population showed a metal-dependent increase in growth and a Ca deficiency-dependent decrease for both Cu- and Ni-treated plants. This population showed to be Ni-tolerant but not Cu-tolerant (Gonnelli et al., 2001) and probably because of the absence of Cu exclusion mechanisms, Ca was accumulated in roots. The increase of Ca concentration in the roots and decrease in the shoots of Ni-treated plants was probably due to Ni exclusion mechanisms that involves other divalent cations such as Ca that cannot be translocated to the shoots.

The growth of non-metallicolous populations was extremely affected by Mg deficiency, especially at root level. Despite the decrease in Mg concentration, going from sufficiency to deficiency, this population showed to concentrate Mg mostly in the roots, probably in attempt to promote the growth of a wider root volume to explore the nutrient solution is search for the missing nutrient. This mechanism was similar to the one adopted by the same population in Ca deficiency stress. It is also noticeable how the allocation of Mg in this population growing in hydroponics was inverse compared to the plants collected in the field, being the root the main allocation compartment.

The growth of the two metallicolous populations were not affected by Mg deficiencies at both root and shoot level. Curiously, the Cu-tolerant population showed a Cu-dependent increase of root growth in Mg deficiency conditions, almost five-fold higher compared to the growth in absence of Cu treatment. A similar root growth increase in response to stresses in the Cu-tolerant population exposed to the same Cu concentration (5 μM) was already reported in previous works (Martellini et al., 2014; Taiti et al., 2016). Being the Mg concentration very

low in the soil of origin, this population probably invests in root growth to search for adequate Mg content in nutrient medium. Despite of this, the population showed to allocate Mg preferentially in the shoots, when in Mg deficiency conditions, probably because of its fundamental role in photosynthetic process. This result demonstrates how this population adapted to the low Mg supply. In fact, a decrease in Mg content in leaves was recorded for non-metallophytes in Cu excess, as for example in *Cucumis sativus* (Alaoui-Sossé et al., 2004) and even in *Silene paradoxa* non-metallicolous population did not vary significantly in the above-mentioned conditions. In the Cu-tolerant population, a Ni-dependent decrease in root and shoot growth and Mg concentration from sufficiency to deficiency conditions could be due to the fact that Ni is less abundant than Cu in the soil of origin and, in the presence of this metal, this population could not respond properly to other kind of stresses.

Interestingly, the PSS serpentine population, that lives in an extreme Mg-enriched soil, not only is unaffected by Mg deficiency in growth, but also does not present a Mg deficiency-dependent variation in Mg accumulation, probably because it physiologically limits Mg uptake to prevent toxic concentrations of the element and thus it is not sensitive to Mg variations in the environment. The metal treatments, especially Ni, increased root and shoot growth of this population, but the growth is reduced when the two stress are applied in combination. This population, when in Mg deficiency conditions, shows a metal-dependent increase of Mg allocation in both roots and shoots probably because metal divalent cations compete with Mg in several biomolecules (for example, Cu substitutes Mg in chlorophylls impairing chlorophyll functionality, see Küpper et al., 2002). In Cu-treated plants of PSS population, Mg translocation to shoots was increased probably in attempt to avoid Cu accumulation in shoots at toxic levels. In fact, this population, not being Cu-tolerant (Gonnelli et al., 2001), does not exclude Cu that hence can freely compete with Mg at shoot level. Instead, Ni-tolerance of this population (Gonnelli et al., 2001) could be the main cause for a major

increase of Mg allocation in the roots, as Ni translocation to the shoots is limited due to the exclusion mechanisms.

4.4.3 Effect of nutrient deficiencies on metal accumulation

In sufficiency conditions, Cu and Ni concentrations in the three populations confirm the exclusion mechanisms of the metal tolerant populations. In all the three populations, both at root and shoot level, the Ca deficiency stress determined an increase in Cu concentrations, from three to five-folds, compared to Mg deficiency stress and to sufficiency conditions. That could be due to competition between Ca and Cu at uptake sites, with Cu having more available sites in Ca scarcity. For this reason, the Cu-tolerant (FC) population, which is a Cu excluder, does not change its Cu inner concentrations, unlike the other two populations do, when in deficiency conditions, both at root and, even more, at shoot level. Increases in root Cu accumulation and Cu translocation to shoots in Ca deficiency conditions was also reported for *Vitis vinifera* (Chen et al., 2013) and *Picea abies* (Österås and Greger, 2006).

Ni concentration, interestingly, increases in the roots of all the three populations, at a higher extent in Mg deficiency rather than in Ca deficiency, whereas in the shoots Ni accumulation increased at a higher extent in Ca deficiency, in comparison to Mg deficiency and to sufficiency conditions. The increase of Ni translocation towards the shoots in Ca deficiency was also reported for rice (Aziz et al., 2014). It was already reported for the Ni hyperaccumulator *Alyssum bertolonii* (Gabbrielli and Pandolfini, 1984) that Ca and, at a higher extent, Mg enhances Ni tolerance and decreases Ni uptake. That could explain the higher Ni root uptake in Mg deficiency stress even in the excluder *Silene paradoxa*. Even in this case, the FC population showed lower values of Ni concentrations, probably due to its Ni-tolerance.

4.4.4. NUE index

The NUE indexes followed the same trend for Ca and Mg in absence of metal treatments. The NUE in non metallicolous population (CVD) decreased in nutrient deficiency, while it increased in the two metallicolous populations, especially at root level. This result demonstrates how the adaptation to metalliferous and nutrient-poor soils by the two metallicolous populations improves nutrient utilization, especially in the organs appointed to their uptake. In the two metallicolous populations it is evident a Cu-induced increase of Ca NUE, that is not present in the non-metallicolous one. Interestingly, the presence of Ni decreases the Ca use efficiency in the Cu-tolerant population.

Regarding Mg use efficiency, the Cu-tolerant population showed an inverse trend than the other two populations, with an increase and a decrease in root NUE, respectively. The Cu-induced increase of Mg use efficiency in roots, under Mg deficiency conditions, was in accordance with the increase of root growth and the Mg accumulation in roots of this population. Interestingly, Ni showed to impair Mg NUE in roots of the three populations, while NUE in shoots remained unaltered. Probably this can be related to the higher Ni accumulation in Mg deficiency conditions in all the three populations at root level that can reach toxic level for the plants. Altogether, these results demonstrated that the plant ability to maximize nutrient utilization is dependent on the soil of origin and on the presence of metals in the nutrient medium.

4.5. Conclusion

Our results suggested a difference between metallicolous and non-metallicolous *Silene paradoxa* populations in facing nutrient deficiency stress, both in absence and presence of metals. The metallicolous populations showed a higher ability in nutrient utilization in deficiency conditions, especially at root level, in a metal-dependent way. The mine waste Cu-tolerant population showed an inverse

behavior and an inverse nutrient allocation trend in respect to the other two populations, especially in Mg deficiency stress. We can conclude that metallicolous populations not only have adapted to toxic heavy metal concentrations but also to nutrient deficiency stress, through mechanisms (for example, implementing high affinity transport system or a higher membrane hyperpolarization) that yet have to be clarified.

CHAPTER 5

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 *Silene paradoxa* populations, from a non-contaminated soil, a serpentine soil and a Cu mine soil respectively, were cultivated in the presence/absence of Ni or Cu 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 Cu mine population grown in the presence of Cu, 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.

5.1 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 (Atkinson and Urwin, 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 Urwin, 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; Bååth 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 et al., 2004; Walters et al., 2005). For example, in wheat metal-induced proteins are

thought to be responsible for Cd-induced resistance against *Fusarium* infection (Mittra et al., 2004) and in *Arabidopsis* Cd 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 non-accumulating 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 non-accumulator 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 Cu-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 toward biotic stress is the recognition of certain molecules derived by potentially pathogenic microbes and known as elicitors or MAMPs/PAMPs (microbe/pathogen-associated molecular pat- terns). 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, þ-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 toward pathogens, phytoalexins are a heterogeneous group of low molecular mass secondary metabolites with antimicrobial activity (Ahuja 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 (Ahuja 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 metallicolous and non-metallicolous populations of *Silene paradoxa* L. grown in presence and in absence of metals.

The species *S. paradoxa* is an excluder pseudo-metallophyte, 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.

5.2 Materials and methods

5.2.1. Plant material and experimental conditions

S. paradoxa L. seeds were collected from plants living on non- contaminated soil (Colle Val D'Elsa), serpentine soil (Pieve Santo Stefano) and a copper mine deposit (Fenice Capanne) 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 (Hoagland and Arnon, 1950) in milliQ-water (Millipore, Billerica, MA, USA)

buffered with 2 mM 2- morpholinoethanesulfonic acid (MES), pH 5.5, adjusted with KOH and different copper ($CuSO_4$) or nickel ($NiSO_4$) 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) $Pb(NO_3)_2$ (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◦ 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.

5.2.2. Determination of element concentration

Element concentrations were determined by digesting oven- dried plant material in a 5–2 (v/v) mixture of HNO_3 (Romil, 69%) and $HClO_4$ (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).

5.2.3. 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 1 L 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).

S. paradoxa leaves were removed from plants and placed directly into boxes containing moist filter paper. 10 µL droplets of water (control) and 3×10^{-4} M CP were applied on the lover 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 6 h, 24 h and 48 h 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).

5.2.4. 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 enables 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 emit- ted. The fluorescence spectrum of CP in solution was also measured (h_{ex} = 365 nm, h_{em} = 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 h_{ex} = 365 nm, h_{em} = 460 nm and slit 5.

5.2.5. Leaves treatments, protein extraction and immune-blot analysis

S. paradoxa leaves were excised from the plant and floated for 5 h on water with gentle shaking. Leaves were infiltrated by means of a hypodermic syringe with 3×10^{-4} M CP. Control leaves were infiltrated with distilled water. Leaves were incubated for 15–60 min at room temperature and then frozen in liquid nitrogen. For protein extraction, leaves were grounded to a fine powder in liquid N_2 and added of 400 µl 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, 1× protease inhibitor cocktail P9599 (Sigma–Aldrich) and 1 mM dithiothreitol (Galletti et al., 2011). After the samples were centrifuged at 14,000 × 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 mem- brane (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 50 mM Tris–HCl pH 7.0 buffer containing 2% SDS and 0.1 M 2-mercaptoethanol for 30 min at 55 ◦C and then incubated with *Arabidopsis thaliana* MPK3 and MPK6 antibodies (Sigma–Aldrich), which were used as quantitative references. Signal detection was performed as above reported.

5.2.6. 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).

5.3 Results

5.3.1. 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 (Table 5.1).

5.3.2. 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 (Table 5.2). As for nickel accumulation, all the populations displayed similar values in roots and in shoots (Table 5.2).

Table 5.1 Biomass production (mg per plant) of the three *Silene paradoxa* populations treated with 5 µM $CuSO_4$ or 5 µM $NiSO_4$ for eight weeks. Values are means ± standard deviation of twelve replicates. Significant differences between the means appear with different letters (*$p < 0.05$, **$p < 0.01$).

