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## Copper tolerance strategies involving the root cell wall pectins in *Silene paradoxa* L.

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### ABSTRACT

New insights were provided on the function of root cell wall pectin concentration and methylation degree in copper tolerance studying contrasting ecotypes of *Silene paradoxa*. A metallicolous copper tolerant population and a non-metallicolous sensitive population were grown in hydroponics and exposed to different CuSO<sub>4</sub> treatments to evaluate copper accumulation in relation to pectin concentration and methylation degree of the root cell wall. In short-term exposure experiments the tolerant population decreased root cell wall pectin concentration and increased their methylation degree, while the sensitive population did not respond. Moreover, a positive correlation between root pectin concentration and metal accumulation in root apoplast and symplast was found. In addition, a negative correlation between pectin methylation degree and apoplastic copper concentration were found to be negatively correlated. In long-term exposure experiments, the sensitive population increased the concentration of pectins with the same methylation degree and consequently the ability of its root cell wall to bind the metal. The opposite phenomenon was shown by the tolerant population. Moreover, pectin methylation degree was higher in the tolerant population in respect to the sensitive one, possibly to limit metal binding to the root cell wall. Therefore, in the copper tolerant population of *S. paradoxa* the generation of metal-excluding root cell walls was suggested to be one of the factors concurring to guarantee a low apoplastic copper accumulation and probably also to limit symplastic copper uptake by the root cells.

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### 1. Introduction

The cell wall is supposed to play a role in the defense response of plants to heavy metals (Krzesłowska, 2011). In fact, some papers indicate that the cell wall is recognized as one of the main compartments for heavy metal accumulation (e.g., Wójcik et al., 2005; Islam et al., 2007; Meyers et al., 2009) as it can contain high amounts of heavy metals (Konno et al., 2005; Kopittke et al., 2008). Sorption of metals in the cell wall represents an important physiological advantage, since it allows their metabolic inactivation (Krzesłowska, 2011).

Plant cell walls are able to bind divalent and trivalent metal cations due to the presence of a number of functional groups such as –COOH, –OH and –SH (Dronnet et al., 1996; Pelloux et al., 2007). However, the essential capacity of the cell wall for binding divalent and trivalent metal cations depends mainly on the amount of polysaccharides rich in carboxyl groups. These polymers are the pectins, largely represented by homogalacturonans (Dronnet et al.,

1996; Fritz, 2007; Pelloux et al., 2007; Caffall and Mohnen, 2009). They are synthesized and methyl-esterified in the Golgi apparatus and transported into the cell wall where they can be enzymatically demethylated (Krzesłowska, 2011). Demethylation of homogalacturonans leads to the formation of pectins with different degrees of methyl-esterification and thus different amounts of free carboxyl groups, able to bind divalent and trivalent metal ions (Dronnet et al., 1996; Fritz, 2007). Therefore, the methyl-esterification of carboxylic groups of the units of galacturonic acid determines the negative charge carried by pectin and eventually the quantity of metal it can bind (Sattelmacher, 2001). In fact, for pectins a negative relationship between methylation degree and binding capacity of some metals, such as lead (Khotimchenko et al., 2007) and aluminum (Schmohl et al., 2000; Mimmo et al., 2009), has been found. Anyway, a direct correlation between root pectin concentration and methylation degree and metal accumulation in root apoplast and symplast *in planta* is still lacking.

The increase in the amount of pectins, especially in those having a low degree of methyl-esterification, has been regarded as a symptom of the defense strategy and plant adaptation to elevated levels of heavy metals in the soil (Krzesłowska, 2011). Results of several studies have provided evidence that plants, in response to the heavy

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metal stress, will increase their cell wall capacity for element accumulation by elevating the amount of polysaccharides, especially pectins, which bind heavy metal ions (Krzesłowska, 2011).

The pectin response to heavy metal treatment has been identified as “wise” (Krzesłowska, 2011), but it is still unclear if it can actually account for naturally evolved plant tolerance to heavy metals. In fact, as far as our knowledge is concerned, such kind of studies have been conducted mainly on selected cultivars of crop plants. For example, an increase in pectins, especially in those with a low degree of methyl-esterification, was found in response to lead in *Allium cepa* root cells (Wierzbicka, 1998), to cadmium in *Linum usitatissimum* hypocotyls (Douchiche et al., 2007), in *Salix viminalis*, (Vollenweider et al., 2006) and in *Oryza sativa* (Xiong et al., 2009). With regards to the response to aluminum, some evidence is in accordance and some other in contrast with the above-mentioned results. In fact, it was found that Al-resistant cultivars of *Cynodon dactylon* accumulated more aluminum in cell walls than the aluminum-sensitive cultivars (Ramgareeb et al., 2004). An aluminum induced increase in pectins was detected in roots of *Cucurbita maxima* (Le Van et al., 1994), *Triticum aestivum* (Tabuchi and Matsumoto, 2001; Hossain et al., 2006), *Zea mays* (Schmohl and Horst, 2000; Schmohl et al., 2000) and *Solanum tuberosum* (Schmohl et al., 2000). On the other hand, the most recent studies have shown that an increase in the level of pectins with a low degree of methyl-esterification and binding  $\text{Al}^{3+}$  within the cell wall are more characteristic of sensitive plant cultivars than those that are tolerant to this metal (Eticha et al., 2005; Amenós et al., 2009; Tolrá et al., 2009).

