Assessing the effect of copper on growth, copper accumulation and physiological responses of grazing species *Atriplex halimus*: ecotoxicological implications.

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ABSTRACT

Tolerance of plants to elevated concentrations of heavy metals in growth media and in its tissues leads to high degrees of metal bioaccumulation, which may pose a risk for humans and animals alike. Therefore, bio-accumulating plants need thorough evaluation from an environmental health point of view. A glasshouse experiment concerning the xerohalophyte *Atriplex halimus* was carried out to determine its tolerance and capacity to accumulate copper. We investigated the effect of Cu from 0 to 30 mmol l^{-1} on the growth, photosynthetic apparatus and nutrient uptake of *A. halimus* by measuring gas exchange, chlorophyll fluorescence and photoinhibition. We also determined total Cu, sodium, potassium, magnesium, phosphorous, and nitrogen content in the plant. Our results indicated that *A. halimus* presented a high tolerance to Cu-induced stress, since the plants were able to survive at concentrations higher than 15 mmol l^{-1} Cu. However, this tolerance was not reflected in its ability to accumulate and tolerate greater amounts of Cu in its tissues, since clear phytotoxicity symptoms were detected at tissue concentrations greater than 38 mg Kg^{-1} Cu. Thus, Cu increment caused a reduction in *A. halimus* growth, which was related to a decrease in net photosynthetic rate. This reduction was associated with the adverse effect of Cu on the photochemical apparatus and the reduction in the absorption of essential nutrients. The high tolerance of *A. halimus* was largely related with the capacity of this species to avoid the absorption of great amounts of Cu. For all the above reasons, *A. halimus* could have the characteristics of a Cu-exclusion plant.

Keywords: Cu-exclusion plant; Fluorescence; Growth; Photoinhibition; Photosynthesis

1. Introduction

Heavy metal pollution is a major ecological concern due to its impact on human health through the food chain and its high persistence in the environment (Sharma and Dubey, 2005). In this context, copper (Cu) is one of the main heavy metal contaminants, resulting from mining, metal processing, fertilizers, fungicides, agricultural, municipal wastes, etc (Kabata-Pendias and Pendias, 2001). Although Cu is an essential micronutrient for plant growth, participating in important biological reactions, namely as an enzymatic cofactor and electron carrier in the photosynthetic and respiratory processes (Andrade et al., 2004), it has been reported to be among the most toxic of heavy metals (Dewez et al., 2005). Excess Cu inhibits plant growth, as well as photosynthetic and respiratory activities (Nalewajko and Olaveson, 1995). Studies have shown that plants grown in Cu-contaminated soil usually accumulate an elevated Cu content in their tissues (Kabata-Pendias and Pendias, 2001). As a result, a series of physiological and toxicological responses take place in plants depending on the Cu concentration in its tissues and the capacity of these plants to tolerate elevated levels of this element.

Atriplex halimus (chenopodiaceae) is a xerohalophyte which is perennial and native to arid and semi-arid Mediterranean regions. This species tolerates harsh conditions such as salinity (Bajji et al., 1998), light stress (Streb et al., 1997) and drought (Martínez et al., 2005). In the joint estuary of the Tinto and Odiel rivers (which is one of the most heavy metal-polluted areas in the world; Sáinz et al., 2002) *A. halimus* grows in anthropized grasslands running parallel to the coastline. These areas contain high levels of trace metal contamination (especially Cu), derived from phosphate-based fertilisers, pyrite roasting and copper smelting plants located near the coast (Elbaz-

Poulichet et al., 1999). Several studies have demonstrated the ability of *A. halimus* to tolerate and accumulate high amounts of cadmium, zinc and lead in its tissues (Lutts et al., 2004; Manousaki and Kalogerakis, 2009; Nedjimi and Daoud, 2009). However, few data are available concerning the effect of copper on the growth and photosynthesis responses of *A. halimus*. Hence, the purpose of this study was to examine the effects of Cu on growth, photosynthetic apparatus and nutrient uptake in *A. halimus* under hydroponic conditions. The specific objectives were to: (1) accomplish a simplified approach to the determination of Cu phytotoxicity thresholds of this species, by analyzing the growth of *A. halimus* in experimental copper treatments ranging between 0 to 30 mmol l^{-1} Cu; (2) ascertain the extent to which the effects on the photosynthetic apparatus (PSII chemistry) and gas exchange characteristics determine plant performance with increasing copper; and (3) examine the response of accumulated copper, sodium, potassium, magnesium, phosphorus and nitrogen to increasing external Cu and how this response affects growth.