Population	Root			Shoot		
	Control	$CuSO_4$	$NiSO_4$	Control	$CuSO_4$	$NiSO_4$
Sensitive	35.7 ± 2.1a	25.4 ± 3.3b*	29.0 ± 2.3b*	169.7 ± 17.2a	105.8 ± 23.0b*	79.8 ± 14.7b**
Copper mine	25.0 ± 5.4	44.3 ± 22.3	29.6 ± 15.0	164.8 ± 19.4	192.4 ± 84.0	139.0 ± 55.9
Serpentine	26.9 ± 4.6	42.5 ± 17.7	24.4 ± 12.6	170.0 ± 66.3	184.1 ± 57.1	150.8 ± 34.0

Table 5.2 Metal accumulation (µg g^{-1} dry weight) in the three *Silene paradoxa* populations treated with 5 µM $CuSO_4$ or 5 µM $NiSO_4$ 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).

Population	Root				Shoot			
	$CuSO_4$		$NiSO_4$		$CuSO_4$		$NiSO_4$	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Sensitive	8.7 ± 4.3	105.9 ± 18.7b*	15.9 ± 3.4	284.6 ± 11.2	5.1 ± 1.0	22.6 ± 2.5b*	2.3 ± 0.7	36.8 ± 5.2
Copper mine	11.2 ± 2.5	61.3 ± 5.2a	14.2 ± 3.7	251.6 ± 25.7	5.2 ± 0.9	15.3 ± 2.3a	2.8 ± 0.5	35.2 ± 3.6
Serpentine	7.4 ± 3.2	185.4 ± 11.6c**	22.8 ± 7.1	302.9 ± 19.1	3.9 ± 0.8	31.2 ± 3.2c*	3.1 ± 0.9	33.2 ± 5.4

5.3.3. Fluorescence of cerato-platanin

The fluorescence spectrum of cerato-platanin is shown in Fig.5.1 together with the spectrum of one of the samples of our experiments. No interference of the two spectra was present at 460 nm, 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 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 generally termed as "phytoalexins" as in Pazzagli et al. (1999), Scala et al. (2004), Lombardi et al. (2013).

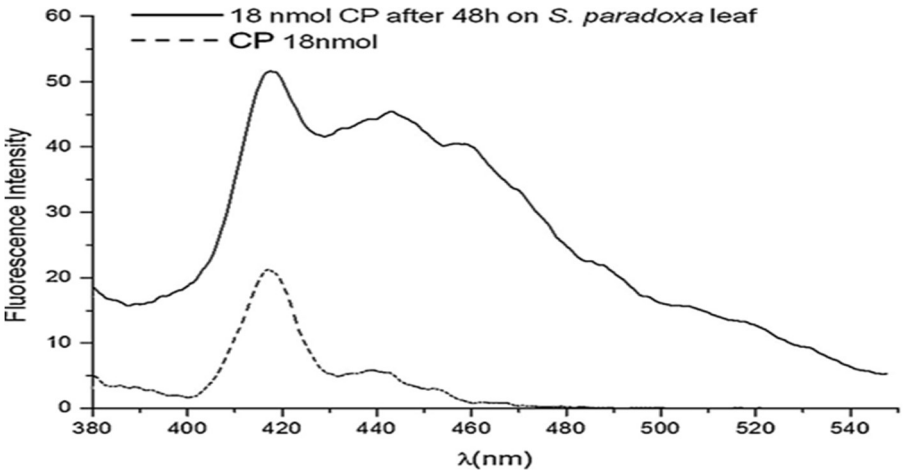

Fig 5.1 Fluorescence emission spectrum of 18 nmol of cerato-platanin (CP) before (dashed line) and after 48 h of incubation on lower surface of *S. paradoxa* leaf (solid line).

5.3.4. Copper and nickel effect on phytoalexin production

Leaves from the three *S. paradoxa* populations were assayed for phenolic compound production after a 24 h treatment with cerato- platanin (Fig.5.2).

When leaves were not exposed to CP, in the sensitive and the Cu mine populations the levels of fluorescence were not significantly different between

control and metal treated plants, whereas in the serpentine 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 Cu mine population displayed a higher value of fluorescence when grown in the presence of Cu and a lower one when grown in the presence of Ni, 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 Cu-treated CP exposed plants in the case of the Cu mine population compared to the sensitive or the serpentine one ($p < 0.05$).

5.3.5. Time dependant-production of phytoalexins

In Fig.5.3 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 Cu in the culture medium. In CP exposed samples, an increment of that value occurred and it was significantly higher in the samples from Cu-treated plants ($p < 0.05$), except for 6 h of exposition. Comparing the populations between themselves, the only significant difference present was between Cu treated-CP exposed plants, being the values of the samples from the Cu 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 Cu 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.5.4. 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 Cu in the culture medium samples from the sensitive plants showed lower values ($p < 0.05$) and those ones from the Cu 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 Cu mine population samples were significantly higher than those ones from the sensitive population samples ($p < 0.01$).

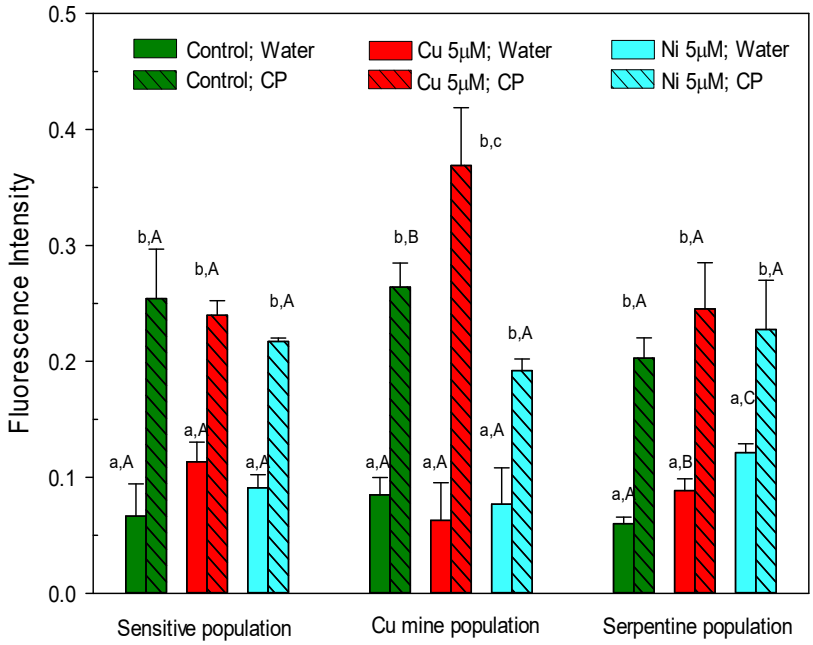

Fig. 5.2 Fluorescence emission at 460 nm of 3 nmol of cerato-platanin (CP) after 24 h of incubation on lower surface of *S. paradoxa* leaves from the three populations. Incubation with 10 µL of water was used as reference. Values are means of three replicates ± standard deviations. Significant differences between the means appear with different letters, small for metal intra-treatment and capital for metal inter-treatment comparisons (at least $p < 0.05$).

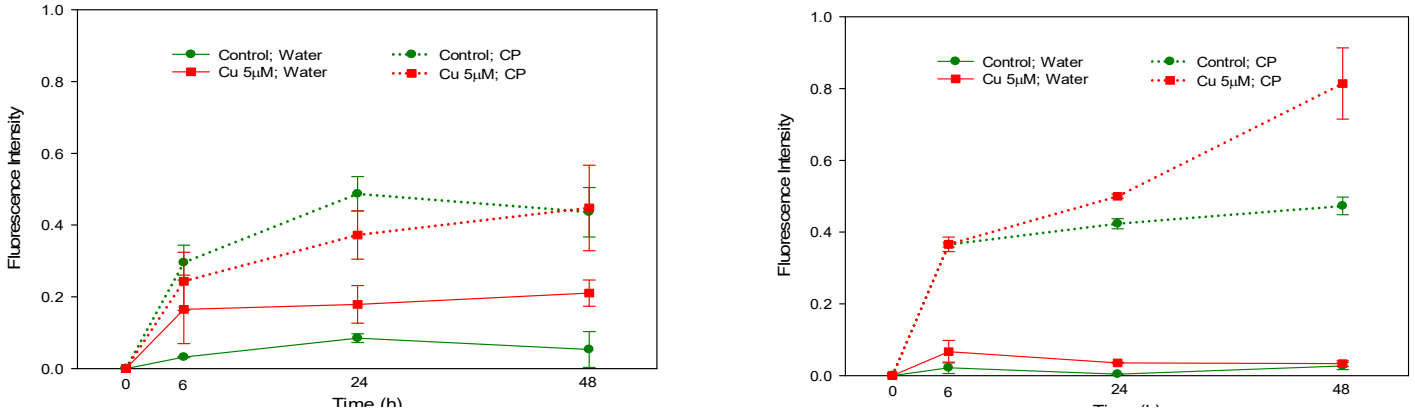

Fig 5.3. Time dependent phytoalexins production upon cerato-platanin (CP) treatment. Fluorescence emission at 460 nm in the sensitive (A) and in the Cu mine (B) populations grown in control and 5 µM $CuSO_4$ solution after incubation with 3 nmol of CP for 6, 24 and 48 h. Incubation with water was used as reference. Values are means of three replicates ± standard deviations.

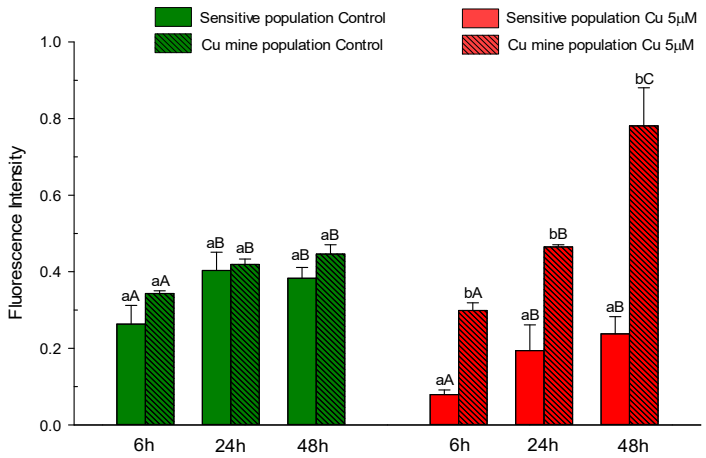

Fig.5.4. Net production of phytoalexins after incubation with 3 nmol cerato-platanin (CP) for 6, 24 and 48 h in the sensitive and in the Cu-mine populations grown in control and 5 µM $CuSO_4$ solution. The net production of phytoalexins was calculated by subtracting the production of phytoalexins induced 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 $p < 0.05$).

5.3.6. Copper concentration dependent-production of phytoalexins

Fig.5.5 reports the fluorescence values from plants cultivated at different Cu concentrations. In CP non exposed plants, the level of phytoalexins did not increased significantly in both the populations. In Cu-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 5 µM $CuSO_4$ 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 Cu in the culture medium (Fig.5.6). In Cu mine plants the net amount of phytoalexins produced increased with increasing Cu 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 ($p < 0.05$).