The results of the above-mentioned studies have provided evidence that, in response to heavy metal stress, plants increase their cell wall capacity for heavy metal accumulation by elevating the amount of polysaccharides, especially pectins, which bind heavy metal ions. Hence, the avoidance of metal ion accumulation in the cell wall as described for some Al-tolerant plants seems to be more an exception than a rule.

As for copper, such kind of studies are mainly concerned with some species of bryophytes and pteridophytes able to accumulate this metal. It was recently shown that cell wall pectins have an important role in Cu accumulation in the fern *Lygodium japonicum* (Konno et al., 2005) and the so-called copper-moss *Scopelophila catarractae* (Konno et al., 2010), which accumulate the highest amount of Cu in their cell wall pectins. Studies concerning copper-induced remodeling of polysaccharides as a higher plant defense strategy to this metal are lacking.

Recently, we investigated the role of the cell wall in the phenomenon of naturally evolved copper tolerance studying metallicolous and non-metallicolous populations of *Silene paradoxa* L. (Colzi et al., 2011). The copper tolerant population of this species was found to realize avoidance at both the symplast and the apoplast level in the roots, involving a low metal binding capacity of the root cell wall. Therefore, the hypothesis that a high binding capacity of cell walls may act to prevent the entry of toxic metals into plant metabolism consequently conferring tolerance (Ernst et al., 1992) was not confirmed. On the other hand, the cell wall hypothesis was from a long time (see for example Verkleij and Schat, 1989) put in doubt by some evidence of a lack of correlation between metal resistance and metal binding capacity of cell wall material. As for a possible metal induced remodeling of the cell wall pectins, our data (Colzi et al., 2011) suggested that in the tolerant population short-term copper treatments could decrease root cell wall pectin concentration and increase pectin methylation degree. Therefore, a low cell wall ability to bind copper was proposed to concur in the generation of the tolerant/excluder phenotype of the *S. paradoxa* copper tolerant population.

In this study we compared two populations of *S. paradoxa* collected from a copper-enriched mine soil and an uncontaminated

one, respectively, evaluating if there can be a direct correlation between root pectin concentration and methylation degree and metal accumulation in root apoplast and symplast. Furthermore, we investigated if in the two populations long term copper exposure can differently affect the development of the root cell wall in terms of pectin concentration and their methylation degree.

## 2. Materials and methods

### 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). Seeds were sown in peat soil and after six weeks seedlings of both populations were transferred to hydroponic culture, in 1-l polyethylene pots (three plants per pot) containing a modified half-strength Hogland's solution composed of 3 mM  $\text{KNO}_3$ , 2 mM  $\text{Ca}(\text{NO}_3)_2$ , 1 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.50 mM  $\text{MgSO}_4$ , 20  $\mu\text{M}$   $\text{Fe}(\text{Na})\text{-EDTA}$ , 1  $\mu\text{M}$   $\text{KCl}$ , 25  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 2  $\mu\text{M}$   $\text{MnSO}_4$ , 2  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.1  $\mu\text{M}$   $\text{CuSO}_4$  and 0.1  $\mu\text{M}$   $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$  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). Nutrient solutions were renewed weekly and plants were grown in a growth chamber (24/16 °C day/night; light intensity 75  $\mu\text{E m}^{-2} \text{s}^{-1}$ , 12 h d<sup>-1</sup>; relative humidity 60–65%).

### 2.2. Short-term exposure test with excised roots

Plants were grown in hydroponic culture for one and for two months before the copper treatment. After the period of pre-culture, the intact plants were transferred to a pre-treatment solution containing 10 mM  $\text{CaCl}_2$  and 5 mM MES with pH adjusted to 5.5, as indicated by Irtelli and Navari-Izzo (2008). 30 min later, the roots of *S. paradoxa* plantlets were excised and incubated for 30 min in a test solution containing copper ( $\text{CuSO}_4$ ) at 0 and 1 mM concentrations and 5 mM MES adjusted to pH 5.5. At the end of incubation in test solutions, samples were washed with milliQ-water and half of them were washed in ice-cold (4 °C)  $\text{PbNO}_3$  (10 mM) for 30 min to remove the adsorbed copper from the root cell wall.

The root material was used for the determination of copper concentration and for the determination of total pectins and the corresponding methylation degree.

### 2.3. Long-term exposure test

Six weeks old seedlings of the two populations were transferred to hydroponic culture containing a nutrient background solution (modified half-strength Hogland's solution, described above) and a series of copper ( $\text{CuSO}_4$ ) concentrations (0, 5, 10 and 15  $\mu\text{M}$ ). Plants were cultivated in a growth chamber (24/16 °C day/night; light intensity 75  $\mu\text{E m}^{-2} \text{s}^{-1}$ , 12 h d<sup>-1</sup>; relative humidity 60–65%) for two months, renewing the growth solutions weekly.

At the end of incubation in test solutions, the root length was measured. Some of the roots were excised and underwent the procedure for the determination of pectins and their methylation degree. The other plant samples were used for the determination of copper accumulation in roots and shoots; half of root samples were desorbed with ice-cold (4 °C)  $\text{PbNO}_3$  (10 mM) for 30 min.