The results of this investigation have implications for heavy metal ecotoxicology involved in the vegetable supply for food, since this species has been considered as an important component of the diet of grazing animals in semi-arid regions (Otal et al., 2010). Moreover, the tolerance of plants to elevated concentrations of heavy metals in growth media and in their tissues may pose a risk to grazing livestock because of the bioaccumulation of high metal concentrations in plants (Kabata-Pendias and Pendias, 2001).

2. Materials and methods

100 *2.1. Plant material*

101

102 Seeds of *A. halimux* were collected in December 2010 from Odiel Marshes and
103 stored in darkness at 4ºC for three months. After the storing period, seeds were placed
104 in a germinator (ASL Aparatos Científicos M-92004, Madrid, Spain) and subjected to
105 an alternating diurnal regime of 16 hours of light (photon flux rate, 400-700 nm, 35
106 μmol m^{-2} s^{-1}) at 25ºC and 8 hours of darkness at 12ºC for a month. Then, seedlings were
107 planted in individual plastic pots (11 cm of diameter) filled with pearlite and placed in a
108 glasshouse with controlled temperature of 21-25ºC, 40-60% relative humidity and
109 natural daylight (minimum and maximum light flux: 250 and 1000 μmol m^{-2} s^{-1},
110 respectively). Immediately afterwards, the pots were allocated in shallow trays with 86
111 mM NaCl solution created by combining 20% Hoagland's solution with the appropriate
112 amount of NaCl (Hoagland and Arnon 1938). Thus, 3 l of the solution were placed in
113 each of the trays (to a depth of 1 cm). The levels in the trays were monitored and they
114 were topped up to the marked level with 20% Hoagland´s solution (without NaCl)
115 whenever necessary to maintain the salt concentration.

116

117 *2.2. Stress treatments*

118

119 In June 2011, after three months of seedling cultures, the pots were allocated to five
120 Cu treatments in shallow trays (six pots per tray, with one tray per Cu treatment): 0, 2,
121 9, 15 and 30 mmol l^{-1} Cu, in the same glasshouse. Copper treatments were established
122 by combining 20% Hoagland's solution and $CuSO_4 \cdot 7H_2O$ of the appropriate
123 concentration. The control, 0 mm l^{-1} Cu treatment, had exactly 0.0005 mmol l^{-1} of Cu,
124 since Hoagland's solution contains a small amount of Cu as an essential trade nutrient.

Cu concentrations were chosen to cover variations recorded by Sáinz et al. (2002) in the salt marshes of the joined estuary of Tinto and Odiel rivers.

At the beginning of the experiment, 2 l of the appropriate solution were placed in each of the trays to a depth of 1 cm. During the experiment, the levels in the trays were monitored and they were topped up to the marked level with 20% Hoagland's solution (without additional $CuSO_4 \cdot 7H_2O$) as a way to limit the change of Cu concentration due to water evaporation of the nutritive solution. In addition, the entire solution (including $CuSO_4 \cdot 7H_2O$) was changed weekly.

2.3. Growth and survival analysis

At the beginning and at the end of the experiment (after 20 days of treatment), three and six entire plants (roots, stems and leaves), from each treatment, respectively, were dried at 80ºC for 48 h and weighed. Also, the number of all fully expanded leaves and total and individual leaf area of *Atriplex halimus* were recorded on the same dates.

The relative growth rate (RGR) in ash-free dry mass of whole plants was calculated using the formula:

$$RGR = (\ln Bf - \ln Bi) \cdot D^{-1} \quad (g\ g^{-1} day^{-1})$$

where Bf = final dry mass, Bi = initial dry mass (average of the three plants from each treatment dried at the beginning of the experiment) and D = duration of experiment (days).

Finally, plant survival was recorded. A plant was considered dead when no green leaves remained at the end of the experiment.

