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Effects of different humic substances concentrations on root anatomy and Cd accumulation in seedlings of *Avicennia germinans* (black mangrove)

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ABSTRACT

Mangrove areas are among most threatened tropical ecosystems worldwide. Among polluting agents Cadmium is often found in high concentrations in mangrove sediments. Humic substances, complex biomolecules formed in soil and sediments during animal and plant residuals decomposition, have a known biostimulant activity and can be adopted to counteract various plant stresses. This study explores, in controlled conditions, the effect of humic substances on *Avicennia germinans* seedlings, with or without cadmium contamination. Humic compounds significantly changed plant root architecture, and, when coupled with cadmium, root anatomy and Cortex to Vascular Cylinder diameter ratio. These modifications led to lower Cd uptake by humic substances-treated plants. Humic substances amendment could be effective, depending on their concentrations, on improving plant health in mangrove areas, for forest recuperation and/or dredged sediments phytoremediation purposes.

1. Introduction

Mangrove forests, the intertidal wetlands confined to tropical and subtropical areas, are special ecosystems with a high level of biodiversity (Field et al., 1998). Mangrove play a key role in the conservation of tropical and subtropical coastlines, supporting a wide variety of ecosystem services (Atkinson et al., 2016) including nutrient cycling, soil formation, wood production, ecotourism. These areas also provide critical nurseries and habitats for fish and crustacean (Lee et al., 2014) which can pass all their life cycle or part of it inside the mangrove. Additionally, containing on average 1023 Mg carbon per hectare, mangroves are among the most carbon-rich forests in the tropics (Donato et al., 2011), thus the conservation of carbon-rich mangroves should be a high-priority component of strategies to mitigate climate change (Atwood et al., 2017). Approximately 150,000 km^2 of mangroves exist worldwide, over two thirds of the forests are located in just eighteen countries among which Indonesia, Brazil and Australia are the top three (Barbier, 2016).

Mangroves are also one of the most threatened tropical ecosystems worldwide, as a consequence of increasing pollution from human activities due to rapid industrialization and urbanization of coastal regions: as a result, the areal extent of mangrove forests has declined by 30–50% over the past half century (FAO, 2007).

Among degradation factors of mangroves forests there is the accumulation of pollutants trough rivers and harbor activities that are often placed close to them (Borja et al., 2012). Pollutants are often illegally discarded on the rivers and consequently on mangroves forests (Maiti and Chowdhury, 2013) producing polluted sediments, often insisting inside the mangrove ecosystems for many years (Bayen, 2012; Bortone et al., 2004; Le Guyader, 2013). This causes significant forest decline, leaving areas characterized by several spots without canopy, as it happening for example, in the mangrove area of Maria Ortiz, a neighborhood of Vitoria, capital city of Espirito Santo State, Brazil (Dobbss, 2012). Moreover, mangrove species might be adopted for the decontamination of the polluted sediments produced from harbor sediment dredging (Pittarello et al., 2017).

Within different classes of pollutants, heavy metals are not readily degradable in nature. Sediments in the mangrove areas represent important natural sinks for heavy metals, involved in the regulation of metal distribution (Chakraborty et al., 2014). However, the total metal

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concentration in sediments is not necessarily an indication for their fate, dispersal and bioavailability. Bioavailability in sediments depends on their chemical speciation which is influenced by buffer capacity of water soluble and exchangeable metal complexes, organic matter and sulphides (Chakraborty et al., 2015a). Metals concentration in mangroves root system is considered as an indicator of metals bioavailability (Chakraborty et al., 2014). There are four typical Brazilian woody mangrove species: *Rizophora mangle*, *Laguncularia racemosa*, *Avicennia germinans* and *Avicennia shaueriana* (Tomlinson, 1986). Woody mangroves species, when able to grow in their own ecosystem, are effective in heavy metal phytostabilization trough metal precipitation in rhizosphere and/or bioaccumulation in roots (MacFarlane et al., 2007), allowing the “self-preservation” of this ecosystem. Some authors showed an interesting potential of *Avicennia* spp. in heavy metals accumulation and translocation to stem (MacFarlane et al., 2003; González-Mendoza et al., 2007). Souza et al. (2015) found that *Avicennia shaueriana* tries to translocate more trace elements to stem and leaves in case of high accumulation in roots, probably to avoid an excess of toxicity in roots tissues. In general, *Avicennia* spp. seems to be promising in sediment phytoremediation, also due to their attitude to exclude salt trough leaves glands: this characteristic leads to a weaker thickening of exodermis cell walls in comparison with other mangroves (Pittarello et al., 2017). Moreover, roots of mangrove species can modify the surrounding sediments redox conditions with oxygen supplied to rhizosphere by aerenchyma (Jacob and Otte, 2003). This influences bioavailability of Cd, which, in surface sediments, is mobile under oxidizing conditions at pH levels below 8 (Chakraborty et al., 2015b).