### 2.4. Determination of copper concentration

Copper concentrations were determined by digesting oven-dried plant material in a 5–2 (v/v) mixture of  $\text{HNO}_3$  (Romil, 69%) and  $\text{HClO}_4$  (Applichem, 70%) in 25 ml beakers at 120–200 °C, after

which the volume was adjusted to 10 ml with milliQ-water. Copper was determined on an atomic absorption spectrophotometer model Analyst 200 (PerkinElmer, Waltham, Massachusetts, USA). Apoplastic copper concentration in roots was calculated as the difference between copper concentration in non-desorbed and desorbed samples.

### 2.5. Pectin determination

At the end of incubation in test solutions, the excised roots were rinsed with milliQ-water and transferred into a 10 ml centrifuge tube. Absolute ethanol (5 ml) was added into the tubes and the samples were mixed with a vortex mixer for 5 min and centrifuged at 15,000 rpm for 5 min. The supernatant was discarded and the samples were extracted three more times with the same amount of ethanol. Roots were transferred to petri dish and dried for 12 h at 35 °C in a conventional oven.

Pectin extraction and determination were carried out according to Yu et al. (1996) with little modifications. Dried root material (about 10 mg) was weighed in test tubes (six replicates for each thesis), 2 ml-aliquots of 98% sulfuric acid and 0.5 ml-aliquots of milliQ-water were added and samples were stirred in a vortex mixer for 5 min. Further 0.5 ml-aliquots of milliQ-water were added dropwise and vortex mixed for 5 min. The samples were filtered on glass wool and adjusted to 10 ml with milliQ-water before use.

Pectin contents of extracts were analyzed by the m-hydroxydiphenyl method; 1 ml of 1:10 diluted extracts was pipetted into a test tube. 6 ml of sulfuric acid/tetra-borate solution (0.0125 M sodium tetra-borate in concentrated sulfuric acid) were added to the samples in an ice water bath and mixed carefully with a vortex mixer. Tubes were heated up in a water boiling bath for 5 min and immediately placed in ice water to cool. 0.1 ml-aliquots of 0.15% m-hydroxydiphenyl (Sigma–Aldrich, St. Louise, MO, USA) were added and the samples were incubated for 15 min at room temperature. The corresponding blank consisted of a solution containing 1 ml of milliQ-water, 6 ml of sulfuric acid/tetra-borate solution and 0.1 ml of sodium hydroxide 0.5%.

The absorbance of the samples was measured at 520 nm using a spectrometer model DR 4000, (Hach Company, Loveland, CO, USA).

Galacturonic acid (Sigma–Aldrich) was used as a calibration standard (concentrations ranged from 10 to 100 µg/ml) and the root total pectin concentration was expressed as galacturonic acid equivalents.

### 2.6. Degree of methylation of pectins

Roots of *S. paradoxa* were prepared in the same way as for pectin determination, by means of four successive washes with absolute ethanol followed by centrifuge and dried for 12 h at 35 °C in a conventional oven. Hydrolysis of methylated pectins and determination of the resulting methanol were carried out according to Klavons and Bennett (1986) with little modifications. About 10 mg of dried root material were weighed in test tubes (six replicates for each thesis), 3 ml of sodium hydroxide (1.5 M) were added and the solution was incubated for 30 min at room temperature. The pectin hydrolyzates were neutralized with phosphoric acid (1 M) to pH 7.0 ± 0.1 and the volume was adjusted to 10 ml with milliQ-water. Aliquots of 1 ml of this solution were mixed in a tube with 1 ml of alcohol oxidase from *Piccia pastoris* (1 UN/ml prepared by diluting 6.8 µl of alcohol oxidase with 10 ml of milliQ-water) and then incubated at 25 °C for 15 min. Afterwards, 2 ml of fluoral-P (0.02 M 2,4-pentanedione in 2.0 M ammonium acetate and 0.05 M acetic acid) were added and vortex mixed. The tubes were placed in a bath water at 60 °C for 15 min and then cooled at room temperature.

Absorbances were measured at 412 nm against a blank containing 1 ml phosphate buffer at pH 7 and 1 ml of alcohol oxidase.

Methanol was used for the calibration curve (concentrations ranged from 0.5 to 5 µg/ml).

Methylation degree (M.D.) was calculated with the following equation:

$$\text{M.D.} = 100 \times \frac{\text{moles of methanol}}{\text{moles of total pectins}}$$

Standard deviation associated to the methylation degree was calculated according to the error propagation rule.

### 2.7. Statistics

Measurements of root length were performed on twelve replicates, the determination of copper and pectin concentration was made on six replicates. Each replicate was measured three times.

Statistical analysis was carried out with one-way and two-way ANOVA using the statistical program SPSS 13.0 (SPSS Inc., Chicago, IL, USA). A posteriori comparison of individual means was performed using Tamhane post hoc test.

## 3. Results

### 3.1. Short-term exposure test with excised roots

Symplastic and apoplastic copper concentrations (Table 1) in excised roots of control plants were not significantly different between one- and two-month-old plants both in CVD and in FC population. Regarding the treated samples (1 mM CuSO<sub>4</sub>), copper accumulation, in both symplast and apoplast, was significantly higher in the younger plants in both populations ( $p < 0.01$ ).