2.4. Gas exchange measurements

Gas exchange measurements were carried out on random, healthy, fully expanded leaves (n = 10, a measurement per plant and four extra taken randomly) using an infrared gas analyzer in an open system (Li-Cor Inc., Lincoln, NE, USA) after 20 days of treatments. Net photosynthetic rate (AN), intercellular CO_2 concentration (C_i), stomatal conductance (gs) and transpiration rate were determined at ambient CO_2 concentration of 380 µmol mol^{-1}, temperature between 20 and 25ºC, 50 ± 5% relative humidity and a photon flux density of (1000 µmol m^{-2} s^{-1}). AN, C_i and gs were calculated using standard formulae of Von Caemmerer and Farquhar (1981). Water use efficiency (WUE) was calculated as the ratio between AN and transpiration rate [mmol (CO_2 assimilated) mol^{-1} (H_2O transpired)].

2.5. Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured in random, fully developed leaves (n = 12, two measurements per plant) using a portable modulated fluorimeter (Mini-PAM, Heinz Walz, Germany) after 20 days of treatments. Light- and dark-adapted fluorescence parameters were measured at dawn (stable, 50 µmol m^{-2} s^{-1} ambient light) and at midday (1600 µmol m^{-2} s^{-1}) to investigate whether Cu concentration affected the sensitivity of plants to photoinhibition.

Plants were dark-adapted for 30 minutes, using leaf–clips designed for this purpose. The minimal fluorescence level in the dark-adapted state (F_0) was measured using a modulated pulse (<0.05 µmol m^{-2} s^{-1} for 1.8 µs) too small to induce significant physiological changes in the plant. The data stored were an average taken over a 1.6

175 seconds period. Maximal fluorescence level in this state (F_m) was measured after
176 applying a saturating actinic light pulse of 10000 µmol m^{-2} s^{-1} for 0.8s. The value of F_m
177 was recorded as the highest average of two consecutive points. Values of the variable
178 fluorescence ($F_v = F_m - F_0$) and maximum quantum efficiency of PSII photochemistry
179 (F_v/F_m) were calculated from F_0 and F_m. This ratio of variable to maximal fluorescence
180 correlates with the number of functional PSII reaction centres and dark adapted values
181 of F_v/F_m can be used to quantify photoinhibition (Maxwell and Johnson, 2000).
182 The same leaf section of each plant was used to measure light-adapted parameters.
183 Steady state fluorescence yield (F_s) was recorded after adapting plants to ambient light
184 conditions for 30 min. A saturating actinic light pulse of 10000 µmol m^{-2} s^{-1} for 0.8 s
185 was then used to produce the maximum fluorescence yield (F_m') by temporarily
186 inhibiting PSII photochemistry. The quantum efficiency of PSII was calculated using
187 light-adapted parameters ($\Phi_{PSII} = (F_m' - F_s)/ F_m'$) according to Mateos-Naranjo et al.
188 (2008a). This parameter measures the proportion of light absorbed by chlorophylls
189 associated to PSII that is used in photochemistry.
190 Fluorescence parameters determined in both light- and dark-adapted states were
191 used to calculate non-photochemical quenching ($NPQ = (F_m - F_m') / F_m'$), which is a
192 parameter that describes mainly the thermal dissipation of energy in the PSII (Maxwell
193 and Johnson, 2000).
194 Chronic (PI_{chr}) and dynamic (PI_{dyn}) photoinhibition were calculated according to
195 Werner et al. (2002) as:
196
197 $$PI_{chr} = \frac{(F_v/F_m)_{max} - (F_v/F_m)_d}{(F_v/F_m)_{max}} \times 100$$
198 $$PI_{dyn} = \frac{(F_v/F_m)_d - (F_v/F_m)_{mid}}{(F_v/F_m)_{max}} \times 100$$

where $(F_v/F_m)_d$ and $(F_v/F_m)_{mid}$ are dawn and midday F_v/F_m values, respectively. $(F_v/F_m)_{max}$ is the maximum F_v/F_m value, which was calculated as the average of dawn measurements of the control 1 day after imposing Cu treatments.

2.6. Chemical analysis of plant samples

In accordance with protocols of Mateos-Naranjo et al. (2008a), at the end of the experiment, leaf and root samples were dried at 80ºC for 48 h and ground. Leaves and roots were carefully washed with distilled water before any further analysis. Then, 0.5 g samples, taken from a mixture of the leaves or the roots belonging to the six plants used for each treatment were digested in triplicate with 6 ml HNO_3, 0.5 ml HF and 1 ml H_2O_2. Cu, Na, K, Mg and P were measured by inductively coupled plasma (ICP) spectroscopy (ARL-Fison 3410, USA). Total N concentrations were determined for undigested dry samples with an elemental analyzer (Leco CHNS-932, Spain).

