

**Paradoxical effects of density on measurement of copper tolerance in *Silene paradoxa* L.**

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## **Abstract**

This work investigated if the assessment of tolerance to trace metals can depend on plant density in the experimental design. A non-metallicolous and a metallicolous population of *Silene paradoxa* were hydroponically cultivated at increasing density and in both absence (-Cu conditions) and excess of copper (+Cu conditions). In -Cu conditions, the metallicolous population showed a lower susceptibility to plant density in comparison to the non-metallicolous one, explainable by a higher capacity of the metallicolous population to exploit resources. In +Cu conditions, an alleviating effect of increasing density was found in roots. Such effect was present at a higher extent in the non-metallicolous population, thus making the populations equally copper tolerant at the highest density used. In shoots, an additive effect of increasing plant density to copper toxicity was reported. Its higher intensity in the metallicolous population reverted the copper tolerance relationship at the highest plant densities used. In both populations, a density-induced decrease in root copper accumulation was observed, thus concurring to the reported mitigation in +Cu conditions.

Our work revealed the importance of density studies on the optimization of eco-toxicological bioassays and of metal tolerance assessment and it can be considered the first example of an alleviating effect of increasing plant number on copper stress in a metallophyte.

## **Abbreviations**

MET, metallicolous population; NONMET, non-metallicolous population;

## Introduction

Interaction among plants is a well-known phenomenon in the plant ecology of stressful environments (Bruno et al. 2003, Liancourt et al. 2005, Lortie and Turkington 2002). Generally, the outcomes of such interactions have been found to be positive at the inter-specific level, with plants showing greater individual fitness or abundance than when they grow alone (Callaway and Walker 1997, Maestre and Cortina 2004, Tielbörger and Kadmon 2000). Despite this, some reports demonstrate that also competition can occur in extreme habitats (Maestre et al. 2003, Pugnaire and Luque 2001). At the intra-specific level, studies about plant-plant interaction in stressful environments are very scarce, without univocal results and only rarely considering populations from contrasting habitats (Dudley and Schmitt 1995, Shaw and Platenkamp 1993, Shaw et al. 1995, Chu et al., 2008).

Surely, among stressful environments, metalliferous soils are largely represented and characterized by high concentrations of trace metals, due to both natural factors and, especially, human activities (Alloway, 2012). Such soils may represent biodiversity hotspots, showing high richness and endemism in plant species (Faucon et al., 2016). To achieve element homeostasis in presence of excessive metal concentrations, plants are able to evolve adaptation at the physiological and molecular levels (Hall 2002, Küpper and Andresen 2016). Therefore, the so-called “metallophytes” (Baker 1987, Ernst 2006) represent a unique and particularly interesting aspect of plant stress biology, considering also that such plants may represent the only valuable choice for the restoration of metal-contaminated soils (Whiting et al. 2004). Metallophytes generally restrict metal entry into the root and translocation to the shoot, or, less frequently, accumulate metals in their shoots at high concentrations, and are thus respectively termed “excluders” and “hyperaccumulators” (Baker 1981, Brooks et al. 1977). Specifically, hyperaccumulators are able to accumulate in the aboveground parts trace metals approaching and even exceeding the concentrations of the macronutrients (Krämer, 2010). Some exclusion and hyperaccumulation physiological mechanisms have been clarified (Ovečka and Takáč 2014, Rascio and Navari-Izzo 2011), but the picture of metal tolerance is far from being complete. About the occurrence of plant-plant interaction at the intra-specific level in metallophytes, there is only one report about a reciprocal planting of a serpentine and a non-serpentine population of *Plantago erecta* (Espeland and Rice 2007). The study showed a mitigating effect of density in the non-serpentine population grown on serpentine soil, a substrate naturally enriched in trace metals (Gonnelli and Renella, 2012), and ascribed such result to changes in metal accumulation, even though plant elemental composition was not investigated. Also in the case of non-metallophytes only one study exists, at least to the best of our knowledge, and it regards the combined effect of plant density and copper excess (Hansi et al. 2014). In that work, the authors showed that increasing plant densities alleviated the toxic effect of copper when growing barley at in the presence of the metal and correlated it to a decrease in shoot copper concentration.