Comparing the two population symplastic copper concentrations (Table 1), no significant differences were observed for the one-month-old roots, while in the two-month-old roots a significantly higher copper accumulation was found in CVD population ( $p < 0.01$ ). As for apoplastic concentrations (Table 1), the copper concentrations were also significantly lower in FC plantlets in both the one- and two-month-old plant experiment ( $p < 0.01$ ;  $p < 0.05$ ).

The percentages of apoplastic and symplastic copper concentrations in excised copper treated roots were calculated. CVD showed significantly ( $p < 0.01$ ) higher percentage of copper in the apoplast of one-month-old plants ( $72.3 \pm 0.5\%$ ) in respect to the two-month-old plants ( $58.1 \pm 1.2\%$ ), while in FC an opposite trend has been observed, being such percentage significantly ( $p < 0.01$ ) higher in the older plants ( $57.6 \pm 2.1\%$  and  $66.2 \pm 2\%$  for one-month-old plants and two-month-old plants respectively). Between the two populations, CVD showed significantly ( $p < 0.01$ ) higher percentage of apoplastic copper in the one-month-old plants and significantly ( $p < 0.01$ ) lower in the two-month-old plants in respect to FC plants.

Total pectin concentrations, methanol concentrations and methylation degrees of pectins are reported in Table 2. Results showed a decrease in all the parameters investigated between one- and two-month-old plant experiments in both the populations and for both control and copper treated plants.

In respect to control plants, copper treated ones did not give any significant variation of total pectin concentrations, methanol concentrations and methylation degrees in CVD population. Conversely, in FC plants a significant decrease of pectin concentrations was observed with copper exposure, both in one- and two-month-old plants ( $p < 0.01$ ). Moreover, the FC population showed a significant increase of the methylation degree after copper treatment in both one- and two-month-old plant experiments.

By comparing the two populations, in the one-month-old plant experiment total pectin concentrations of control plants were significantly higher in FC than in CVD population, while in the treated plants there were no significant differences (Table 2). The total pectin concentrations in two-month-old plants resulted

**Table 1**  
Copper accumulation in excised roots of two populations of *S. paradoxa* (CVD non-mine population and FC mine population) after copper (CuSO<sub>4</sub>) exposure for 30 min. Values are means of six replicates ± standard error, significant differences between the means appear with different letters, small for the intra-population and capital for the inter-population ones (\**p* < 0.05; \*\**p* < 0.01).

Treatment (CuSO <sub>4</sub> mM)	Symplastic Cu concentrations (μg g <sup>-1</sup> d.w.)		Apoplastic Cu concentrations (μg g <sup>-1</sup> d.w.)	
	1 month	2 months	1 month	2 months
CVD				
Control	17.2 ± 0.6 aA	18.8 ± 0.7 aA	1.3 ± 0.3 bA	1.6 ± 0.7 bA
1	467 ± 14 cB**	290 ± 24 dC**	1219 ± 97 eB**	403 ± 22 cB**
FC				
Control	16.0 ± 0.8 aA	13.7 ± 0.9 aB**	1.5 ± 0.6 bA	2.8 ± 1.3 bA
1	445 ± 39 cB**	171 ± 29 dD**	605 ± 77 eC**	336 ± 19 fC*

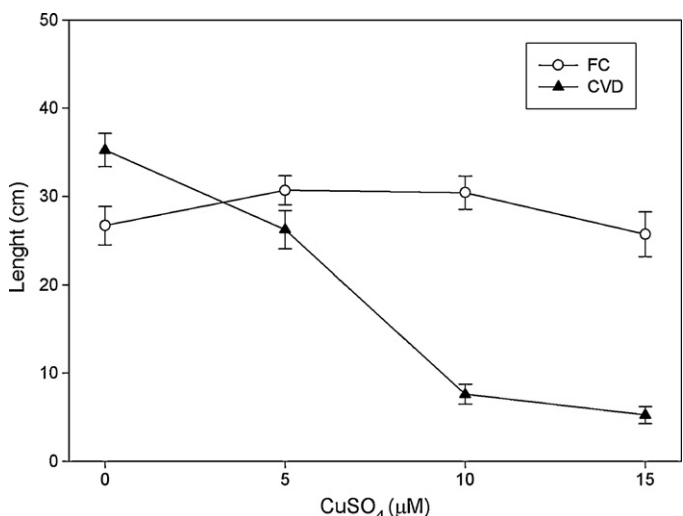
**Table 2**  
Total pectin concentrations, methanol concentrations resulting from hydrolysis of methylated pectins and the corresponding methylation degrees found in excised roots of two populations of *S. paradoxa* (CVD non-mine population and FC mine population) after copper (CuSO<sub>4</sub>) exposure for 30 min. Values are means of six replicates ± standard error, significant differences between the means appear with different letters, small for the intra-population and capital for the inter-population ones (\**p* < 0.05; \*\**p* < 0.01).