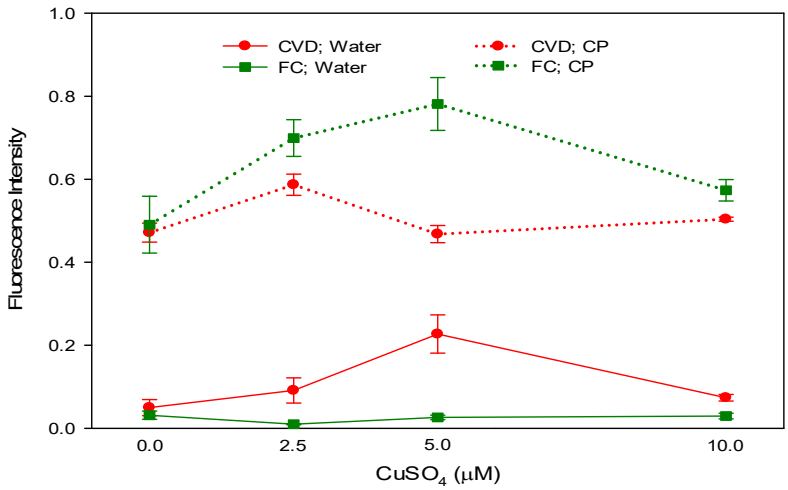

Fig.5.5. Concentration-dependent phytoalexins production in *S. paradoxa* sensitive (CVD) and Cu mine (FC) populations after 48 h of incubation with 3 nmol of cerato-platanin (CP). Incubation with water was used as reference. Values are means of three replicates ± standard deviations.

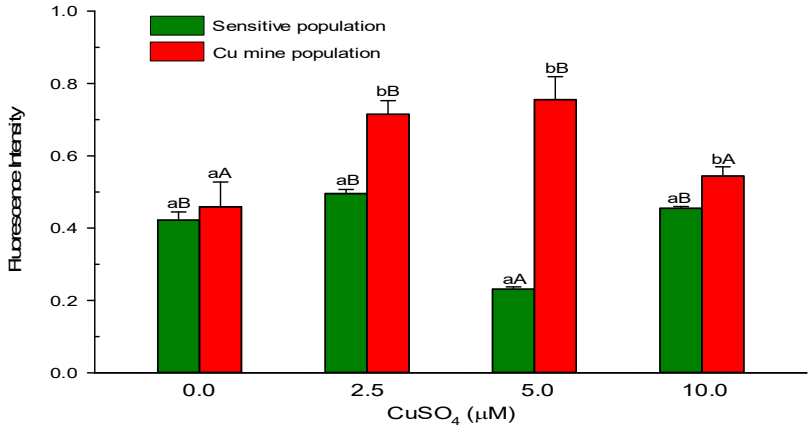

Fig. 5.6 Net production of phtytoalexins after 48 h of incubation with 3 nmol of cerato-platanin (CP) in sensitive and Cu mine populations grown at different Cu concentrations. The net production of phytoalexins was calculated by subtracting the production of phytoalexins induced by water from phytoalexins production induced by cerato-platanin at various Cu 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 $p < 0.05$).

5.3.7. 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 defense 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 defense signaling (Pitzschke et al., 2009). Immunoblot analysis of CP-treated leaves showed that either in Cu mine and sensitive plants treated with water the activation of kinases was not detectable (Fig.5.7A and C). On the contrary, the treatment with CP induced an increase in kinases phosphorylation and such increase was more

evident in both sensitive and Cu mine plants grown in Cu containing medium when compared to the same plants grown in control medium (Fig.5.7B and D). Results also showed that the kinase activation was one of the first signal in defense 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 Cu mine population grown in 5 µM copper a basal level of kinase activation was observed also without CP treatment (Fig.5.7D).

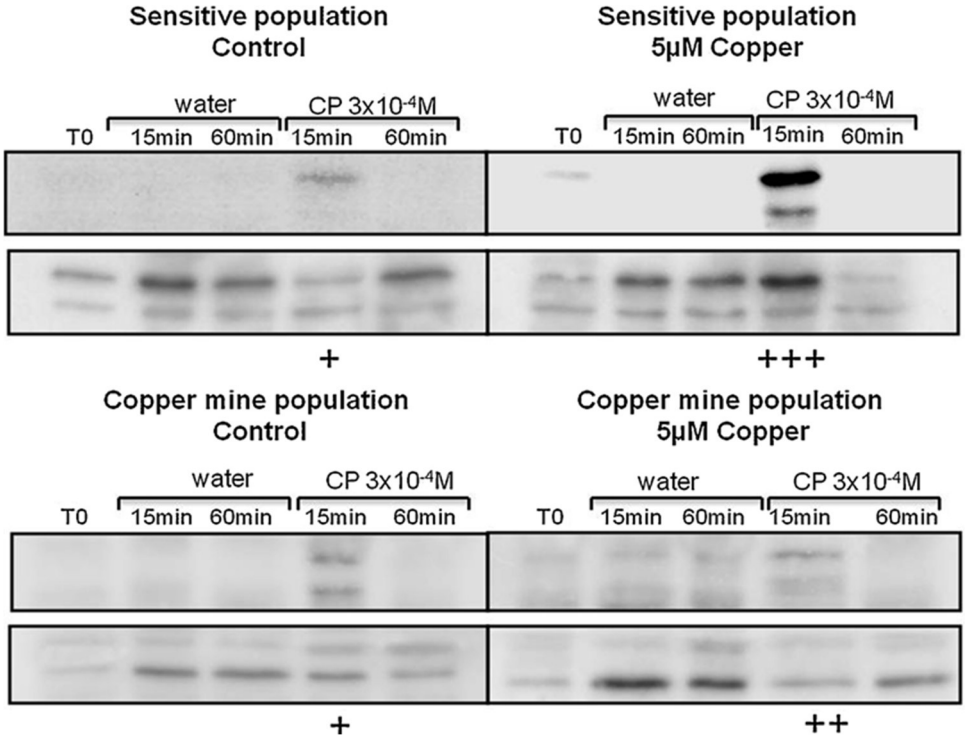

Fig. 5.7 MAPKs activation in *Silene paradoxa* leaves treated with 3 nmol of cerato-platanin (CP). Phosphorylation of MAPK in sensitive (A) and copper mine (C) leaves grown in water (left panel) and respectively (B and D) in Cu medium (right panel) visualized with anti ERK1/2 antibodies. In each figure 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.

5.4 Discussion

5.4.1. 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 (Table 5.1). 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 (Table 5.1), even if their features of Cu and Ni tolerance were reported to be different. The serpentine population was shown to be Ni-tolerant but not Cu-tolerant, whereas the mine population was shown to be Cu-tolerant and Ni co-tolerant (Gonnelli et al., 2001). Probably, the Cu concentration used was too low to reveal the sensitivity of the serpentine population to this metal in respect to the mine population, but increasing it to that point could provoke an effect on the sensitive population so deleterious to render the experimental design not suitable to the proposed aim. As far as metal accumulation is concerned (Table 5.2), metal concentrations were similar among the populations in most of the cases. In fact, the only significant difference was the low Cu concentration shown by the Cu mine population and generated by its well-known behavior as a Cu excluder (Gonnelli et al., 2001; Colzi et al., 2011;2012).

Cerato-platanin was used as biotic stimulus to detect the ability of metallicolous and non-metallicolous *S. paradoxa* populations 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 and Solomon, 2013). The emission fluorescent spectra obtained (Fig.5.1) 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 *S. paradoxa* (Fig.5.2). The presence of Cu or Ni 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 Cu, it showed a level of phytoalexin production higher than in control conditions and higher than the other two populations. Intriguingly, the presence of Ni did not induce a similar response. Therefore, at least for the populations studied, a metal specificity for such particular behaviour 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 dependent 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 Cu-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 Cu-tolerant populations. Considering that Cu-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 behaviour was different when the Cu metallicolous population was grown in control conditions and similar to that one of the sensitive population. The mine population seemed to be able to "sense" the presence of Cu in its environment. Probably, at normal Cu 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, Cu 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.

5.4.2. Characterization of copper effect on phytoalexin production

The different behavior that the Cu-tolerant population had shown when grown in the presence of that metal was confirmed by time-dependence experiments (Fig.5.3). The cerato-platanin imposed production increased with the exposition time in a similar way for the sensitive plants, irrespectively of the presence of Cu in the culture medium, and for the non-treated metallicolous plants. All the values of the previous samples were lower than the Cu-treated cerato-platanin exposed metallicolous samples. This behaviour 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 Cu, in the tolerant population compared with the sensitive one (Fig.5.4).

In Cu 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 (Figs.5.5 and 6). Till 5 μM CuSO4, the previously hypothesized "metal sensing" of the mine population seemed to increase its effect following the Cu concentration, the more the metal was present in the environment, the more phytoalexins needed to be produced under fungal attack. At the highest Cu concentration used, the level of phytoalexin production surprisingly fell to control level. Plant growth and Cu accumulation were monitored also in this kind of experiment, giving expected results (a higher Cu-imposed effect on plant growth for the sensitive population, along with a higher Cu 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).

5.4.3. 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 *S. paradoxa* from non-contaminated and copper mine soil, either grown in the presence/absence of 5 μM $CuSO_4$, showed that CP activated MAPKs in both the populations and that such activation was larger when plants were grown in the presence of Cu (Fig. 5.7). 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 defense at the Cu 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 Cu excess, can affect the defensive machinery system of a particular metallicolous population of *S. 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.

5.5. Conclusions

The intensity of response to biotic stress, in terms of phytoalexin production after PAMP exposition, shown by the excluder pseudo-metallophyte *S. 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 Cu 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 Cu-tolerant plants grown in the presence of Cu. 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.

CHAPTER 6

Under fungal attack on a metalliferous soil: ROS or not ROS? Insights from *Silene paradoxa* L. growing under copper stress

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Abstract

We investigated how the adaptation to metalliferous environments can influence the plant response to biotic stress. In a metallicolous and a non-metallicolous *Silene paradoxa* population the induction of oxidative stress and the production of callose and volatiles were evaluated in the presence of Cu and of the PAMP fungal protein cerato-platanin, separately and in combination. Our results showed incompatibility between the ordinary ROS-mediated response to fungal attack and the acquired mechanisms of preventing oxidative stress in the tolerant population. A similar situation was also demonstrated by the sensitive population growing in the presence of Cu but, in this case, with a lack of certain responses, such as callose production. In addition, in terms of the joint behaviour of emitted volatiles, multivariate statistics showed that not only did the populations respond differently to the presence of Cu or biotic stress, but also that the biotic and abiotic stresses interacted in different ways in the two populations. Our results demonstrated that the same incompatibility of hyperaccumulators in ROS-mediated biotic stress signals also seemed to be exhibited by the excluder metallophyte, but without the advantage of being able to rely on the elemental defense for plant protection from natural enemies.

* *these two authors contributed equally to the work*

6.1 Introduction

Heavy metal-adapted plants represent an interesting and suit- able model system for exploring the naturally occurring interaction between abiotic and biotic stress. Although it is known that, in non- adapted plants, metal ions can confer “metal-induced fortification” (Mithöfer et al., 2004; Walters et al., 2005) and defend the plant against fungal infection (Cabot et al., 2013), information on the relationships between pathogens and metal excess in metallophytes remains relatively scarce.