In any case, due to the increasing spread of metal pollution in the environment over the last century, growing attention has been dedicated to the assessment of their toxicity on living organisms, plants

included. Actually, higher plants have been proposed, already from a long time, as excellent model for toxicity testing in the case of inorganic contaminants (Wang, 1991). Nonetheless, in eco-toxicological or in physiological studies the effects of trace metals are evaluated without including different levels of plant densities in a standardized manner (see for example Charles et al. 2011, Sinkkonen et al. 2009). Conversely, in the case of organic pollutants, the occurrence of positive interactions in their presence has been firmly established and resolved in the consideration that the toxin dose received by a plant is inversely related to plant density (Weidenhamer 2006). Therefore, due to the many literature examples, at the inter-specific level, about plant-plant interaction in stressful environments and the two above-mentioned reports at the intra-specific level about element excess (Espeland and Rice 2007, Hansi et al., 2014), a significant occurrence of a density-dependent response of plants in the case of trace metal stress cannot be excluded, thus insinuating possible doubts on the results of the element toxicity assessment in relation to the experimental conditions used. In addition, also in physiological studies on plant adaptation to metalliferous environments the density effect is never taken into consideration, thus opening the possibility to either interesting findings or misevaluations in the phenomenon of metal tolerance and, at the same time, further claiming for intensive research on such topic. Nevertheless, density study can be of fundamental importance also for the implementation of the new emerging “agromining” practices (van der Ent et al., 2015).

Here, we propose a hydroponic experiment in presence of excessive copper concentration to study the effect of increasing plant density on the assessment of metal toxicity in a metallicolous and a non-metallicolous population of a facultative metallophyte. Our study is the first to take into consideration at the same time plant density, copper effect, different populations and metal accumulation in both roots and shoots. To this aim, we chose two contrasting populations of the metallophyte *Silene paradoxa* L. (Caryophyllaceae), a species living in non-contaminated areas and showing metal-tolerant populations on metalliferous soils as well (Chiarucci et al. 1995). In previous studies, the metallicolous population of *S. paradoxa* from the Fenice Capanne mine (Tuscany, Italy) was found to be tolerant to some elements present in excess in the mine dump in comparison to a population from a non-contaminated soil (Arnetoli et al. 2008a, 2008b, Colzi et al. 2014, Gonnelli et al 2001). In the present study, copper was chosen as the environmental toxin because copper tolerance has already and widely been assessed in such metallophyte of the excluder type, like most of the copper tolerant plants are (Lange et al., 2017), and some of its copper exclusion mechanisms have already been clarified (Bazihizina et al. 2015, Colzi et al. 2011, 2012, 2015).

Our study tested the hypothesis of a possible relationship between metal effect and plant density in hydroponics experiments, evaluated its dependence on changes in the amount of metal accumulated by the plant and, through the comparison between a metallicolous and a non-metallicolous population, it investigated whether the experimental design could have any effect on the expression of metal tolerance.