Treatment (CuSO <sub>4</sub> mM)	Total pectin concentrations (μmol galacturonic acid equivalents g <sup>-1</sup> d.w.)		Methanol concentrations (μmol CH <sub>3</sub> OH g <sup>-1</sup> d.w.)		Methylation degrees (%)	
	1 month	2 months	1 month	2 months	1 month	2 months
CVD						
Control	596 ± 44 aA	376 ± 15 bA	206 ± 35 aA	52.5 ± 3.7 bA	34.9 ± 5.2 aA	14.1 ± 1 bA
1	657 ± 39 aA	425 ± 25 bA	184 ± 25 aA	60.8 ± 4 bA	28.8 ± 4 aA	14.5 ± 0.8 bA
FC						
Control	799 ± 38 aB**	423 ± 21 bA	245 ± 22 aA	63.3 ± 4.6 bA	30.7 ± 2.2 aA	15 ± 0.5 bA
1	606 ± 22 cA	325 ± 13 dB**	223 ± 22 aA	57.6 ± 2.6 bA	36.8 ± 1.9 aB*	18 ± 0.8 cB**

significantly lower in treated FC plants as compared to treated CVD ones. No significant differences were found in methanol concentration between the treatment and the populations. The methylation degrees of copper treated plants were higher in FC plants than in CVD ones in both the experiments (one-month-old: *p* < 0.01; two-month-old: *p* < 0.05).

### 3.2. Long-term exposure test

Based on the root growth response, the FC plants exhibited a significantly higher copper tolerance than the CVD plants (*p* < 0.01) (Fig. 1). In FC plants, there was no significant decrease in root



**Fig. 1.** Root length of two populations of *S. paradoxa* (○, Colle Val D'Elsa non-mine population (CVD); ▲, Fenice Capanne mine population (FC)) (mean of twelve replicates ± standard error) after long-term exposure to increasing copper concentrations (0, 5, 10, 15 μM CuSO<sub>4</sub>) for two months.

growth. On the contrary, in the CVD plants, all the concentrations produced a significant decrease of root length (*p* < 0.01).

Copper concentrations in roots and shoots of long-term exposed plants are shown in Table 3. The copper concentrations in roots and shoots of both populations increased proportionally with increasing copper exposure, except for CVD shoots that showed a saturation trend. Symplastic copper concentrations were significantly lower in FC as compared to CVD at all the external copper concentrations used (*p* < 0.05), while apoplastic copper concentrations were lower only at the two highest external copper concentrations used (*p* < 0.05).

Regarding the percentage of apoplastic copper concentrations in long-exposure copper treated roots, FC showed significantly (*p* < 0.01) higher values at higher copper concentrations (10 CuSO<sub>4</sub> μM – 46.6 ± 1.5%; 15 CuSO<sub>4</sub> μM – 46.7 ± 1.8%) in respect to the lowest copper concentration (5 CuSO<sub>4</sub> μM – 9.7 ± 2.4%). Similarly, in CVD the percentage of copper in the apoplast was significantly (*p* < 0.01) lower in the lowest copper concentration (5 CuSO<sub>4</sub> μM – 3.7 ± 0.7%) in respect to higher copper concentrations (10 CuSO<sub>4</sub> μM – 22.7 ± 0.6%; 15 CuSO<sub>4</sub> μM – 40.4 ± 1.7%). In FC, comparing with CVD population, the percentage of apoplastic copper concentration was significantly higher for all the concentrations (at least *p* < 0.05).

Copper concentrations in shoots were much lower than in roots even if they increased with increasing copper treatments (Table 3). Shoot copper concentration was significantly lower in the FC mine population compared to CVD (*p* < 0.05), except for the highest copper treatment.

Total pectin concentrations, methanol concentrations and methylation degrees after long-term copper exposure treatments, are shown in Table 4. Results indicated that copper treatments gave an inverse trend for the two population. In FC plants, a significant decrease was observed at the two highest copper treatments, while in CVD population a significant increase of total pectin concentrations was found with the two highest copper concentrations (*p* < 0.05) in respect to control. Significant differences were found between the two populations in total pectin concentrations, as they



**Table 3**

Copper accumulation in roots and shoots of two populations of *S. paradoxa* (CVD non-mine population and FC mine population) after long-term exposure to increasing copper concentrations (0, 5, 10, 15  $\mu\text{M}$   $\text{CuSO}_4$ ) for two months. Values are means of six replicates  $\pm$  standard error, significant differences ( $p < 0.05$ ) between the means appear with different letters, capital for the inter-population and small for the intra-population ones. b.d.l. = below detection limit.

Treatments ( $\text{CuSO}_4$ $\mu\text{M}$ )	Root symplastic Cu concentrations ( $\mu\text{g g}^{-1}$ d.w.)	Root apoplastic Cu concentrations ( $\mu\text{g g}^{-1}$ d.w.)	Shoot Cu concentrations ( $\mu\text{g g}^{-1}$ d.w.)
CVD			
Control	19 $\pm$ 1 aA	b.d.l.	9.9 $\pm$ 0.3 aA
5	258 $\pm$ 34 bA	10 $\pm$ 5 aA	42 $\pm$ 2 bA
10	839 $\pm$ 56 cA	246 $\pm$ 12 bA	97 $\pm$ 7 cA
15	663 $\pm$ 17 dA	449 $\pm$ 52 cA	81 $\pm$ 3 dA
FC			
Control	26 $\pm$ 4 aA	b.d.l.	7.6 $\pm$ 0.5 aA
5	121 $\pm$ 2.5 aB	13 $\pm$ 9 aA	32 $\pm$ 2 bB
10	204 $\pm$ 8 bB	178 $\pm$ 10 bB	62 $\pm$ 3 cB
15	404 $\pm$ 78 cB	354 $\pm$ 38 cB	96 $\pm$ 5 dB