Aerenchyma is a spongy tissue present in root mangrove aerial extensions termed ‘pneumatophores’ and in absorbing roots (Hogarth, 1999). These tissues guarantee oxygen supply in anaerobic substrates ensuring O_2 diffusion into below ground root portion (Youssef and Saenger, 1996). The part of this O_2 stored inside the roots that exceeds the need for aerobic respiration, is released through the aerenchyma into the surrounding rhizosphere. This mechanism is called radial oxygen loss (ROL) in wetland plants (Armstrong, 1978; Colmer and Pedersen, 2008). In case of trace elements contamination several mangroves species like *Aegiceras corniculatum*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*, increase the thickness of outer cortex and lignification in cell walls with the consequence to reduce ROL (Cheng et al., 2010). Pi et al. (2009) found that *Avicennia marina* roots present the thinner outer cortex both in mature zone, 8 cm from root tip, and close to the tip, in comparison with other seven mangrove species, although shows the highest resistance to waterlogging. Souza et al. (2015) found root anatomical changes in *Avicennia shaueriana* in four mangrove forests of Espirito Santo State (Brazil), characterized by different levels of oxygen deficiency in sediments: seems that in *A. shaueriana* pneumatophores and absorbing roots cortex and aerenchyma area were inversely correlated with oxygen concentration in sediments.

Cadmium (Cd) is a non-essential heavy metal, released into the environment by traffic or industrial activities like mining, electroplating, manufacturing of plastics, alloy preparation. It also derives from batteries that contain Cd and it is a by-product of mineral fertilizers. Cd is considered highly toxic for animals and humans due to its solubility in water depending on its speciation. In particular, due to their longevity, humans can accumulate Cd in their organs by eating contaminated plants and animals (Kirkham, 2006): for this it is important to implement all possible actions in order to remove Cd from food chain or block its circulation in soil and water (Grant et al., 1998). In humans Cd intoxication can lead to kidney, skeletal, respiratory and reproductive systems damages (Godt et al., 2006).

While Cd can sometimes be found in high concentrations in mangrove sediments (Nascimento et al., 2006), bioaccumulation of Cd in edible animals can be high in mangrove systems even at low Cd loading in the sediment (Chakraborty et al., 2015b; Chakraborty et al., 2015a).

In plants Cd exerts its toxic effects competing with several essentials nutrients and/or impairing transportation mechanisms: its competition with Fe causes chlorosis (Das et al., 1997), it decreases water content (Sanita di Toppi and Gabrielli, 1999) and causes root tips browning with a consequent growth inhibition (Kahle, 1993). Interestingly, as found by Baryla et al. (2001) in *Brassica napus*, Cd does not directly impair antenna photosystem but decreases number of chloroplasts per cell and changes cell size, suggesting that Cd interferes with chloroplast replication and cell division. Furthermore, although brassicas grew in highly enriched CO_2 atmosphere (4000 $\mu L\, CO_2\, l^{-1}$), growth inhibition by Cd was not reversed, probably because low stomatal conductance was not the main effect of Cd toxicity. First one of these two insights are confirmed by Di Cagno et al. (1999) who evidenced that in *Heliantus annus* seedlings treated with Cd^{2+} the photochemical efficiency of photosystem II (PSII) was not altered although, after 7 d of treatment leaf area, chlorophyll content and CO_2 assimilation rate decreased both in young and mature leaves. Water deficit was clear in *Heliantus annus* exposed to excess concentrations of Cd (Kastori et al., 1992). In *Cajanus cajan*, 20 mM Cd^{2+} inhibited by 87% CO_2 exchange rate and the extent of inhibition increased with duration of exposure. Stomatal conductance decreased in parallel with transpiration rate. After 10 days of treatment, wilting occurred (Sheoran et al., 1990). Costa and Morel (1994) found that in hydroponic, Cd above 100 μM caused stomatal closure in lettuce, while the opposite effect was obtained with concentrations up to 0.1 μM.

Cd is mainly accumulated in roots but depending on plant species, plant organs, its concentration and time to exposure, it can be significantly translocated to shoots and use same N and P pathway to reach shoots and reproductive organs. In wheat (*Triticum turgidum*) Cd accumulation in grains increased significantly with N and P applications, indicating an environmental effect on Cd phytoavailability (Grant and Bailey, 1998; Di Cagno et al., 1999). On the opposite, in *Heliantus annus* Simon (1998) evidenced N, P, K, Ca, Mg, Cu, Fe, Mn and Zn uptake was not influenced by lower or higher Cd concentrations; Cd accumulation in roots shoots and leaves was directly correlated with its concentration in the medium and the main accumulation was in roots, up to 13.69 $mg\, kg^{-1}$ depending on the concentration. In aquatic species, *Bacopa monnieri* maximum Cd accumulation (906.5 $mg\, kg^{-1}$ DW) was found in roots exposed to 200 μM Cd for 144 h (Singh et al., 2006).

Cd attitude to being accumulated in plants roots and reproductive organs can be a great risk for animal and human health, however, several woody species can be successfully adopted in Cd-contaminated soil to accumulate the metal in the plant organs aiming to soil phytoremediation. For example Robinson et al. (2000) employed poplar and willow clones in soils containing a range of Cd concentrations among 0.6 and 60.6 $mg\, kg^{-1}$ dry soil obtaining a Cd accumulation in willows clones up to 167 $mg\, kg^{-1}$ while poplars accumulated up to 209 $mg\, kg^{-1}$. *Avicennia marina,* in Cd low contaminated sediment (1.23 $mg\, kg^{-1}$) achieved to translocate to leaves 1.04 $mg\, kg^{-1}$ (Usman et al., 2013).