A high soil element concentration is generally tolerated by adapted plants through the restriction of metal uptake and/or translocation (such plants are termed “excluders” according to Baker (1981). In contrast, a few species called metal “hyper- accumulators” (Van der Ent et al., 2013) accumulate metals in their shoots at extraordinarily high concentrations. Their self-defense against biotic stress is increased by the accumulation of high metal concentrations in the plant tissues and is called “elemental defense” (Horger et al., 2013; Cheruiyot et al., 2013). However, in the majority of metallophytes, which tend to be excluders (Paton and Brooks, 1996; Chipeng et al., 2010; Gall and Rajakaruna, 2013), this phenomenon is expected to be highly improbable. In fact, their tissue metal concentrations, even if higher than in the same plant species grown in non-contaminated soil, are most likely not sufficient to cause the elemental defense effect. Metal-adapted plants generally show a high sensitivity to soil pathogens, but there are certain exceptions (see Springer, 2009a and 2009b for studies on serpentine plants). According to the “phytosanitary effect” (Poschenrieder et al., 2006), some metals can prevent the growth and development of pathogens in the substrate or on the plant surface. Therefore, a lower pressure of pathogenic fungi on metal- loaded soils could be hypothesized to explain the higher susceptibility of metallophytes to pathogens (Tadros, 1957), but such a phenomenon has never been clearly demonstrated.

To the best of our knowledge, the only report that illuminates the intriguing and yet unexplored topic of the interaction between heavy metal stress and biotic stress in the case of metal-adapted plants is our previous investigation on phytoalexin production, induced by biotic stress, in metallicolous and non-metallicolous populations of the excluder *Silene paradoxa* L. grown in the presence and in the absence of metals (Martellini et al., 2014). The species *S. paradoxa* is generally found in non-contaminated areas and occasionally on metalliferous soils (Chiarucci, 2003). Different responses (Martellini et al., 2014) to a biotic stimulus depending on the site of origin and on the presence of metals in the culture medium were found in a population from a copper mine dump compared to a population from a calcareous soil and a population from a serpentine outcrop. In particular, the non-catalytic fungal protein cerato-platanin (CP), a well-known pathogen-associated molecular pattern (PAMP) that is widespread in filamentous fungi (Pazzagli et al., 1999; Lombardi et al., 2013; Baccelli et al., 2014; Pazzagli et al., 2014), was reported to induce a high production of phytoalexins in the mine population only when grown in the presence of copper. These results suggested that, in specific cases, the adaptation to metalliferous environments can affect the plant response to biotic stress (Martellini et al., 2014).

Reactive oxygen species (ROS) play a central role in plant disease resistance (Fobert and Despre´s, 2005; Torres et al., 2006), as the PAMP-induced oxidative burst (Navarro et al., 2004) is required for downstream plant defences. As in biotic stress, heavy metals, redox active or not, can induce ROS production (Garnier et al., 2006; Miethke and Marahiel, 2007; Choudry et al., 2013), in this case as a direct effect of their toxicity. Therefore, a potential conflict between ROS functionality and suppression may be created in a metalliferous environment where plants can be subjected to simultaneous biotic and heavy metal stress. In the case of hyperaccumulating plants, the reconciliation of this dilemma was found to be impossible (Fones et al., 2013). Metal hyperaccumulation was shown

to be incompatible with defensee signalling through ROS, and normal defence responses were found to become progressively uncoupled from ROS signalling in *Noccaea caerulescens* (Fones et al., 2013). In this context, nothing is known about metal-excluding plants that cannot rely on the "elemental defence" due to their lower metal tissue concentration and their need to adapt to heavy metal-induced oxidative stress. In the case of the above-mentioned *S. paradoxa* populations, it was found that the Cu mine population was able to limit Cu-induced oxidative stress (Gonnelli et al., 2001), making this system particularly suitable for the aim proposed in this paper. In fact, in hyperaccumulators, the impossibility of reconciliation between ROS functionality and suppression has also been suggested to result from by their development of antioxidant-based metal tolerance mechanisms (Fones et al., 2013). Callose (1,3-b-glucan) deposition is a well-known response to fungal attack (see, for example, Nielsen at al., 2012 and Ellinger et al., 2013) induced by oxidative burst (Vellosillo et al., 2010). Callose biosynthesis is promoted by ROS formation after PAMP recognition (Galletti et al., 2008; Luna et al., 2011) and has been found to be affected in hyperaccumulators due to their uncoupling of the signal (ROS) and their response in terms of anti-pathogen defences (Fones et al., 2013), with the accumulated metal providing an alternative defence against many pathogens (Fones et al., 2010). Callose is also synthesized in response to heavy metal stresses, as callose layers can block apoplast metal migration in the root, thus limiting uptake (Krzesłowska, 2011).

The emission of VOCs is another well-known plant response to biotic stress. Among the so-called green leaf volatiles (GLVs), C6- aldehydes can protect plants from infections due to their toxicity toward phytopathogenic bacteria and fungi (Ble´e, 2002). Specifically, after fungal infection, compounds such as Z-3-hexenal, E-2- hexenal, Z-3-hexenol, E-2-hexenol, and Z-3-hexenyl acetate were found to be emitted from *Zea mays* (Piesik et al., 2011), *Arabidopsis thaliana* (Kishimoto et al., 2008) and *Phaseolus vulgaris* (Ongena et al., 2004)

and are able to inhibit fungal growth (Scala et al., 2013). In addition, pathogen-induced VOC-terpenoids were shown to possess direct antimicrobial properties in vitro (Hamilton- Kemp et al., 1988) and to be produced after fungal attack, such as in the case of volatile monoterpenes, which are already known to play a role in plant defence (Kishimoto et al., 2006a; Neilson et al., 2013), in some cases very similar to the role of C6-aldehydes (Kishimoto et al., 2006b) or of various sesquiterpenes (Ahuja et al., 2012; Niinemets et al., 2013). Pathogen-induced ROS formation mediates the oxidation of polyunsaturated fatty acids, converted enzymatically or non-enzymatically to oxylipins, which induce the expression of genes involved in the biosynthesis of secondary metabolites such as VOCs (Mitho€fer et al., 2004). Similarly, metal- induced ROS formation can trigger the emission of VOCs (Mithofer et al., 2004), which serve as active scavengers of the ROS themselves due to their general antioxidant characteristics (Loreto and Schnitzler, 2010).

This study tests the hypothesis that adaptation to a metalliferous environment can affect plant responses to fungal attack, especially in terms of pathogen-induced oxidative stress in the excluder *S. paradoxa*. This investigation is particularly intriguing given that non-accumulators can hardly take advantage of any elemental defence, even if, in the case of herbivory, certain Lepidoptera have been found to be sensitive to metal concentrations in artificial diets below the minimum hyperaccumulator level (Cheryuiot et al., 2013). Thus, we investigated the different responses to biotic stress of a metallicolous *S. paradoxa* population compared to the non-metallicolous population by growing the plants in presence of copper, exposing them to CP, and determining the production of malondialdehyde (MDA), H_2O_2, callose and volatile organic compounds (VOCs).

6.2 Materials and methods

6.2.1. Plant material and experimental conditions

Seeds of *S. paradoxa* were collected in Tuscany (Italy) from plants growing in the Colle Val D'Elsa area (CVD population, non- contaminated soil) and in the Fenice Capanne area (FC population, Cu mine deposit). For site and population description see Chiarucci (2003), Gonnelli et al. (2001) and Pignattelli et al. (2012). Seeds were randomly collected choosing at least 50 adult plants of the same size over the entire spatial distribution of the populations (about 0.5 km^2 for the non-contaminated soil and about 0.2 km^2 for the mine dump). Seeds from the different plants were mixed together prior to the experiments. After germination in peat soil, the 6-week-old plantlets (192 plantlets for population) were transferred to 1-L polyethylene pots containing a half-strength Hoagland's solution (Hoagland and Arnon, 1950) in milliQ-water (Millipore, USA) buffered with 2 mM MES, pH 5.5, adjusted with KOH. For half of the plants, the nutrient solution was supplied with 5 μM $CuSO_4$. We chose this concentration since it had previously been proved to be the most effective in disclosing the differences in the responses to the applied stresses between the populations (Martellini et al., 2014). Hydroponic solutions were renewed once a week and for eight weeks the plants were grown in a growth chamber (for growth conditions see Pignattelli et al., 2012).

6.2.2. Leaf treatment with cerato-platanin

Heterologous CP was obtained from the yeast *Pichia pastoris*, transformed with the pPIC-*cp* plasmid to permit the recovery of the protein from the cultural filtrate (Pazzagli et al., 2009). A single purification step by RP-HPLC was needed to obtain the pure protein in high yield (60 mg from 1L of cultured medium). Pure heterologous CP was compared with the native one both for biological activity and structure. Primary and secondary structure were checked by mass

spectrometry and circular dichroism analysis, respectively. Biological activity was measured through its ability to induce the synthesis of phytoalexins from the lower surfaces of *Arabidopsis* leaves.

The *S. paradoxa* plants were uniformly sprayed with 10 ml of a solution of 0.3 m M CP (or of milliQ-water in case of reference plants) and incubated for 24 h in a glass jar. After the incubation time VOCs emission was evaluated and plants were sampled for the other analyses.

6.2.3. MDA and H_2O_2 concentration assays

Leaf malondialdehyde (MDA, end product of lipid peroxidation) and H_2O_2 concentrations were determined following the method of Velikova et al. (2000). Fresh samples (about 1 g, 12 plants per population per concentration) were homogenized with 4 ml of 0.1% (w/v) trichloroacetic acid in ice bath. The homogenate was centrifuged at 12,000 × g for 20 min and supernatant was used for both the determinations that were performed at room temperature. For MDA analysis 1 ml of 20% (w/v) trichloroacetic acid comprised of 0.5% (w/v) thiobarbituric acid was added to 1 ml aliquot, heated at 95 °C for 30 min and cooled in an ice bath to end the reaction. The tubes were centrifuged at 10,000 × g for 10 min, and the supernatant absorbance was recorded at 532 nm and then at 600 nm for the subtraction of the non-specific absorption. The amount of MDA was calculated through the extinction coefficient of 155 mM^{-1} cm^{-1}. As for H_2O_2 assay, 1 ml of the supernatant was added to 1 ml of 10 mM potassium phosphate buffer (pH 7.0) and 2 ml of 1 M KI. The reaction mixture was incubated for 1 h in darkness and absorbance measured at 390 nm H_2O_2 concentrations were calculated based on the supernatant absorbance at 390 nm and on a standard curve with known concentrations (Alexieva et al., 2001).

6.2.4. Callose determination

Callose extraction and quantification (12 plants per population per concentration) was performed according to Smith et al. (2011), employing curdlan (Sigma) as standard and expressing the concentration of callose as mg curdlan equivalent (CE) g^{-1} FW.

6.2.5. VOCs determination

The VOC measurements were performed using a system similar to that previously used by Danner et al. (2012). Briefly, shoots were isolated from the root system using a synthetic rubber-based sealant (Terostat IX, Henkel, UK) placed around the base of the stem to exclude the influence of water evaporation and ambient air. The shoots of the plants were uniformly sprayed with 10 ml of a solution of 0.3 mM CP or with milliQ-water and subsequently were transferred to a glass jar. The VOCs emitted by treated and non-treated plants were sampled directly from the glass jar, that was equipped with two Teflon tubes, respectively the air inlet and outlet, located on opposite sides. The inlet tube was connected to a zero-air generator (Peak Scientific) and the outlet to the PTR-ToF- MS (8000, Ionicon Analitic GmbH, Innsbruck, Austria). Care was taken to maintain constant temperature and humidity during measurements as chemical reactions are very sensitive to changes of such parameters (Mancuso et al., 2015). VOC analysis was performed 24 h after the treatment in an air conditioned room at 25 ± 1°C. Prior to the analyses stomatal conductance was measured as described in Bazihizina et al. (2015) and no significant differences were found among the different samples of each population.