**Table 4**

Total pectin concentrations, methanol concentrations resulting from hydrolysis of methylated pectins and the corresponding methylation degrees found in roots of two populations of *S. paradoxa* (CVD non-mine population and FC mine population) after long-term exposure to increasing copper concentrations (0, 5, 10, 15  $\mu\text{M}$   $\text{CuSO}_4$ ) for two months. Values are means of six replicates  $\pm$  standard error, significant differences ( $p < 0.05$ ) between the means appear with different letters, small for the intra-population and capital for the inter-population ones.

Treatments ( $\text{CuSO}_4$ $\mu\text{M}$ )	Total pectin concentrations ( $\mu\text{mol}$ galacturonic acid equivalents $\text{g}^{-1}$ d.w.)	Methanol concentrations ( $\mu\text{mol}$ $\text{CH}_3\text{OH g}^{-1}$ d.w.)	Methylation degrees (%)
CVD			
Control	392.3 $\pm$ 24.5 aA	48.4 $\pm$ 4.3 aA	12.33 $\pm$ 0.8 aA
5	469.0 $\pm$ 34.0 aA	56.5 $\pm$ 4.7 aA	12.0 $\pm$ 0.5 aA
10	512.8 $\pm$ 62.5 bA	70.1 $\pm$ 9.4 bA	13.7 $\pm$ 0.8 aA
15	605.6 $\pm$ 74.7 cA	67.4 $\pm$ 11.2 abA	11.14 $\pm$ 1.2 aA
FC			
Control	440.1 $\pm$ 23.1 aA	71.4 $\pm$ 8.1 aB	16.2 $\pm$ 1.6 aB
5	405.4 $\pm$ 26.9 abA	62.5 $\pm$ 5.2 aA	15.4 $\pm$ 1.3 aB
10	306.8 $\pm$ 27.6 bB	60.8 $\pm$ 8.3 aA	17.7 $\pm$ 1.2 aB
15	320.2 $\pm$ 28.5 bB	58.8 $\pm$ 5.5 aA	15.9 $\pm$ 0.8 aB

were lower in FC population in respect to CVD one at the two highest concentration used. As for methanol concentration (Table 4), the only significant difference was observed in CVD population exposed to 10  $\mu\text{M}$   $\text{CuSO}_4$ , where it was significantly higher as compared to the control and to 5  $\mu\text{M}$   $\text{CuSO}_4$ . The methanol concentration did not differ between the two populations, except for the control plants, being FC methanol concentration higher than in CVD. Even the methylation degree did not change increasing copper concentration in the culture medium. Comparing the two populations, the methylation degrees were higher in FC plants than in CVD plants ( $p < 0.05$ ).

#### 4. Discussion

Plants with different ages were used to compare copper accumulation in roots containing different concentrations and methylation degree of pectins, as the latter are inserted into the wall as highly methyl-esterified polymers subsequently deesterified to varying degrees during the development of the plant itself (Mohnen, 2008). After a short-term copper treatment for the evaluation of plant responses to acute metal stress (Table 1), copper accumulation was significantly higher in one-month-old plants than in two-month-old plants in both the populations and in both apoplast and symplast. At the same time, the tolerant population showed a lower copper accumulation in respect to the sensitive one, for apoplastic copper statistically significant in plants of both the two ages and for symplastic copper only in the older plants. Probably, the time and the concentration used were not adequate to generate also a significant difference in symplastic copper accumulation in younger plants. Therefore, the excluder nature, both at the symplastic and the apoplastic levels, of the copper tolerant population of *S. paradoxa* (Gonnelli et al., 2001; Colzi et al., 2011) was confirmed and a higher copper accumulation in young

plants assessed. This latter result can be due to the fact that young roots can be more efficient than older roots at taking up elements from the solution, probably because a proportion of roots becomes more and more inactive in uptake functions during development. Consequently, the root copper absorption function might vary with the morphology and the architecture of the root system, as already suggested by Perriguet et al. (2008) for cadmium in maize.

Calculating the percentage ratio between copper concentration in apoplast and symplast, an interesting result came out. In the sensitive population, plants of both ages allocated copper preferentially in the apoplast, but to a greater extent in one-month-old plants. Therefore, in this population, when the root structure allowed a higher total amount of copper to be taken up, its allocation was preferentially apoplastic. The tolerant population showed the opposite trend: even if the percentage of apoplastic copper was always higher than the symplastic one, the plants that preferentially allocated copper in the apoplast were the two-month-old ones. These plants were also the ones with the lower total amount of copper taken up. This result could suggest an interesting hypothesis: in both populations root developed in a way that tended to diminish copper accumulation, but in a population-dependent way. The copper tolerant population underwent a kind of root development devoted to favor the exclusion of copper from the symplast, thus preventing the onset of the stress as metal tolerant plants generally show (Hall, 2002). In other words, in such development the decrease of copper accumulation was mainly dependant on the decrease of the symplastic copper concentration, whereas in the sensitive population such decrease was mainly dependant on the apoplastic component. In fact, older tolerant plants showed the actual lowest value of symplastic copper. Probably, this condition may be achieved also by the lowest copper accumulation displayed in their apoplast and not only by a reduced copper uptake into the

root cells, being this latter strategy already demonstrated in copper tolerant *S. vulgaris* (Van Hoff et al., 2001).