2.7. Statistical analysis

Statistical analysis was carried out using Statistica v. 6.0 (Statsoft Inc.). Pearson coefficients (r) were calculated to assess correlation between different variables. Data were analysed by means of a one-way analysis of variance (*F*-test). Data were first tested for normality with the Kolmogorov-Smirnov test and for homogeneity of variance with the Brown-Forsythe test. Significant test results were followed by Tukey tests for identification of important contrasts. Differences between measurements of fluorescence at dawn and midday were compared by the Student test (*t*-test).

3. Results

3.1. Growth and survival analysis

Total dry mass decreased with increasing Cu concentration (above: r = -0.75 and belowground biomass: r = -0.79, $p < 0.01$; Fig. 1A) and was directly correlated with the reduction in relative growth rate (RGR; Fig. 1B). Moreover, total leaf area decreased with Cu concentration (r = -0.80, $p < 0.01$; Fig. 1C), which was associated with the reduction in the number of leaves (with values of 128 ± 18, 119 ± 8, 97 ± 8, 86 ± 12 and 50 ± 8 leaves per plant for 0, 2, 9, 15 and 30 mmol l^{-1} Cu, respectively) and individual leaf area (Fig. 1D).

In addition, all plants exposed to the 30 mmol l^{-1} Cu concentration treatment were unable to survive for the full 20 days of the experiment.

3.2. Gas exchange

AN decreased with increasing Cu concentration after 20 days of treatment (r = -0.84, $p < 0.01$; Fig. 2A) and AN values recorded at 0 and 2 mmol l^{-1} Cu were significantly higher than at the other Cu concentrations (Anova, $p < 0.001$). There was also a strong correlation between AN and RGR (r = 0.97, $p < 0.01$).

Gs showed the same pattern as AN, decreasing with increasing Cu concentration (r = -0.87; $p < 0.01$; Fig. 2B). Contrarily, Ci increased with Cu concentration (r = 0.89, $p < 0.01$; Fig. 2C), but no statistical differences were recorded between 0, 2 and 9 mmol l^{-1} Cu treatments (Anova, $p > 0.05$).

Finally, WUE decreased with increasing Cu concentration (r = -0.84, $p < 0.01$; Fig. 2D), but no differences were recorded between 0, 2 and 9 mmol l^{-1} Cu treatments (Anova, $p > 0.05$).

3.3 .Chlorophyll fluorescence

Values of F_v/F_m at dawn and midday decreased with external Cu concentration (dawn: r = -0.88, $p < 0.01$; midday: r = -0.94, $p < 0.01$; Fig. 3A). Also, F_v/F_m values were significantly higher for the control and 2 mmol l^{-1} Cu than for the other Cu treatments at dawn and midday after 20 days of treatment. Furthermore, F_v/F_m was always lower at midday, the reductions resulting mainly from a lower Fm at midday than at dawn (data not presented). Fv/Fm values at dawn were lower than control in all treatment except for 2 and 9 mmol l^{-1} Cu (Fig. 3A).

Similarly, Quantum efficiency of PSII (Φ_{PSII}) at dawn and midday followed a similar pattern that F_v/F_m (dawn: r = -0.86, $p < 0.01$; midday: r = -0.75, $p < 0.01$; Fig. 3B). In addition, Φ_{PSII} at midday was lower than at dawn (t-test, $p < 0.05$; Fig. 3B) and was directly associated with a lower photochemical quenching at midday than at dawn (qP, data not presented).

Non-photochemical quenching (NPQ) did not show a relationship with Cu concentration at dawn, whereas this parameter increased with copper concentration at midday (r = 0.66, $p < 0.01$; Fig. 3C), ranging between 0.8 ± 0.1 and 2.7 ± 0.3 for 0 and 30 mmol l^{-1} Cu solution, respectively.

The percentage of chronic and dynamic photoinhibition increased with the addition of Cu to nutrient solution (chronic: r = 0.98, $p < 0.01$ and dynamic r = 0.99, $p < 0.001$; Fig. 4). However, if compared with the control, the greater values of dynamic and

chronic photoinhibition were recorded at 15 and 30 mmol l^{-1} Cu (Fig. 4).