## **Materials and methods**

### **Plant material and experimental conditions**

*Silene paradoxa* seeds were collected at the Fenice Capanne (MET, metallicolous population, Tuscany, Italy) mine waste, characterized by excessive concentrations of As, Cu, Fe, Pb and Zn (Pignattelli et al. 2012, total Cu  $1443 \pm 658 \mu\text{g g}^{-1}$  d.w.), and at Colle val d'Elsa (NONMET, non-metallicolous population, Tuscany, Italy) uncontaminated soil (Arnetoli 2004, total Cu  $33.4 \pm 3.5 \mu\text{g g}^{-1}$  d.w.) during summer 2014 and stored at 4°C until use. The soil total Ca, Mg and K concentrations were about 4.1%, 2.0% and 0.5% at Colle val d'Elsa and 3.3%, 0.5% and 0.1% at Fenice Capanne mine waste, respectively (Mascaro et al. 2001, Rabatti 2015). Although the experiment was not performed aseptically, the seeds were submitted to a disinfection treatment in order to avoid the spreading of contaminants on the surface of culturing media. The treatment consisted of soaking the seeds in a 0.1% HgCl<sub>2</sub> solution for 8 min followed by three rinses with sterile deionized water. The seeds were then put to germinate on filter paper, wet with sterile distilled water, and placed within plastic germination boxes; the boxes were kept in a climatic chamber at 24±1 °C in darkness. The seedlings were collected after two weeks, when they were about 1 cm tall (about 0.01 mg dry weight, presence of the cotyledons only), and transferred in small plastic pots (5.4 mm diameter, 5.4 mm height, ca. 23 cm<sup>2</sup> area, ca. 100 cm<sup>3</sup> vol) containing 7g of sterile perlite (pellet diameter 0.4-0.6 cm). The pots were filled with 60 ml of sterilized Hoagland's solution at half concentration, pH adjusted to 5.6 with KOH 0.1 mol L<sup>-1</sup> (-Cu conditions), and, for the copper treatment (+Cu conditions), the solution contained CuSO<sub>4</sub> 10 μ mol L<sup>-1</sup>. As suggested by Hansi et al. (2014), the hydroponics setup presents the advantage of minimizing the possibility that uncontrolled environmental factors could affect the metal availability and consequently its toxicity, fundamental in the proposed kind of study. The plantlets were transferred in different pots at increasing density, ranging from 1 to 50 plants per pot, to recreate increasing degrees of intra-specific plant-plant interaction. The plantlets were arranged in 10 replicates for growth at density 1 and 3 replicates for growth at density 5, 15, 25 and 50. The experiments were repeated twice. The cultures were incubated in a growth chamber at 23±1 °C with a 16 h photoperiod (100 μmol m<sup>-2</sup> s<sup>-1</sup> provided by Osram Cool Daylight 30W lamps) and a relative humidity of 60/65%. Since this study was devised to be an investigation of density-dependent phytotoxicity, where plants grow in a given substrate volume containing a finite amount of phytotoxin, as indicated by Weidenhamer (2006), in order to re-establish the solution level, every 4-5 days a small amount of sterile distilled water was added. Nutrient solution was not used in order both to maintain finite the amount of excess copper and to avoid the addition of nutrients, which could have prevented competition for mineral resources. Plantlets were cultivated for 4 weeks, as previous pilot experiments proved it to be an adequate period to reveal both a significant effect of increasing density on the size of still healthy plants and significant differences between the two populations. At the end of the growth period, the plantlets (with four leaves formed) were gently removed from the medium and the roots were soaked for 5 min in an ice-cold (4°C) Pb(NO<sub>3</sub>)<sub>2</sub> (10 mM) solution to remove the adhering metal from the cell walls. Dry weight of shoots and roots were recorded

for each sample. Measurements were performed on 20 samples per treatment with the samples randomly selected in treatments with plant densities of 5, 15, 25 and 50 plantlets.

### **Determination of copper concentration**

Root and shoot copper concentrations were determined by atomic absorption spectrometry (AAAnalyst 200, Perkin Elmer). Oven-dried plant material (approximately 0.1 g) was digested, as in Pignattelli et al. (2013), in a 5–2 (v/v) mixture of HNO<sub>3</sub> (Romil, 69%) and HClO<sub>4</sub> (Applichem, 70%) in 25 ml beakers at 120–200°C. After digestion, the volume was adjusted to 10 ml with Milli-Q-water and the element concentration measured.

### **Statistics**

Prior to performing 1- and 2-way ANOVAs, data sets were previously checked for normality using the Shapiro-Wilk test ( $p < 0.05$ ). If results indicated that data did not follow the required Normal parametric model for classical ANOVA, the Kruskal-Wallis test was applied with at least  $p < 0.05$  for the one-way analysis (Kruskal and Wallis 1952, Wilcox 2012), to decide whether the compared population distributions were or not identical without assuming their normality. The homogeneity of the variance was previously checked by applying the Levene's test, while pot-hoc analysis was obtained through the application of the Dunn test (multiple comparisons using rank sums) (Dunn 1964). All statistical analyses were performed using the R statistical software environment (R Core Team 2015). In particular, for the two-way analysis of variance the R package WRS2 (robust statistical methods, <https://cran.r-project.org/web/packages/WRS2>) was used, based on robust measures of location and associate post-hoc tests.