To find if in *S. paradoxa* the above-mentioned results could be correlated to a different composition of the cell wall, its pectin concentration and methylation degree were evaluated in the different populations (Table 2). One-month-old plants showed root pectin concentration higher than two-month-old plants, confirming that the pectin concentration decreases during cell wall and whole plant development and differentiation (Scheller et al., 2007). Not only the pectin concentration was different but also the methylation degree. In fact, in accordance with the fact that pectins are deposited highly methyl-esterified and then are demethylated (Mohsen, 2008), young plants showed a methanol concentration and a methylation degree higher than older plants. Once more, a consideration on a different kind of root cell wall development between the two populations can be made. The control plants of the tolerant population showed a higher age-dependant decrease in pectin concentration (from 800 to 420  $\mu\text{mol}$  galacturonic acid equivalents  $\text{g}^{-1}$  d.w. in tolerant plants and from 600 to 380  $\mu\text{mol}$  galacturonic acid equivalents  $\text{g}^{-1}$  d.w. in the sensitive plants) and a lower age-dependant decrease of the methylation degree (from 30% to 15% in the tolerant population and from 35% to 14% in the sensitive population), thus seeming to develop roots with a supposed lower capacity of binding metals during plant growth.

Comparing the changes induced by the short-time copper treatment between the populations, data confirmed the results obtained by Colzi et al. (2011). In the tolerant population copper treatment provoked a significant decrease in total pectin concentrations, whereas the statistically significant difference found in pectin methylation degree between the two populations depended mainly on the pectin decrease observed in the tolerant population, rather than on an increase of their methylation. Thus, in the presence of copper, the tolerant population of *S. paradoxa* seemed to be able to reduce the metal binding sites of the cell wall and, as a consequence, the apoplast copper concentration. In fact, the structural and ionic characteristics of the apoplast can affect the ion composition of the medium that bathes the cell membrane (Grignon and Sentenac, 1991) and thus the amount of metal that enters the cell. The differences found in pectin composition and methylation degree that generated apoplasts with different cation exchange capacity of the root (Sattelmacher, 2001) could be one of the factors that concur to a lower copper accumulation also in the symplast. So, at least for short-time exposure experiments, in *S. paradoxa* the remodeling of polysaccharides for copper tolerance strategy did not seem in favor of an increase of pectins and a decrease of methylation degree, as found for selected cultivars of crop plants or the so-called copper mosses (as reviewed in Krzesłowska (2011)) and identified as a “wise” response.

Comparing results on copper accumulation and pectins, a positive correlation can be found, that is the higher the pectin concentration, the higher the copper accumulation for both the apoplast and the symplast. Until now, a direct correlation between root pectin concentration and metal accumulation in root apoplast and symplast *in planta* has not been found. Correlations between different methylation degrees and copper accumulation can be drawn comparing only treated one-month-old plants, as they displayed not statistically different pectin concentration. In this condition, *S. paradoxa* tolerant population plants showed a higher methylation degree and a lower apoplastic copper concentration, thus suggesting a negative correlation between these two parameters *in vivo*, as it was for example suggested by Mimmo et al. (2009) studying aluminum sorption in extracted pectins.

Long-term exposure experiments were performed to evaluate if the presence of different copper concentration in the culture medium could affect differently in the two populations the development of root cell walls, in terms of their pectin composition and

methylation degree. The concentrations used in that experiment allowed a normal plant development for the tolerant population and an impaired development for the sensitive one (Fig. 1). As far as metal accumulation is concerned (Table 3), both apoplastic and symplastic copper concentrations increased with increasing metal concentration in the culture medium. Only at the higher concentration used, the symplastic copper accumulation did not follow this trend in the sensitive population. This condition could just be the result of copper toxicity, impairing every cellular function, ion uptake included. Comparing the populations, even in this kind of experiment, the tolerant population confirmed its excluder nature, for both copper accumulation in the symplast and in the apoplast, in respect to the sensitive population. The percentage of copper allocation in root symplast and apoplast was compared between the populations excluding the lowest concentration used, as it generated a very low accumulation of copper in the apoplast, too far from a result that can be reliably discussed. Results indicated that, increasing copper medium concentration, the tolerant population did not change its allocation pattern probably as a consequence of an efficient and active exclusion from both the compartments at the same time. Increasing copper medium concentration, the sensitive population tended to allocate the metal preferentially in the apoplast. Whatever the reason of the sensitive population was to significantly allocate more copper in this compartment, it did not succeed in protecting the plant from copper toxicity. Furthermore, this behavior could also be one of the causes of the lower root elongation displayed by the sensitive population, as, besides inhibiting meristematic cell divisions, metals bound to the cell wall contribute to its stiffening, thus being one of the main causes of plant growth inhibition observed under heavy metal stress (Hall, 2002; Patra et al., 2004; Poschenrieder et al., 2008).