VOCs were determined by injection of the mixture of the head space in the drift tube of the PTR-ToF-MS via a heated (60°C) peek inlet tube with a flow rate of 100 standard cubic centimeters per minute (sccm) for 3 min. The time of sampling for each TOF acquisition channel was 0.1 ns, for a mass spectrum between *m/z* ¼ 30e210. In all measurements the drift tube conditions were: drift

voltage 600 V, temperature 110°C, pressure 2.25 mbar, voltage of extraction at the tube end (Udx) 35V relative to an E/N value of 140 Td (1 Td ¼ 10^{-17} Vcm^{-2}). Internal calibration was based on *m/z* ¼ 29.997 (NO^+), m/z ¼ 59.049 (C2H5O2þ), *m/z* ¼ 180.937 ($C_6H_4C_{l3}^+$). To obtain a high mass accuracy, an off-line calibration was per- formed by following the procedure described in Cappellin et al. (2010) with 3 points. This procedure enabled us to achieve a high accuracy in the mass determination for the considered mass range, which was sufficient for the sum formula identification as clearly demonstrated by Cappellin et al. (2011). An example of the obtained mass spectra is given in Fig.6.1. For a detailed explanation of the PTR-TOF-MS technology see Blake et al. (2009).

Raw data were acquired with the TofDaq software (Tofwerk AG, Switzerland) using a 20 ns dead time for the correction of Poisson, and the extraction of peaks was performed according to the methodology of Cappellin et al. (2011), employing a modified Gaussian peak shape. For peak quantification data were corrected with the duty cycle and the signals were normalized to the primary ion signal (count per second, cps, to normalized count per second, ncps) (Taiti et al., 2015). For VOC identification and data modelling we used an average signal intensity recorded for 60 s, which allowed the acquisition of 60 average spectra with a high mass accuracy. For the identified signals all of the *m/z* were tentatively assigned to the mass formulae reported, relying on the high instrumental mass accuracy and resolution. Moreover, the tentative identifications through the integration of previous knowledge of the VOCs emitted by plants, where analyses were performed with PTR-MS instruments, have been improved.

6.2.6. 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).

The joint behaviour of the various VOCs in the different matrices was analyzed by using robust Principal Component Analysis (rPCA), a methodology often used for exploratory data analysis or as a dimension reduction technique (Krzanowski, 1988). It is a modification of the widely used statistical procedure and works well with respect to anomalous observations (Hubert et al., 2005). In particular, it is very useful when the data set is highly dimensional, i.e. if there are a lot of variables and a consistent number of cases clustered in groups, as in our study. In PCA, with a minimal loss of information, a set of $k < p$ new uncorrelated variables, called principal components, was constructed. These principal components can reveal latent structures in the data and be used in further analysis or interpretation of acting biochemical processes.

All the samples were considered together in order to compare their behaviour simultaneously and to evaluate their relationships and the effect of their differences on the VOC variability. Statistical analysis was performed with Matlab_R2014b developing dedicated algorithms based on Hubert et al. (2005). To our knowledge, this is the CVD plants the net production of MDA significantly decreased in the presence of copper. In the FC plants, the net MDA production was similar in the control and 5 μM $CuSO_4$ treated plants. The CVD population showed significantly higher values of net MDA production compared to the FC population in non-treated plants.

Measurements of H_2O_2, MDA, callose and VOCs were performed on six replicates. Each replicate was measured three times.

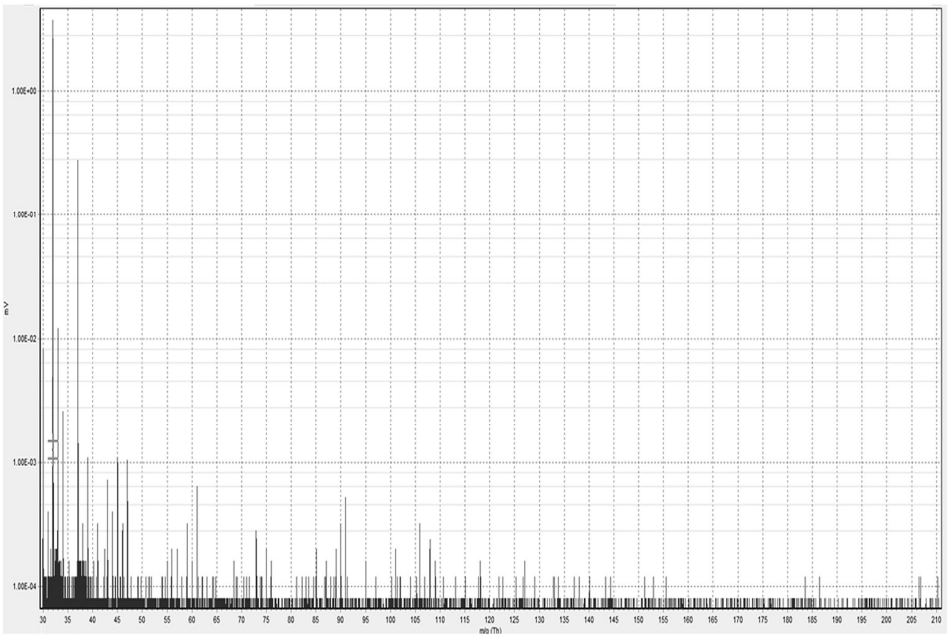

Fig.6.1. Example of a typical PTR-ToF-MS profile obtained by volatile analysis of *Silene paradoxa* plants (sample of CVD population grown under control conditions and exposed to CP).

6.3 Results

6.3.1. MDA production

Plants from the two *S. paradoxa* populations were assayed for MDA production after a 24 h treatment with CP (Fig.6.2A). When leaves were not exposed to CP, the shoot levels of MDA were significantly higher in Cu-treated plants compared to the controls only in the CVD population. When comparing the two populations, the FC plants showed significantly lower concentrations of MDA in the presence of copper compared to the CVD plants ($p < 0.05$).

After exposure to CP, all the samples showed significantly higher values of MDA concentrations. Comparing these data for metal-exposed and non-exposed plants, the MDA values obtained were not dependent on the growth conditions. Comparing the populations, the FC plants showed lower levels of MDA than the CVD plants for each type of sample ($p <0.05$).

An estimation of the net production of MDA induced by CP, independently of the amount induced by the copper treatment, was calculated by subtracting the values of MDA of the non CP-exposed samples from the values of the corresponding CP-exposed samples. The obtained values are reported in Fig. 5.2B. In the CVD plants the net production of MDA significantly decreased in the presence of copper. In the FC plants, the net MDA production was similar in the control and 5 μM $CuSO_4$ treated plants. The CVD population showed significantly higher values of net MDA production compared to the FC population in non-treated plants.

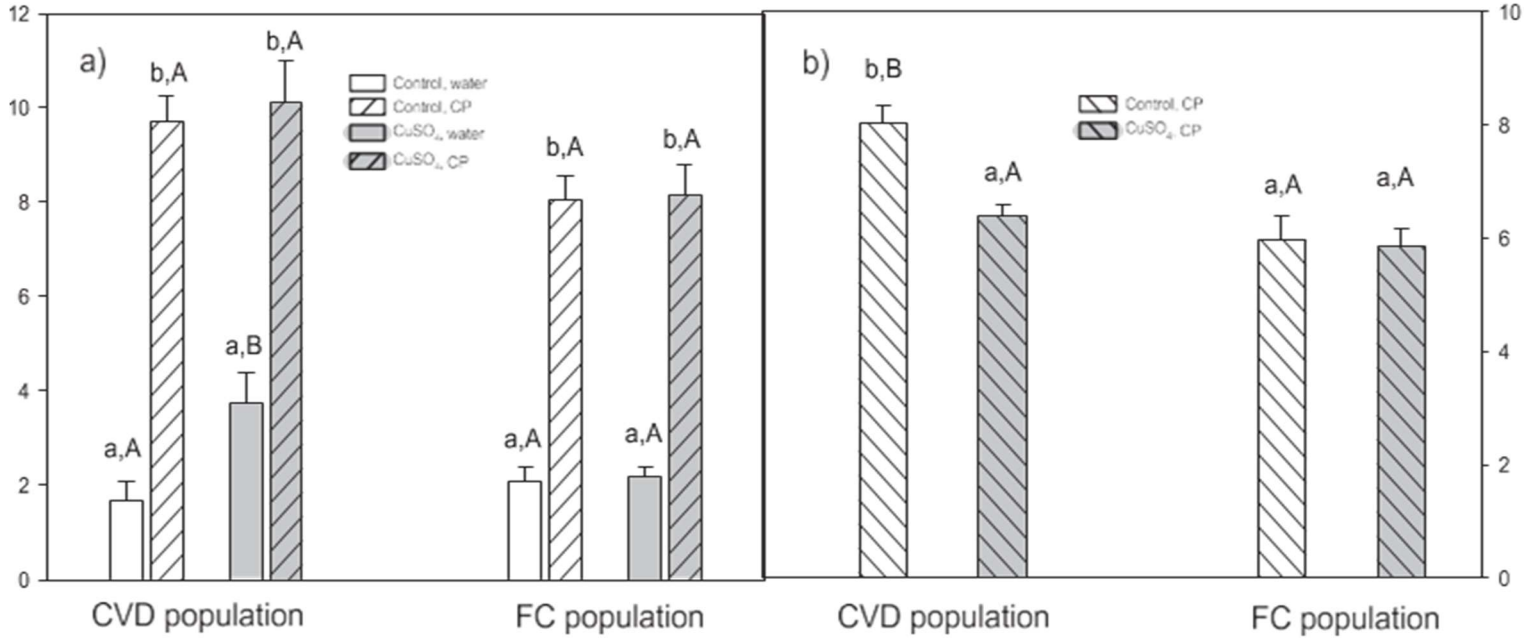

Fig. 6.2 A) Effect of 24 h of incubation with 0.3 mM CP on malondialdehyde concentration in CVD sensitive and FC copper mine populations grown in absence and presence of 5 mM CuSO4. Values are means of six replicates ± standard deviations. Significant differences between the means appear with different letters, small for metal intra-treatment and capital for metal inter-treatment comparisons (at least $p < 0.05$). B) Net production of malondialdehyde after 24 h of incubation with 0.3 mM CP in CVD sensitive and FC copper mine populations grown in absence and presence of 5 µM $CuSO_4$. For calculating the net malondialdehyde production, the production of malondialdehyde induced by water was subtracted from the malondialdehyde production induced by CP. Values are means of six replicates ± standard deviations. Significant differences between the means appear with different letters, small for intra-population and capital for inter-population comparisons (at least $p < 0.05$).

6.3.2. H_2O_2 production

The accumulation of H_2O_2 in Cu- and CP-treated *S. paradoxa* plants is shown in Fig. 6.3A. In the CVD population, Cu exposure provoked a significant increase in H_2O_2 level that was not present in the FC population. After CP treatment, a significant increase in the concentration of this molecule was present only in non-Cu- exposed plants and at a higher level in the case of the CVD population. Comparing the populations, the CVD plants showed a higher concentration of H_2O_2 than the FC population ($p < 0.05$), with the exception of control plants not exposed to CP.

In the CVD plants, the net production of H_2O_2 was higher in the controls than in the copper-treated plants (Fig. 6.3B). In the FC plants, a net H_2O_2 production was present only in control conditions and to a significantly lower extent than in the CVD plants.

6.3.3. Callose production

In non CP-exposed plants the presence of Cu in the culture medium significantly increased the shoot concentration of callose in the CVD population, whereas no metal-imposed difference in the level of this molecule was found in the FC population (Fig.6.4A).