## **Results**

### **Effects of increasing density and copper treatment on plant growth**

Figure 1 reports the plant dry biomass at increasing plant density, from 1 to 50 plants per pot, in -Cu and in +Cu conditions. In -Cu conditions, considering the whole plant weight (normalized to the plant biomass at density 1 for a direct comparison of the result of increasing density on the two populations, Fig 1A), the negative effect of plant-plant interaction on plant biomass was significant from the lowest density applied (5 plants) for the NONMET population. The MET population was less sensitive to increasing plant density under -Cu conditions. At the highest density, the final reduction in plant biomass was about 40% and 25% for the NONMET and MET populations, respectively. The interaction population\*density was shown to be significant ( $p < 0.05$ ).

In both roots and shoots in -Cu conditions (Fig. 1B and C), increasing plant density led to a decrease in dry biomass, significantly from density 5 in the NONMET population and from density 25 in the MET population. Two-way ANOVA showed significant results for the interaction population\*density only in

the case of roots ( $p < 0.01$ ). In NONMET plants the plant-plant interaction effect was more marked in shoots (about 40% of reduction compared to about 20% in roots at the highest densities), while in MET plants it was so in roots (about 50% of reduction compared to about 20% in shoots at the highest densities).

In +Cu conditions, significantly lower values of the whole plant dry weight, normalized on density 1, were obtained with respect to -Cu conditions, for both populations and all the plant densities (at least  $p < 0.05$ , except density 25 for NONMET and density 15 for MET, Fig. 1A). In such conditions, the plant total biomass decreased more sharply in the NONMET population as compared to the MET population, but a similar reduction of about 50% was reached at the two highest densities.

Considering the two organs separately (Fig. 1B and C), significantly lower values of root and shoot dry weight were obtained in comparison to -Cu conditions, for both populations and all the plant densities (at least  $p < 0.05$ ). In the NONMET population, increasing plant density from 1 to 50 plants per pot in +Cu conditions caused a significant decrease in shoot weight starting from density 5, whereas it induced a significant increase in root weight from density 15 (Fig 1B and C). In the MET population, a significant decrease in both root and shoot weight was found from density 15. In shoots, the final decrease was similar in both populations, about 65% and 55% in NONMET and MET population respectively. The root biomass of both populations showed a significant interaction between the +Cu condition and plant density ( $p < 0.01$ ) according to a two-way ANOVA, whereas in shoots it was significant only in MET population ( $p < 0.01$ ). Between the populations, a two-way ANOVA showed significant results for the interaction population\*density in the case of both root and shoot biomass ( $p < 0.01$ ). The interaction among density, population and +Cu conditions for average plant biomass was found to be significant as well ( $p < 0.01$  for both roots and shoots).

Plant height at the end of the experiment was also measured. In both -Cu and +Cu conditions, increasing density had a non-significant effect on plant height, slightly increasing it in both populations and, especially, in the NONMET one (data not shown).

To calculate a reliable tolerance index, the root and shoot biomass of both NONMET and MET plants in +Cu condition was normalized to their counterparts in the -Cu condition at each density. Results showed that increasing plant density alleviated the effect of +Cu conditions on the root biomass decrease, significantly from density 5 and density 25 in the NONMET and MET population, respectively (Fig. 2). Values of root tolerance indices were always higher in the MET population with respect to NONMET plants, with a significant interaction population\*density ( $p < 0.05$ ). Conversely, in shoots increasing plant density increased the reduction in biomass induced by +Cu conditions, significantly from density 5 for the NONMET plants and from density 25 for the MET plants. Up to density 15, the tolerance index values were higher in the MET population, and, at higher density, they were higher in NONMET plants. The interaction population\*density was significant ( $p < 0.05$ ).

### **Effects of increasing density on copper accumulation**

In -Cu conditions, plant-plant interaction did not cause any remarkable change in the copper concentration of roots and shoots of both populations (Tab. 1). No differences in the values of the shoot-to-root ratio were recorded as well.

In +Cu conditions, root copper accumulation decreased as plant density increased from 1 to 50 plants per pot in a significant way from density 15 and 25 in NONMET and MET populations, respectively. Copper concentrations were always significantly higher in the NONMET plants than in the MET plants. In shoots, increasing plant density did not have a significant effect on copper accumulation, even if in both populations the values of metal concentrations tended to decrease. As in roots, the NONMET population showed significantly higher copper accumulation than the MET population. The shoot-to-root ratio slightly increased following plant density, but without significant differences in both populations.