3.4. Chemical analysis of plant samples

Our mineral analysis data show that Cu tissue concentrations were greater in the roots than in the leaves (two-way Anova, $p < 0.05$; Fig. 5A), and increased with external Cu concentration (roots: r = 0.94, $p < 0.05$; leaves: r = 0.96, $p < 0.01$; Fig 5A). In contrast, leaf Na, P and N decreased with increasing Cu concentration (Fig. 5B, 5D and 5F), whereas in root tissues P and N concentrations were lower in the presence of Cu (ca. 5.4 mg/g and 2.1 % for all Cu treatment of P and N respectively) respect to the control (Fig. 5D and 5F). A similar trend was recorded for K and Mg concentrations in roots, whereas their concentration in the leaf showed few differences respect to the control (Fig. 5C and 5E).

4. Discussion

The upper critical level of an element is the lowest concentration in tissues at which it has toxic effects (Kabata-Pendias and Pendias, 2001). Unlike some Cu tolerant plant species (metalophytes), in which Cu content in leaves can be as high as 1000 mg Kg^{-1} (Marschner, 1999), the critical toxicity level of copper in leaves of *A. halimus* is 20 mg Kg^{-1} to 30 mg Kg^{-1} dry matter (Kabata-Pendias and Pendias, 2001). In the present study, the Cu concentration in *A. halimus* tissues increased significantly with metal addition, the Cu concentration reaching values between 21 and 120 mg Kg^{-1} and between 114 and 273 mg Kg^{-1} for shoots and roots, respectively, for plants treated with a range of Cu concentration from 2 to 30 mmol l^{-1} Cu. These values are much higher than those

299 suggested as normal in tissues and thus could be toxic for plants of *A. halimus*.

300 Copper toxicity thresholds of *A. halimus* were analysed in relation of its survival,
301 growth, photosynthetic responses and Cu concentration pattern in tissues. Thus, on the
302 basis of *A. halimus* survival, our results indicate that lethal concentration of copper
303 (LC50, the metal concentration that kills 50% of plants) was between 15 and 30 mmol l^{-1}
304 Cu because no individuals were able to survive when subject to an Cu concentration of
305 30 mmol l^{-1} for the full 20 days. Paschke and Redente (2002) determined LC50 of
306 copper for six grass species used in restoration activities in concentrations close to 5
307 mmol l^{-1} Cu. On the other hand, in relation to biomass responses analyses, the effective
308 concentration of Cu (EC50, substrate Cu concentration resulting in 50% biomass
309 reduction) was more than 15 mmol l^{-1} Cu. At this concentration, *A. halimus* showed a
310 37% of biomass reduction after 20 days of treatment. As for LC50, the value of EC50 of
311 *A. halimux* is considerably higher than those reported by several authors for many
312 different species (Paschke and Redente, 2002), which indicates that *A. halimus* has a
313 great capacity to grown in a copper contaminated medium. On the other hand, the
314 analysis of Cu tissue concentration indicated that the phytotoxicity thresholds (PT50,
315 tissue concentration of a plant resulting in 50% biomass reduction) were between 38-
316 120 mg Kg^{-1} and between 200-270 mg Kg^{-1} for shoots and roots of *A. halimus*,
317 respectively. Contrarily to the recorded for LC50 and EC50, this PT50 value is
318 considerably lower than those reported for other species. Paschke and Redente (2002)
319 indicated PT50-shoots as high as 737 mg Kg^{-1} for slender wheatgrass and 10,792 mg
320 Kg^{-1} for redtop. Hence, the wide tolerance of *A. halimus* on the range of Cu
321 concentration tested in this experiment is not reflected in its ability to accumulate and
322 tolerate greater amounts of Cu in its tissues. This response was different to the reported
323 previously by Lutts et al. (2004), who found that *A. halimus* is also tolerant to both Cd

and Zn, but contrarily to the Cu accumulation response; it may accumulate these elements in high amount in the aboveground tissues. These specific metal discrepancies could be attributed to different tolerance mechanisms. Several mechanisms have been suggested to account for metal tolerance in plants, such as metal sequestering in tissues or cellular compartments that are insensitive to metals. Restriction of upward movement into shoots (an avoidance mechanism) and translocation of excessive metals into old leaves shortly before their shedding can also be considered as tolerance mechanisms, as can the increase in metal-binding capacity of the cell wall (Verklejj and Schat, 1990). The analysis of Cu toxicity thresholds of *A. halimus* indicates that this species could be considered as a Cu-excluding plant, since, as had already been underlined by Wei et al. 2005, it was able to survive and growth in high copper polluted medium, denoting a high Cu tolerance. However, Cu concentration in its aboveground was low, in spite of the elevated concentration in roots.