Comparing the populations, the CVD and FC plants showed different concentrations of callose, but the difference was not significant. When the leaves were exposed to CP, the callose concentration increased in all the samples. In both populations, this increase was significant both in the control condition and in the presence of 5 μM $CuSO_4$. The callose concentrations were higher in the FC plants than in the CVD plants when exposed to CP ($p < 0.05$).

Regarding the estimation of the net CP-induced production of callose (Fig.6.4B) in the CVD plants, this value significantly decreased in the presence of Cu in the culture medium, whereas the FC plants showed the opposite behaviour. The FC population showed significantly higher values of net callose production than the CVD population in the case of Cu-treated plants.

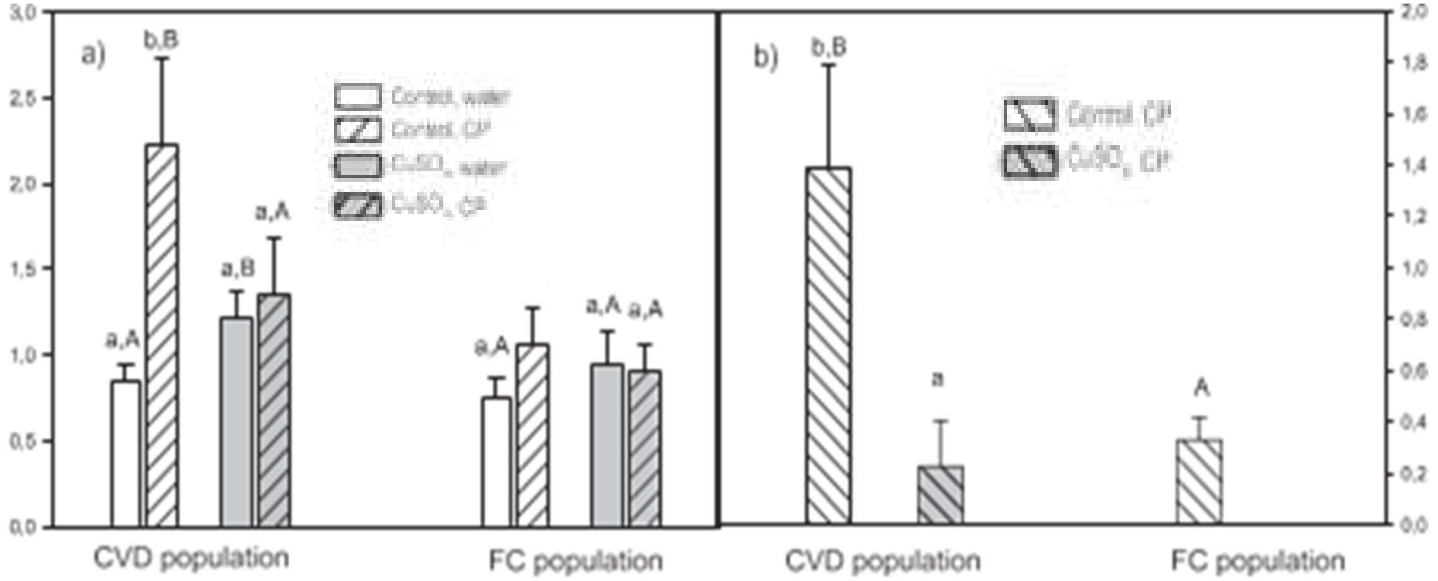

Fig. 6.3. A) Effect of 24 h of incubation with 0.3 mM of CP on hydrogen peroxide concentration in CVD sensitive and FC copper mine populations grown in absence and presence of 5 µM $CuSO_4$. Values are means of six replicates ± standard deviations. Significant differences between the means appear with different letters, small for metal intra-treatment and capital for metal inter-treatment comparisons (at least $p < 0.05$). B) Net production of hydrogen peroxide after 24 h of incubation with 0.3 mM CP in CVD sensitive and FC copper mine populations grown in absence and presence of 5 µM $CuSO_4$. For calculating the net hydrogen peroxide production, the production of hydrogen peroxide induced by water was subtracted from the hydrogen peroxide production induced by CP. Values are means of six replicates ± standard deviations. Significant differences between the means appear with different letters, small for intra-population and capital for inter-population comparisons (at least $p < 0.05$).

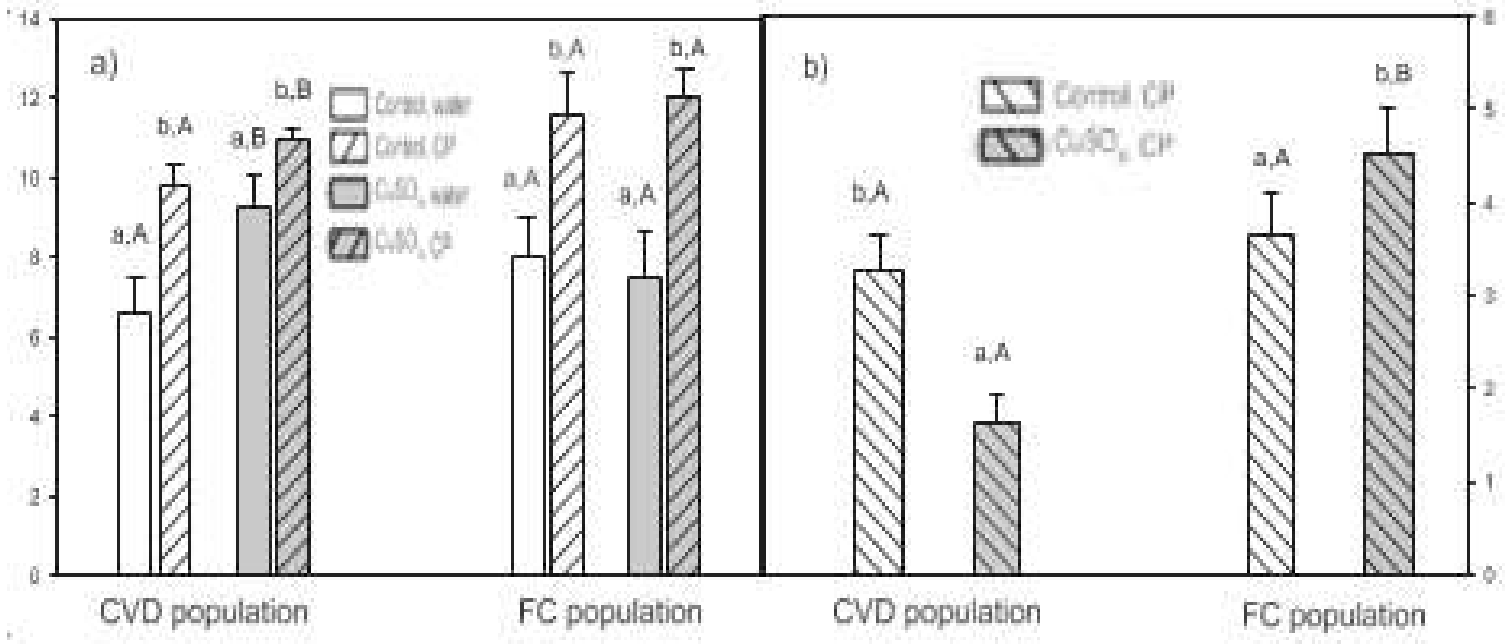

Fig. 6.4. A) Effect of 24 h of incubation with 0.3 mM CP on callose concentration in CVD sensitive and FC copper mine populations grown in absence and presence of 5 µM CuSO4. B) Net production of callose after 24 h of incubation with 0.3 mM CP in CVD sensitive and FC copper mine populations grown in absence and presence of 5 mM CuSO4. Values are means of six replicates ± standard deviations. For calculating the net production of callose, the production of callose induced by water was subtracted from the callose production induced by CP. Significant differences between the means appear with different letters, small for intra-population and capital for inter-population comparisons (at least $p < 0.05$).

6.3.4. VOCs determination

VOC spectra from each plant were obtained by PTR-ToF-MS, using the data set comprising peaks from *m/z* ¼ 20 to *m/z* ¼ 220. The raw data were filtered by the elimination of peaks with average intensity <1 ncps.

Table 6.1 reports the most interesting protonated masses together with the molecular formula, the tentative identification and the signal intensity normalized by the leaf area (expressed as normalized counts per second) for each sample. Bibliographic citations listed in Table 6.1 refer to volatile compounds identified in other studies by PTR-MS technologies showing the same molecular mass.

Some interesting differences were obtained for the signal intensity values of the different volatiles (Table 6.2A and B) and for the calculation of their net values of intensity (Table 6.3).

In the CVD population, copper treatment induced a significant increase in *m/z* 33.033, tentatively identified as methanol, whereas in the FC plants, Cu induced a decrease, although to a non-significant extent. Cerato-platanin exposure increased raised the *m/z* 33.033 emission in all the samples, with the highest levels reached in the CVD population. The net *m/z* 33.033 emission generally followed the same trend, with values from the CVD population always significantly higher than values from the FC population. For *m/z* 57.033, 81.069 and 99.080, putatively associated with E-2-hexenal and Z-3-hexenal; *m/z* 101.096, putatively hexanal; *m/z* 85.085, putatively hexanol; and *m/z* 43.018, putatively hexyl acetate derived from the fragmentation of C6-aldehydes, Cu treatment generally did not induce any change in their signal intensity in the CVD population, whereas a Cu-mediated increase was found in some cases in the FC population. Under CP exposure, the CVD population showed no change in the level of these VOCs, whereas in the FC population, the treatment induced a significant increase in the signal intensities of most of the masses. This result was obtained in control conditions and especially in Cu-treated plants, as also shown by the calculation of the volatile net emission. The volatile with *m/z* 137.132,

putatively monoterpenes, showed the same results as the previously mentioned compounds. Copper was able to increase the emission of the putative sesquiterpenes (*m/z* 205.195), in both populations, though to a significant extent only in the FC population. Their emission was similarly increased by CP exposure, significantly only in the FC population and only in the presence of Cu, as shown by the calculation of its net emission.

The signal at *m/z* 63.027, putatively dimethylsulfide, showed only a Cu-mediated increase in the CVD plants and only a CP- induced increase, to a lower extent in the presence of Cu, in the FC plants.

Comparing the populations of plants in control conditions, the level of VOC emission was significantly different for all the identified *m/z* peaks except 99.080, 101.096 and 205.195 ($p < 0.05$). When the plants were exposed to CP, the differences were also significant for the first two of the above volatiles. In Cu-treated plants, only the levels of *m/z* 63.027 and 99.080 showed no any difference between them, whereas when the samples were also exposed to CP, *m/z* 85.085 was the only similar one ($p < 0.05$).

Regarding the rPCA for VOCs emitted by the plants, Fig. 6.5 shows the biplot of the first extracted component versus the second obtained from the analysis of the joint behaviour of the above- mentioned volatiles for all the experimental groups. The biplot was able to explain approximately 88% of data variability and discriminated the volatile emissions of diverse treatments and populations, which were located in different parts of the graph with little overlap. The first component (77%) was positively associated with *m/z* 57.033, 63.027, 43.018, 81.069, 99.080, 101.096, 137.132 and 205.195 and negatively associated with *m/z* 33.033. The second component (11%) was positively associated only with *m/z* 85.085.