## Discussion

Despite their interesting and intriguing aspects, experiments on the effect of increasing density on plant growth can be problematic to conduct and to interpret due to the intrinsic artefactual nature of the culturing conditions themselves. In this work, we demonstrated that there can be variation in the expression of metal responses depending on plant density,

In -Cu conditions, our results showed an expected negative effect of increasing density on total plant biomass in both non-metallicolous and metallicolous populations of *S. paradoxa* (Fig. 1), due to increasing competition (Silvertown and Charlesworth 2001, White et al. 2007). Unexpectedly, the metallicolous population showed a lower susceptibility to increasing plant density with respect to the non-metallicolous population. This result might be explained by the smaller plant size in the metallicolous population (see for example Colzi et al. 2011, 2012 for *S. paradoxa*). Small size may be an adaptation to the manifold constraints of the metalliferous soils, including drought and nutrient stress (Ernst, 2006).

Similarly to -Cu conditions, in +Cu conditions a density-induced decrease in the total plant biomass was also found in both populations (Fig. 1). Interestingly, a significant interaction between copper and density was found only in the metallicolous population. In this population, Cu stress apparently enhanced the negative effect of increasing density. Therefore, the effect of the combined stress factors in the metallicolous population resulted synergistic, while in the non-metallicolous population it was simply additive. Consequently, even if the non-metallicolous population was the more sensitive to increasing density, starting to be negatively affected already at the lowest values, at the highest densities both populations were similarly affected, thus revealing a paradoxical effect of plant density on the assessment of metal tolerance in the studied metallophyte. Possible hypotheses on which mechanisms for adaptation to the metalliferous environment, induced by the presence of copper in the metallicolous population and responsible for such unexpected results, are hard to draw. In any case, a possibility could

be represented by the copper-induced decrease in the metal binding capacity of the root apoplast, already reported as an exclusion mechanism in the metallicolous population (Colzi et al 2011, 2012). Such remodeling of the root cell walls could have decreased the accumulation of the other mineral nutrients, thus resulting in a lower plant size. Specific adaptations to the paucity of nutrients of the original environment (Rabatti 2015) could in part compensate for the copper-induced decrease in cell wall metal binding, guaranteeing a satisfying trade-off between trace metal exclusion and nutrient acquisition. Another concurring hypothesis could regard a possible copper-induced production of root exudates in different quantity and quality in the two populations. Actually, the metallicolous population was reported to exude organic acids, phenols and flavonoids under copper excess (Quartacci et al., 2009). Such molecules can chelate metals, thus possibly decreasing their availability, and, at the same time, some of them can have allelopathic activity (Bertin et al., 2003, van Dam and Bouwmeester, 2016), therefore a higher production in the metallicolous population could have contributed to the reported results. In any case, a future analysis of the hydroponic solutions at the end of experiment, in terms of changes in pH and presence of root exudates, could give interesting perspectives to examine the above-mentioned hypotheses, as well as the analysis of the complete elemental composition of the plants.

One of the most intriguing results is the increase in root mass in response to density in the +Cu conditions the non-metallicolous population. Actually, the presence of a toxin in the soil can be a case in which increasing density generates increase in biomass (Weidenhamer, 2006). The only study on copper influence on the size-density relationship (Hansi et al. 2014) reported facilitation in two crop plants, but it was performed only on shoots and, furthermore, it did not show any evidence of increased biomass. Contrary to roots, no enhanced growth at increased plant density in +Cu conditions was found in shoots of the non-metallicolous plants. It is possible that the effect of plant density on copper toxicity in roots was not strong enough to provoke a result also in shoots. Therefore, the effect of the combined stresses seemed to be facilitative in roots and simply additive in shoots. This result could have been generated by the above-mentioned hypothesis on the effect of the copper-inducible tolerance mechanisms on plant growth. Similar to our results, facilitation was reported as very important only for the non-serpentine population of *Plantago erecta* grown on a serpentine soil and not for the serpentine population (Espeland and Rice 2007).