The excluder phenotype was realized also for shoot copper accumulation as the tolerant population showed the lower values. Only at the highest concentration used, the sensitive population showed a lower shoot copper accumulation. This result could probably derive from an impairing of the root to shoot transport system due to a too high copper toxicity.

The differences found in copper-induced changes in root cell wall composition (Table 4) could concur to explain the above-mentioned data. The most intriguing result was that in the two *S. paradoxa* populations copper-induced responses were both opposite and at different degrees, the sensitive population showing the widest response, probably just because it was the more copper-stressed. Particularly, the tolerant population decreased the root pectin concentration, even if to a low extent, whereas a statistically significant increase in total pectin concentration in the sensitive population was found following the increase of copper in the culture medium. To our knowledge, this was the first time that a similar result was found studying naturally evolved tolerant population of an excluder plant (for detailed references on plant cell wall responses to heavy metals see for example Krzesłowska (2011)).

Regarding the methanol concentration, it increased in the sensitive population, even if not always in a significant way for all the concentrations used. This increase was proportional to the increase in the total pectin concentration, so that the methylation degree did not change. In the tolerant population, the presence of copper in the culture medium decreased the methanol concentration, even if in a non-significant way in respect to the control, thus not leading to any variation in the methylation degree. Therefore, in the presence of increasing copper concentrations, the sensitive population seemed to respond by increasing the ability of the root cell wall to bind the metal rising the concentration of pectins with the same methylation degree. The opposite behavior was shown by the tolerant population. This response could be fundamental to guarantee a lower apoplastic copper concentration, that could be one of the factors concurring also to limit copper uptake by the root

cells. Moreover, other important results regarded the methylation degree, that was always higher in the tolerant population in respect to the sensitive one, possibly to lower the ability of the root cell wall to bind copper. That situation was found even in the control plants, in which, in addition, also the methanol concentration was higher, probably to constitutively allow the formation of root cell walls with a lower ability to bind any metal.

In respect to the paper of Krzesłowska (2011) and the root cell wall response there proposed, with the only exception of some of the studies on aluminum, *S. paradoxa* tolerant plants seemed to perform the opposite strategy, that was restricting the immobilization of copper in the root cell wall. In our opinion, both the responses can be “wise” and adopted by plants, depending on the level of tolerance that needs to be reached. The tolerance level of metallophytes is undoubtedly higher than the one of crop species selected cultivars, as the former live in very stringent environments realizing adaptive metal tolerance strategies extremely effective in the protection from high heavy metal stress. Indeed, also crop plants can be characterized by different levels of constitutive metal tolerance, just having some variation in their metal homeostatic network. However, this feature cannot be sufficient to enable them to cope with extremely high metal concentrations in the environment, as metallophytes do. Both quantitative and qualitative reasons can be produced: an inadequate variation level of metal tolerance or the identification of a response that represented just an accidental mechanism allowing some cultivars to achieve a different level of tolerance. Moreover, another consideration deserves to be made: a simple tolerance strategy, based on the cell wall working like a “filter” immobilizing elements to exclude them from the symplast, would have scarce success. Although the root cell wall is directly in contact with metals in the soil solution, adsorption onto the cell wall must be of limited capacity and thus have a limited effect on metal activity at the surface of the plasma membrane. Therefore, it is hard to explain metal tolerance with such a mechanism (Ernst et al., 1992). Moreover, a root cell wall with a high capacity of metal binding will be unfavorable for growing in a metalliferous soil, as the high level of root metal binding that would be realized will inhibit the root growth itself promoting cell wall stiffening (Eticha et al., 2005; Yang et al., 2008). Therefore, the decrease in pectin levels operated by the mine population can be really considered as one of the adaptations to naturally realize exclusion for living in copper contaminated environments. This assumption is in accordance with some recent results obtained for aluminum (Eticha et al., 2005; Amenós et al., 2009; Tolrá et al., 2009). On the other hand, a confirmation of such hypothesis can be indirectly provided by the behavior of copper hyperaccumulator plants that is exactly the other way round, such as their tolerance strategy: hyperaccumulators have high metal levels in their cell walls as reviewed in Krzesłowska (2011).

## 5. Conclusions

Exclusion of copper showed by the metalcolous population of *S. paradoxa* seemed to be realized through a fine remodeling of some of the root cell wall polysaccharides. Pectins concentrations and their methylation degree changed in different ways in the sensitive and the tolerant populations in both short- and long-term exposure experiments. The common feature was that the tolerant population always seemed to generate root cell walls with a low ability of binding copper through a decrease of pectin concentration and an increase of their methylation degree. This physiological adjustment concurred to generate metal-excluding root cell walls in the tolerant population of *S. paradoxa*. This condition resulted in a lower accumulation of copper in the root apoplast and probably

was also one of the factors concurring to the limitation of copper accumulation in the symplast.

Therefore, the metallophyte root response to excessive metal concentrations is still far to be exhaustively elucidated and is undoubtedly fundamental to unravel some physiological strategies concurring to metal tolerance. Furthermore, information on this topic can be of fundamental importance also to optimize the possible use of metalcolous plants in phytoremediation techniques.

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