Despite the high Cu resistance demonstrated by *A. halimus*, the increase in Cu concentration in the medium affected the growth of this species. Compared to the control, the reduction in RGR with 2, 9, 15, 30 mmol l^{-1} Cu were 8, 21, 37 and 98%, respectively, and this response was apparent in total leaf area, which was associated with the reduction in the number of leaves and individual leaf area. Inhibition of growth and biomass reduction are general responses of higher plants to Cu excess (Kabata-Pendias and Pendias, 2001) and these effects are often the result of limitation in photosynthesis, mineral nutrition and water balance.

The photosynthetic apparatus is particularly susceptible to copper, resulting in a decrease in the activity of photosystem II and electron transfer rates (Mallick and Mohn, 2003). The effects of Cu on AN and gs were very clear across the whole range of Cu concentrations, except in 2 mmol l^{-1} Cu, where AN values were similar to the control

349 treatment. The decreased in AN caused an overall decline in WUE, especially in the
350 highest Cu concentration treatment. The decline of A may be ascribed to stomatal
351 and/or non-stomatal limitations (Flexas and Medrano, 2002; Perez-Martin et al., 2009);
352 thus, Cu stress can affect photosynthesis in terms of CO_2 fixation, electron transport,
353 photophosphorylation and enzyme activities. Therefore, if the limitation of AN in *A.*
354 *halimus* were due to gs, there should be a reduction in Ci. However, Ci increased with
355 increasing Cu concentration. This increase of Ci may be explained by modifications of
356 Rubisco activities of *A. halimus*, as has been previously described for *Spartina*
357 *densiflor*a in response to Cu stress (Mateos-Naranjo et al., 2008b). The inhibition in
358 enzyme activity in the presence of heavy metals could be due to substitution of Mg in
359 the active site of RuBisCO subunits by metal ions (Siedlecka and Krupa, 2004). Thus,
360 we recorded an overall decline in tissue Mg concentration with the increase in Cu in the
361 growth medium, especially in root tissues. The reduction in the absorption of essential
362 mineral elements has been described as one of the effects of heavy metals on plants
363 (Kabata-Pendias and Pendias, 2001). In this regard, we also reported that the presence
364 of Cu affected the concentration of the macroelements, Na, K, Mg, this leading to
365 dysfunctions and structural changes arising from the lack of these and other essential
366 elements (P and N). For example, an excess of Cu inhibits the activity of phosphatase,
367 thereby diminishing the availability of P (Tyler, 1976), and P concentration in *A.*
368 *halimus* tissues. Lin and Wu (1994) found that in *Lotus purshianus*, an excess of Cu
369 reduced the concentration of P in both root and leaf tissues. Our results likewise
370 demonstrate adverse effects of Cu on N metabolism. In this respect, we observed a
371 reduction in N concentration in leaves with the increase of Cu in nutrient solutions,
372 which could be linked with a decrease in chlorophyll content. Brahin and Mohamed
373 (2011) described a clear reduction of chlorophyll a and b content of *A. halimus* exposed

374 to medium copper concentration of 2 mM. The reduction in chlorophyll content of *A.*
375 *halimus* could as well entail a decline in the photosynthetic function.
376 On the other hand, the decrease in AN could be due to the different effects of Cu
377 on the integrity or function of the photochemical apparatus of *A. halimus*. Plants
378 undergo light stress when they absorbe more light than they can use in photosynthesis.
379 This situation appears when light intensity rises excessively or when photosynthesis
380 rates diminishe due to adverse situations like drought, salinity, extreme temperatures or
381 toxicity. Light stress leads to photoinhibition, thereby causing a reduction in the normal
382 photosynthesis rate and damage in the photosynthetic apparatus. The reduction in
383 Fv/Fm values at midday indicated that *A. halimus* experienced photoinhibition (dynamic
384 photoinhibition) at the higher light flux. This photoinhibition was assumably triggered
385 by a lower proportion of open reaction centres (lower values of Fm) resulting from a
386 saturation of photosynthesis by light. Also, ΦPSII decreased as a consequence of the
387 decrease in qP and the increase in NPQ (up to 2.5 mmol l^{-1}), which indicates that the
388 plants dissipated light as heat. Increased thermal energy dissipation is considered a
389 photoprotective mechanism that preserves photosynthetic reaction centers from light-
390 induced damage (Maxwell and Johnson, 2000).
391 Furthermore, Fv/Fm and ΦPSII were clearly affected by Cu stress in the three
392 highest Cu concentration treatments at midday, this suggesting that Cu excess enhances
393 photoinhibition induced by light stress. Our data showed that Fv/Fm values at dawn
394 remained lower than control parameters for unstressed plants in all treatments except for
395 2 and 9 mmol l^{-1} Cu (Björkman and Demming, 1987), a consequence of the high
396 chronic photoinhibition recorded in the treatments with the highest Cu concetrations.
397 Photoinhibition is an important event that affects photosynthetic productivity and,
398 therefore, plant growth (Melis, 1999).