Most interestingly, due to the different effects of the stress combination in the shoots of the two populations, additive or synergistic, at the two highest densities the non-metallicolous population seemed to be more tolerant than the metallicolous one. Such result can be fully appreciated by directly comparing the tolerance indices of the root and shoot biomasses (Fig. 2). Actually, such calculation clearly demonstrated that the tolerance index varied according to density within each population. Even more interestingly, it shows that the elevated copper tolerance of the metallicolous population (Colzi et al. 2011, 2012, Gonnelli et al. 2001) was evident for almost all the density values in roots and for only the lower values in shoots.

The pattern of copper accumulation (Tab. 1) showed that in -Cu conditions the metal concentration in the plant organs did not decrease according to increasing plant-plant interaction. This suggests that competition for Cu is unlikely to be the cause of the density-dependent decrease in plant growth. Furthermore, the similar copper concentrations showed by the two populations proposed that, at least for the experimental conditions used, the constitutive copper tolerance mechanisms did not interfere significantly with the acquisition of such nutrient. Further research under sub-optimal copper supply could clarify the possible occurrence of an increased copper requirement in the metallicolous population. Actually, in *Mimulus guttatus* differences in copper requirement were found between highly tolerant and less tolerant plants, even though too small to explain the apparent cost of tolerance (Harper et al, 1997, 1998), whereas a high need of copper was demonstrated in *Haumaniastrum katangense* (Chipeng et al., 2010) and in some metallicolous populations of *Crepidiorhapon tenuis* (Faucon et al., 2012). In +Cu conditions, at any plant density, root and shoot metal concentrations were always higher in the non-metallicolous population than in the metallicolous one, thus confirming its well-known excluder behavior (Gonnelli et al. 2001). In roots of the two populations, a density-induced decrease in copper accumulation was found, which may explain the facilitative effect of plant density. In pot experiments, the effects of a toxin decrease at high plant densities because of lower doses of toxin accumulated by plants, the so-called dilution hypothesis (Weidenhamer et al. 1989). Despite the decrease in root copper concentration in both populations, the lower density-induced facilitative effect showed by the metallicolous population could be explained by its metal tolerance itself, making the population experience the presence of copper, and its subsequent accumulation, as a less limiting condition. At the species level, facilitation was already reported to be most significant when plants are growing under conditions far from their environmental optima (Bertness and Callaway 1994, Liancourt et al 2005). Our results may suggest that concept could be extended to the population level, as already suggested by Espeland and Rice (2007) for a serpentinophyte.

In both populations, the density-induced lower metal accumulation in roots was not able to generate a significantly lower level of copper concentration also in the shoots, thus explaining a part of the lack of the facilitative effect in the latter organs. On the contrary, Hansi et al. (2014) showed density-induced facilitation through copper dilution in barley shoots, but without reporting data on roots. In *S. paradoxa*, the very low metal shoot translocation shown by the plant could have concealed the effect of the differences in copper accumulation occurred at the root level. Therefore, the apparent inversion of the tolerance relationship displayed by the plant shoots at the highest densities could not be explained by a different decrease in copper accumulation in the two populations.

Our results can have fundamental importance for a more realistic re-design of eco-toxicological bioassays, which are urged to include experiments at densities actually occurring in nature. Furthermore, they are essential for trace metal studies in general, since we demonstrated that there can be experimental conditions in which not only the effect of element excess varies according to the density applied, but

also a density-induced paradoxical inversion of the tolerance relationships between different populations of a same plant species can occur.

### **Author contributions**

C. G. designed the experiments, M. C. performed the experiments, M. D. B. analyzed the element concentration, I. C., A. B. and A. C. analyzed the data, E. P. edited the English language, C. G. interpreted the data and wrote the manuscript. All authors commented on the results and the manuscript.

### **Acknowledgements**

This work was supported by the University of Firenze (Fondi di Ateneo 2014 and 2015).