Protonated theoretical mass *m/z*	**Protonated chemical formula**	**Tentative identification**	**References**
33.033	CH_5O^+	Methanol	*Taiti et al., 2015*
43.018	$C_2H_2O\text{-}H^+$	Fragments of hexyl acetate	*Brilli et al., 2011*
57.033	$C_3H_5O^+$	Fragments of hexanals	*Brilli et al., 2012*
63.027	$C_2H_7S^+$	Dimethylsulfide (DMS)	*Mancuso et al., 2015*
81.069	$C_6H_9^+$	Monoterpene fragments or fragments of hexanal	*Taiti et al., 2015; Brilli et al., 2011*
85.065	$C_5H_9O^+$	Pentenone	*Brilli et al., 2011*
99.080	$C_6H_{11}O^+$	Hexenals	*Brilli et al., 2012*
101.096	$C_6H_{13}O^+$	Hexenols + Hexanal	*Brilli et al., 2012*
137.132	$C_{10}H_{17}^+$	Monoterpenes	*Taiti et al., 2015*
205.125	$C_{15}H_{25}^+$	Sesquiterpenes	*Taiti et al., 2014*

Table 6.1. Protonated masses, molecular formula, tentative identification and references of the investigated volatile compounds.

Table 6.2 Effect of 24 h of incubation with 0.3 mM CP on VOCs emission (in ncps) in CVD sensitive (A) and FC copper mine (B) populations grown in absence and presence of 5 mM $CuSO_4$. Values are means of six 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 $p < 0.05$).

Protonated theoretical mass *m/z*	Control		Cu- treated	
	H_2O	CP-treated	H_2O	CP-treated
A				
33.033	40.6 ± 2.6 aA	159.1 ± 26.9 bA	260.2 ± 48.1 aB	475.7 ± 29.7 bB
43.018	1.5 ± 0.4 aA	1.8 ± 0.6 aA	5.4 ± 0.7 aB	5.0 ± 3.3 aA
57.033	1.5 ± 0.5 aA	2.5 ± 1.4 aA	2.8 ± 1.4 aA	2.9 ± 1.1 aA
63.027	1.3 ± 0.2 aA	1.4 ± 0.3 aA	3.6 ± 0.7 aB	3.4 ± 1.7 aA
81.069	1.4 ± 0.4 aA	2.4 ± 0.8 aA	3.6 ± 0.7 aA	2.9 ± 0.7 aA
85.065	5.7 ± 0.8 aA	6.5 ± 2.2 aA	9.0 ± 2.3 aA	9.8 ± 3.6 aA
99.080	1.5 ± 0.4 aA	1.3 ± 0.3 aA	1.5 ± 0.5 aA	1.7 ± 0.5 aA
101.096	2.4 ± 0.7 aA	2.1 ± 1.0 aA	2.4 ± 0.7 aA	2.4 ± 0.9 aA
137.132	1.1 ± 0.4 aA	1.2 ± 0.5 aA	1.2 ± 0.4 aA	1.4 ± 0.6 aA
205.195	1.3 ± 0.1 aA	1.6 ± 0.4 aA	1.3 ± 0.2 aA	1.7 ± 0.6 aA
B				
33.033	57.6 ± 7.1 aA	94.3 ± 10.6 bA	38.2 ± 2.4 aA	74.2 ± 32.1 bA
43.018	57.8 ± 13.1 aA	107.3 ± 22.4 bA	77.2 ± 3.7 aB	173.2 ± 0.2 bB
57.033	5.9 ± 0.7	8.8 ± 1.1	11.0 ± 0.5	33.2 ± 0.6

	aA	bA	aB	bB
63.027	5.4 ± 1.7	54.4 ± 1.4	4.6 ± 1.6	20.8 ± 3.8
	aA	bB	aA	bA
81.069	4.6 ± 1.1	7.0 ± 1.1	10.7 ± 0.6	32.7 ± 6.8
	aA	aA	aB	bB
85.065	1.6 ± 0.5	2.1 ± 0.1	4.6 ± 0.7	9.1 ± 0.7
	aA	aA	aB	bB
99.080	1.6 ± 0.3	3.0 ± 0.6	1.8 ± 0.2	9.7 ± 0.1
	aA	bA	aA	bB
101.096	3.6 ± 1.6	6.1 ± 2.1	5.3 ± 0.2	19.2 ± 1.7
	aA	aA	aA	bB
137.132	2.9 ± 1.5	4.1 ± 1.8	4.0 ± 0.9	13.3 ± 0.4
	aA	aA	aA	bB
205.195	1.4 ± 0.3	1.5 ± 0.3	2.3 ± 0.1	3.0 ± 0.2
	aA	aA	aB	bB

	CVD		FC	
Mass *m/z*	Control	$CuSO_4$	Control	$CuSO_4$
33.033	159.1 ± 28.9 aB	215.5 ± 29.8 aB	36.8 ± 10.7 aA	54.2 ± 9.1 aA
43.018	-	-	49.6 ± 22.4 a	96.0 ± 0.3 b
57.033	-	-	2.9 ± 1.5 a	22.2 ± 0.6 b
63.027	-	-	49.0 ± 1.4 b	16.2 ± 3.8 a
81.069	-	-	-	22.0 ± 6.9
85.065	-	-	-	4.5 ± 0.7
99.080	-	-	1.4 ± 0.4 a	7.8 ± 0.1 b
101.096	-	-	-	13.9 ± 1.7
137.132	-	-	-	9.3 ± 0.5
205.125	-	-	-	0.6 ± 0.2

Table 6.3. Net emission of VOCs (in ncps) after 24 h of incubation with 0.3 mM CP in CVD sensitive and FC copper mine populations grown in absence and presence of 5 mM CuSO4. For calculating the net VOC production, the emission of VOCs induced by water was subtracted from the VOC emission induced by CP. Values are means of six replicates ± standard deviations. The symbol - was used when the two terms of the subtraction were not significantly different. Significant differences between the means appear with different letters, small for intra-population and capital for inter-population comparisons (at least $p < 0.05$).

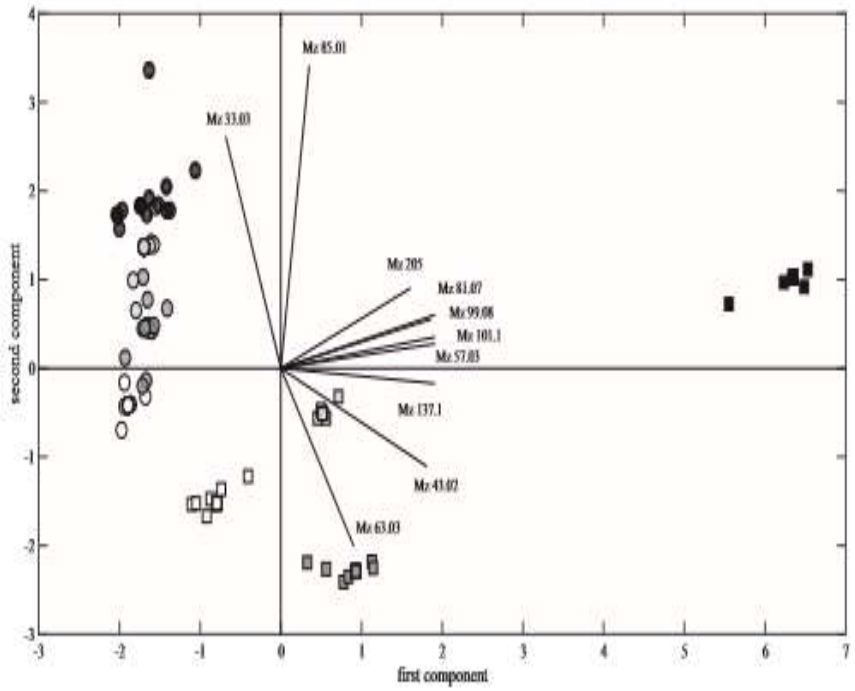

Fig. 6.5. Biplot obtained by the analysis of the joint behaviour of the studied VOCs (circle = Colle Val D'Elsa population, square = Fenice Capanne population, white symbols =control, light grey symbols = CuSO4 5 µM, dark grey symbols = CP, black symbols = CuSO4 5 µM + CP).

6.4 Discussion

In Cu-exposed plants, the increase in metal-induced lipid peroxidation, evaluated through MDA determination, was non-existent in the tolerant population and significant in the sensitive one. A similar result has previously been reported and attributed to higher ascorbate concentrations and the activity of antioxidant enzymes present, both constitutively and inducibly, in the tolerant population by Gonnelli et al. (2001). Treatment with the fungal PAMP was able to induce lipid peroxidation in all the different samples. Interestingly, the presence of Cu in the culture medium did not significantly affect the level of lipid peroxidation after CP exposure. Thus, this report is the first direct evaluation of possible changes in pathogen-induced lipid peroxidation in the presence of this

metal, though previous works have documented an interaction between Cu and fungal infection, possibly through an imbalance in plant antioxidative responses (see for example Chmielowska et al., 2010). In the sensitive population, the lack of an additive effect of PAMP treatment on the levels of lipid peroxidation could likely be due to a Cu-mediated activation of the antioxidant defences, preventing a further oxidative burst. In the tolerant population, the lower levels of MDA and, generally, of its net production could be due to the high antioxidant arrangement of this population both in the absence and in the presence of the metal (Gonnelli et al., 2001). These hypotheses were confirmed by the direct analysis of H_2O_2. In the sensitive population, the Cu-induced increase in ROS could be ascribed to the autoxidation of the metal (Schutzendubel and Polle, 2002), whereas in the tolerant population, the lack of a metal-mediated increase in H_2O_2 was most likely due to its higher ability to prevent Cu toxicity. When the plants from the sensitive population were exposed to CP, a significant increase in H2O2 level was present only in control conditions. Cerato-platanin has already been shown to induce H2O2 production in *A. thaliana* (Baccelli et al., 2014), most likely through NADPH oxidase RBOHD activation and O_2^- formation (Kadota et al., 2014). The CP-induced increase in MDA could be explained by the reaction between O_2^- and H_2O_2 and the subsequent generation of OH^-, which is known to be the main mediator of lipid peroxidation (Farmer and Mueller, 2013). In metal-exposed plants, the PAMP- induced ROS production was most likely scavenged by Cu-activated antioxidant defences. In any case, although the ROS concentrations were supposedly lower, they seemed to be sufficient to increase MDA concentration in these conditions without evident H_2O_2 accumulation. The tolerant population showed a lower susceptibility to CP treatment in terms of MDA production, most likely explained by lower H_2O_2 accumulation due to the constitutively active antioxidant machinery. A similar constitutive incompatibility between adaptation to metalliferous environments and the "ordinary" ROS-mediated plant defence response has been previously found in

hyperaccumulators (Fones et al., 2013) and ascribed to high levels of glutathione and ascorbate (Hall, 2002; Freeman et al., 2004) and of antioxidant enzymes (Boominathan and Doran, 2003).