5. Conclusion

Our data suggest that *A. halimus* is Cu-resistant plant, since all plants were able to survive at concentrations greater than 15 mmol l^{-1}, but metal concentration achieved in plant tissues were generally kept at low levels. Thus, there were clear phytotoxicity symptoms at Cu tissue concentration greater than 38 mg Kg^{-1}. In this sense, the addition of Cu to the nutrient solution affected the growth of *A. halimus*. Differences in growth rates over the range of Cu studied can be largely accounted for by effects on net photosynthesis; Cu has a marked effect on the photochemical (PSII) apparatus, as well as on water balance and on the absorption of essential mineral nutrients. Therefore, the high resistance of *A. halimus* was largely related with the capacity of this species to avoid the absorption of great amounts of Cu in its tissues. This capacity renders *A. halimus* a species with the basic characteristics of a Cu-exclusion plant. This Cu-exclusion condition should reduce health risks, for Cu concentration in plant aboveground tissues that did not affect yield (< 38 ppm) was lower than the maximum tolerable level of 40 ppm of copper (MTL; the maximum dietary level of a specific mineral that will not cause any adverse effects when fed for a specific period of time on a animal) stabilised for NRC (2005). On the other hand, our results have great interest for Cu remediation purposes, since if Cu-excluding mechanisms of *A. halimus* could be discovered and metal-excluding genes could be transplanted to crops, it would be highly useful for the safety of agricultural products in copper contaminated areas.

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Fig. 1. Growth analysis of *Atriplex halimus* in response to treatment with a range of Cu concentrations over 20 d. Total dry mass (above- and belowground biomass) (A), relative growth rate, RGR (B), total leaf area (C) and individual leaf area. Values represent mean ±SE, n = 6. Different letters indicate means that are significantly different from each other (Tukey test, $p < 0.05$).

Fig. 2. Net photosynthetic rate, AN (A), stomatal conductance, gs (B), intercellular CO_2 concentration, Ci (C) and water use efficiency, WUE (D) in randomly selected, fully expanded leaves of *Atriplex halimus* in response to treatment with a range of Cu concentrations over 20 d. Values represent mean ±SE, n = 12. Different letters indicate means that are significantly different from each other (Tukey test, $p < 0.05$).

Fig. 3. Maximum quantum efficiency of PSII photochemistry, F_v/F_m (A), quantum efficiency of PSII, Φ_{PSII} (B) and non-photochemical quenching (C) at midday (●) and at dawn (○) in randomly selected, fully expanded leaves of *Atriplex halimus* in response to treatment with a range of Cu concentrations over 20 d. Values represent mean ±SE, n = 10. Different letters indicate means that are significantly different from each other (Tukey test, $p < 0.05$).

Fig. 4. Total, chronic and dynamic photoinhibition in randomly selected, fully expanded leaves of *Atriplex halimus* in response to treatment with a range of Cu concentration over 20 d. Values represent mean ±SE, n = 10. Different letters indicate means that are significantly different from each other (Tukey test, $p < 0.05$).

548 **Fig. 5.** Total copper, Cu (A), total sodium, Na (B), total potassium, K (C), total
549 phosphorous, P (D), total magnesium, (E) and total nitrogen (F) concentrations for
550 above- (○), and belowground biomass (●) of *Atriplex halimus* in response to treatment
551 with a range of Cu concentrations over 20 d. Values represent mean, n = 6. Different
552 letters indicate means that are significantly different from each other (Tukey test, $p <$
553 0.05).