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## Tables

Table 1. Copper concentration ( $\mu\text{g g}^{-1}$  dry weight) in roots and shoots and shoot-to-root ratio of two populations of *S. paradoxa* cultivated for 4 weeks at increasing plant density in control conditions (-Cu) or in presence of  $10\mu\text{mol L}^{-1}$   $\text{CuSO}_4$  (+Cu). NONMET: Colle val d'Elsa, non-metallicolous population; MET: Fenice Capanne, metallicolous population. Values are means of six replicates  $\pm$  standard error. Means sharing the same letter are not significantly different (small letters: intrapopulation; capitals: between populations) (1-way ANOVA, at least  $p < 0.05$ ).

Plant density	Root				Shoot				Shoot-to-Root			
	NONMET		MET		NONMET		MET		NONMET		MET	
	-Cu	+Cu	-Cu	+Cu	-Cu	+Cu	-Cu	+Cu	-Cu	+Cu	-Cu	+Cu
<b>1</b>	11.6	342.8	12.6	147.2	3.9	38.6	3.7	22.0	0.33±	0.11±	0.29±	0.15±
	± 1.9	± 25.2	± 1.2	± 6.84	± 0.5	± 3.7	± 0.6	± 2.8	0.04	0.01	0.04	0.02
	aA	bB	aA	bA	aA	aB	aA	aA	aA	aA	aA	aA
<b>5</b>	12.4	338.9	11.5	147.6	3.8	34.5	3.2	20.1	0.31±	0.1 ±	0.27±	0.13±
	± 2.3	± 15.6	± 1.7	± 9.3	± 0.4	± 3.9	± 0.5	± 2.2	0.03	0.01	0.04	0.01
	aA	bB	aA	bA	aA	aB	aA	aA	aA	aA	aA	aA
<b>15</b>	11.8	323.8	11.4	140.8	4.2	37.9	3.6	20.6	0.37±	0.12±	0.32±	0.14±
	± 1.7	± 24.3	± 1.3	± 7.5	± 0.3	± 2.5	± 0.5	± 3.1	0.03	0.01	0.03	0.02
	aA	aB	aA	bA	aA	aB	aA	aA	aA	aA	aA	aA
<b>25</b>	12.5	286.8	12.0	116.7	3.6	40.5	4.0	19.8	0.28±	0.14±	0.31±	0.17±
	± 1.1	± 15.1	± 1.6	± 5.8	± 0.3	± 2.1	± 0.6	± 2.2	0.026	0.01	0.04	0.02
	aA	aB	aA	aA	aA	aB	aA	aA	aA	aA	aA	aA
<b>50</b>	12.7	267.3	12.6	109.2	3.8	35.7	3.5	19.8	0.33±	0.13±	0.29±	0.18±
	± 1.2	± 15.3	± 1.5	± 9.9	± 0.6	± 1.6	± 0.4	± 1.9	0.047	0.01	0.03	0.02
	aA	aB	aA	aA	aA	aB	aA	aA	aA	aA	aA	aA

## Figure captions

Fig. 1. Effect of increasing plant density on growth of two populations of *S. paradoxa* cultivated for 4 weeks in -Cu conditions or +Cu conditions ( $10\mu\text{mol L}^{-1}$   $\text{CuSO}_4$ ). A) Total plant dry biomass relative to control values (= density 1). B) Root dry biomass. C) Shoot dry biomass. NONMET: Colle val d'Elsa, non-metallicolous population, circle; MET: Fenice Capanne, metallicolous population, triangle; white symbol: -Cu conditions, black symbol: +Cu conditions ( $10\mu\text{mol L}^{-1}$   $\text{CuSO}_4$ ). Values are means of 20 replicates  $\pm$  standard error. For each population, significant differences between the mean values of the plant biomass at increased densities in comparison to density 1 appear with asterisks (1-way ANOVA, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

Fig. 2. Tolerance indices of the two populations of *S. paradoxa* in +Cu conditions ( $10\mu\text{mol L}^{-1}$   $\text{CuSO}_4$ ) as % of the dry biomasses of -Cu conditions at each density. A) Roots. B) Shoots. NONMET: Colle val d'Elsa, non-metallicolous population, black circle; MET: Fenice Capanne, metallicolous population, black triangle). Values are means of 20 replicates  $\pm$  standard error. For each population, significant differences between the mean values of the tolerance indices at increased densities in comparison to density 1 appear with asterisks (1-way ANOVA, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