Regarding callose production, the presence of Cu in the culture medium was able to trigger this response only in the sensitive population. A similar behaviour has been previously reported in *S. paradoxa* roots as an ineffective attempt of the sensitive population to block metal migration through the plant (Colzi et al., 2015). Here, an analogous Cu-imposed situation could also be envisaged for shoots. Cerato-platanin exposure was able to induce callose production in all the samples, but to different extents. In the sensitive population grown under control conditions, the above- mentioned CP-induced increase in ROS concentration could be regarded as the main trigger for the evident callose biosynthesis. Callose production was less marked in the presence of Cu, most likely because the metal-activated scavenging of ROS quenched a portion of the signal molecules for this response. To the best of our knowledge, this report is the first to describe the lack of a ROS- triggered response in metal-treated plants. No information on this topic is available on either crop plants or metallophytes, with the sole exception of the above-mentioned case of hyper- accumulators, showing a similar condition of missing ROS burst and callose deposition (Fones et al., 2013). In the tolerant population the callose response was evident not only under control conditions but also in the presence of Cu. This population, to maintain high callose deposition in presence of a lower CP-induced production of H_2O_2, could most likely rely, at least in part, on a ROS- independent signalling pathway for PAMP-induced callose production, such as through salicylic acid and auxin (Ellinger and Voigt, 2014) and/or ABA (Anderson et al., 2004). In metal-treated tolerant plants, the callose deposition, in the complete absence of net H_2O_2 production, could suggest that the presence of Cu triggered an enhancement of the non ROS-mediated signal. This mechanism, worthy of further study, could in part solve the difficult dilemma between the use of a ROS-based response and the constitutively

acquired ability to counteract oxidative stress. In this context, in non-metallophytes, Cd has been shown to protect *A. thaliana* against *Botrytis cinerea* infection though the activation of the jasmonate-ethylene and salicylic acid signalling pathways (Cabot et al., 2013). *Silene paradoxa* plants were able to produce several VOCs in all the different culture conditions. A Cu-mediated increase in methanol emission was found in the sensitive population, whereas the opposite trend occurred in the tolerant one. This report is the first to describe a possible relationship between Cu-treatment and methanol emission in plants. Considering that the main source of methanol emission is cell wall pectin demethylesterification (Oikawa et al., 2011), similar Cu-induced changes in pectin concentration, increased in the sensitive population and decreased in the tolerant one, could be envisaged in *S. paradoxa* shoots, as already found in the roots (Colzi et al., 2011, 2012). Methanol emission increased following plant exposure to CP. In fact, pathogen attack is known to regulate plant pectin methylesterase expression (Lionetti et al., 2012), and PAMP signals seem to be involved in their activation (Bethke et al., 2014). The net methanol emission was lower in the tolerant population, suggesting a lower CP-induced activation of pectin methylesterases. This result could be partly due to the above-mentioned lower H_2O_2 production, as this ROS is reported to increase the activity of such enzymes (Lionetti et al., 2012). Assuming that the pathogen induction of pectin methylesterases can make the cell wall more prone to degradation by pectin hydrolases (Hok et al., 2010), a lower PAMP- induced pectin methylesterase activation could be regarded as a protective mechanism against infections.

Regarding GLVs, Cu-treatment did not exhibit any significant effect on the signal intensities of the C6-aldehydes E-2-hexenal, Z-3-hexenal and hexanal, except for a slight induction in certain cases in the tolerant population. To date, significant inductions of C6- aldehydes emission mediated by Cu alone have never been reported (Winter et al., 2012; Rostàs et al., 2013). Interestingly, CP exposure was able to induce the release of such GLVs, known to be emitted by

plants to counteract fungal growth (Scala et al., 2013), only in the tolerant population and to a higher extent in the presence of Cu. To the best of our knowledge, this work is the first report of the PAMP-induced production of such compounds. The responsiveness to CP treatment found only in the tolerant population could most likely be considered further evidence that the adaptation to metalliferous environments can realistically affect plant response to biotic stress. Such over-emission of volatiles could have the same postulated adaptive meaning as the previously reported phytoalexin over-production (Martellini et al., 2014), suggesting a greater susceptibility of such populations to pathogen attack. The reported CP-induced increase in GLVs could have been generated by an increase in salicylic acid, which is known to induce the lipoxygenase pathway (Blée, 2002), as a normal ROS-based mechanism for the signalling of volatile biosynthesis (Mithöfer et al., 2004) can hardly be invoked. Cerato-platanin was shown to induce the production of salicylic acid (Baccelli et al., 2014), and the presence of Cu could have generated a useful additive effect. In any case, this dramatic increase in C6-aldehydes in such conditions could be among the factors concurring in the explanation of the already reported over-production of phytoalexins, as such compounds are known to directly induce phytoalexin synthesis (Kishimoto et al., 2006a; Ahuja et al., 2012). The same results, implying similar considerations, were also obtained for hexanol, derived from the reduction of C6-aldehydes and known to induce certain plant defences (Bate and Rothstein, 1998), as well as for volatile monoterpenes and sesquiterpenes. In this latter case, a marked Cu-induced increase in their emission was also present, but the meaning of this response, though previously reported in several plant species (Obara et al., 2002; Mithöfer et al., 2004; Attaran et al., 2008) remains to be elucidated. Furthermore, the tolerant population showed a higher emission of terpenes, thus suggesting a possible relationship between such compounds, which are well known to possess antioxidant capacities (Holopainen and Gershenzon, 2009), and Cu tolerance.

A particular induction pattern was shown by the emission of dimethylsulfide, a VOC enzymatically produced by plants (Bentley and Chasteen, 2004) in response to various environmental variables (Fall et al., 1998). To date, there has been no evidence of its role in the type of stresses here studied, but, its emission was triggered by Cu in the sensitive population and by CP in the tolerant one, in this case with a lower intensity in the presence of metal.

A different joint behaviour of emitted VOCs in the two populations was revealed by rPCA. The first component of the biplot was characterized by a similar joint behaviour of all the volatiles, except methanol and hexanol, and mainly influenced cases from the tolerant population. The second component was associated with changes in methanol and hexanol and affected cases from the sensitive population. The two population cases were not only discriminated in control conditions but also progressively discriminated in the presence of the stresses, even more so when applied in combination. In fact, cases from the sensitive population shifted upwards following the methanol and hexanol vectors, whereas cases from the tolerant population shifted towards the right and were discriminated by the other types of volatiles. In the sensitive population, the biotic and abiotic stresses, separately and in combination, seemed to produce a movement of the cases in the same direction, whereas in the tolerant population, the two stresses applied separately seemed to perturb the cases in two different directions. The movement of the cases generated by the co-presence of copper and CP seemed to mainly follow the direction imposed by the presence of the biotic stress and not the metal stress, to which the population is tolerant. Therefore, in terms of emitted VOC joint behaviour, not only did the populations seem to behave differently in the presence of abiotic or biotic stress, but a different type of interaction of the two stresses in the two different populations was revealed.

6.5 Conclusions

Our results suggest incompatibility between the ordinary ROS- mediated response to fungal attack and the acquired mechanisms preventing oxidative stress in the metallicolous population of *S. paradoxa*. The excluder metallophyte showed the same behaviour as the hyperaccumulating species, although it could not take advantage of metal therapy for protection from natural enemies. Further studies on a wider variety of oxidative stress parameters and on a higher number of metallicolous populations from other types of metalliferous environments could clarify possible alternative and undiscovered strategies adopted by plants to counter biotic stress in the presence of metals.

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CHAPTER 7

General remarks

Silene paradoxa growing in a mine waste in Fenice Capanne (Grosseto)

Firstly, this work confirmed the usefulness of the facultative metallophyte *Silene paradoxa* as a model in comparative studies on metal tolerance and adaptation mechanisms, thanks to the multiple ecotypes that this specie presents.

The specific metal tolerances of these populations were widely determined (Gonnelli et al., 2001) while the exclusion tolerance mechanism of this specie was already clarified in several reports (Gonnelli et al., 2001; Colzi et al., 2011;2012). Comparing populations differing by their sites of origin and hence by metal-tolerance, i.e. metal sensitive population (CVD), Cu-tolerant mine population (FC) and Ni-tolerant serpentine population (PSS)-the last one appearing only in Chapter 4 and partially in Chapter 5-, the present thesis improved knowledge on: 1) Cu-imposed modification in root morphology and composition and in photosynthetic parameters 2) metal-dependent differences in response to macronutrient deficiency 3) interaction between heavy metal stress and production of defense response to biotic stress.

Results showed the existence of Cu-induced production of mucilage and lignin at root level that can concur to the tolerance mechanisms in the Cu-tolerant *Silene paradoxa* population. Furthermore, in such population the presence of the metal was able also to increase root lignification and to promote an early differentiation of the vessels. All these Cu-imposed features could contribute to the exclusion mechanisms evolved by this species to limit Cu uptake and translocation. The observation of Cu-induced precipitates in the root walls of such population was the first observed in presence of this metal.

At photosynthetic level, differences between the CVD and the FC populations have been demonstrated to be not only of different degree, with FC population being more efficient, but also of different nature. Results showed how the photosynthetic activity of FC population is limited predominantly by Cu-imposed stomatal closure, whereas the CVD population experienced any kind of limitation, especially biochemical. The lower Cu concentrations accumulated in the Cu-tolerant population could be one of the factors concurring to the reported

differences in photosynthetic activity, but also a higher capacity of internal detoxification and compartmentalization of the metal could not be excluded.

The three *Silene paradoxa* populations above-mentioned were grown in Ca and Mg deficiency and exposed to Cu and Ni. Results indicated differences in growth, nutrient allocation and nutrient use efficiency among the three populations. These differences could be due to the different soils of origin (presenting different nutrient deficiencies) and to the specific metal-tolerances (Cu and Ni for FC population, only Ni for PSS population, Gonnelli et al., 2001). The metallicolous populations (FC and PSS), compared to the CVD, showed a higher ability to maximize nutrient utilization in deficiency conditions, especially at root level. Interestingly, the FC population showed a significant different response compared to the other two populations tested, both in samples grown in hydroponics and collected in the field, and, curiously, this result was recorded also for the following experiments on interaction between heavy metal stress and biotic stress.

In fact, after exposition of the three populations to Cu and Ni and to a biotic elicitor (PAMP fungal protein cerato-platanin), the FC population over-produced defense responses to pathogen attack (phytoalexins) in a Cu-dependent way. That effect probably was a consequence of a stronger activation of defensive mechanisms that started by the activation of MAPKs and concluded with the secretion of fluorescent phenolic compounds on the lower surface of the leaves.

Finally, we wondered if *Silene paradoxa* relies on ROS as signaling molecules to produce defense responses to biotic stress. In fact, unlike hyperaccumulator species, excluder species (as *Silene paradoxa*) cannot rely on the "elemental defense" towards pathogen attack, but on the other hand, they have to fight the oxidative burst due to the ROS production as a direct effect of metal toxicity. Results on malondialdehyde, H_2O_2, callose, and volatiles production showed incompatibility between the ordinary ROS-mediated response to fungal attack and the acquired mechanisms of preventing oxidative stress in the FC

population. Therefore, this population demonstrated that the same incompatibility of hyperaccumulators in ROS-mediated biotic stress signals also seemed to be exhibited by the excluder metallophyte, but without the advantage of being able to rely on the elemental defense for plant protection from natural enemies. Future works could clarify the alternative and yet undiscovered pathways (i.e. phytohormones) leading to production of defense responses to biotic stress.

To resume, in the present work, we successfully clarified some of the mechanisms that undergo the different responses to biotic and abiotic stresses corresponding to adaptations to different soils of origin and to development of different metal-specific tolerances. The obtained results can be used for further investigations on the effect of interaction between environmental stresses in the excluder metallophyte *Silene paradoxa* and can be extended to other metallicolous populations from other metalliferous environments and even to other metal-adapted species.

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