Phytoremediation efficiency is affected by multiple environmental factors, such as soil texture, pH, redox conditions, cation exchange capacity, microorganisms, Ca/Fe/Mn/Al/P contents and the presence of natural organic matter (Pittarello et al., 2017). Within natural organic matter, humic substances (HS) are known to affect both plant physiology (Nardi et al., 2009) and metal availability. In particular, environmental availability of metals can be increased or decreased by exogenous humic substances in relationship with metal, pH and soil characteristics (Wiszniewska et al., 2016): Chakraborty (2010) reported that at pH < 6 humic acids are aggregated due to neutralization of negative charges by H^+ leaving Cd mainly in bioavailable form in the solution; at pH > 6 humic acids disaggregate offering more complexing sites for Cd^{2+} ions forming inert complexes when pH increases up to 7.

Cd-humic matter interaction have several other consequences: Cd can be found as weak complexes with dissolved or sedimentary organic matter in field conditions (Martínez and McBride, 1999; Tuschall Jr. and Brezonik, 1981; Muller, 1999; Chakraborty, 2010; Almås et al., 2000; Chakraborty et al., 2012a, 2012b; Eggleton and Thomas, 2004); more specifically Cd forms thermodynamically less stable complexes with humic substances (Chakraborty and Chakrabarti, 2008).

Although metals prefer to associate with sedimentary organic binding phase, Chakraborty et al. (2016) explained the low Cd concentration in the organic binding phases of the studied sediments trough the hard soft acid base theory: the soft acid Cd^{2+} prefer softer bases like S- and N-residues in organic binding phases to form highly stable complexes. The concentrations of soft base ligands in humic substances are very little and cannot bind all the Cd so the remaining Cd binds with –COOH and –OH groups, resulting in low stability complexes bioavailable in the system (Chakraborty and Chakrabarti, 2008; Chakraborty et al., 2016). The formation of Cd weak complexes happens also due to the competition with Cu, Ni, Fe and Al for the binding sites of organic phases in sediments.

HS are complex biomolecules formed through tissue transformation, during animal and plant residuals decomposition, due to soil chemical and microbial activities (Canellas et al., 2010). They are composed by supermolecules with high apparent molar mass that can be disassembled by small amounts of organic acids: within the net of the supermolecules, products of soil microbial activities, like auxins, could be entrapped and then released under the action of roots organic acids (Piccolo, 2002). Humic compounds were found to influence root architecture and nutrient uptake, enhancing plant yield (Nardi et al., 2009; Herder et al., 2010). This action is exerted by HS as a function of their degree of hydrophobicity, depending on the number of functional acidic groups as carboxyl and phenol (Canellas et al., 2012). These plant responses to HS treatments ascribe HS in the class of plant biostimulants (Calvo et al., 2014).

Many works related to humic compounds as biostimulants are focused in finding the optimal dose of humic and/or fulvic acids in order to maximize root development, nutrient use efficiency and resistance to diseases and abiotic stresses, mainly for horticultural implementations (Canellas et al., 2015; García et al., 2016). In ex-situ phytoremediation of dredged sediments, soil rich in organic matter processed by earthworms is employed to foster plants establishment on contaminated sediments (Doni et al., 2013; Doni et al., 2015). Seedlings of *Rizophora mangle* and *Laguncularia racemosa*, in a very early stage of root development, treated with different concentration of humic matter extracted from their own mangrove sediment, were established in a contaminated and degraded mangrove area: during the two months before planting seedlings treated with humic matters exhibited 50% more than control in roots length, while two years after planting in contaminated sediments treated plants showed 20% more in stem length and an higher percentage of survivors in comparison with not treated plants (Dobbss, 2012). The feasibility of amending mangrove areas with nutrient solutions containing N and P has demonstrated by Feller et al. (1999) obtaining positive plant responses. This approach could be employed to treat polluted areas with humic compounds solutions to trigger biostimulant mechanisms in mangrove plants.

This work investigates the effects on *Avicenna germinans* of Cd pollution in the presence of humic substances in controlled conditions.

The research aimed to:

1) Find potential changes in HS optimal dose depending on plant developmental age;
2) Describe the effects of Cd contamination and HS biostimulant activity on mangrove root anatomy;
3) Evaluate which HS dose might be adopted to enhance plant growth in presence of Cd stress.

These results could help defining a phytostabilization and a phytoextraction strategy involving HS, both for the protection and restauration of mangrove ecosystems and for contaminated dredged sediments clean up.

2. Materials and methods

2.1. Plant and sediment sampling

Mature and healthy propagules of *Avicennia germinans* and sediment samples were collected in mangrove forest of Benevente river, close to Anchieta town in Espirito Santo State, Brazil; sediment sampling points are S 20°46′18″ W 40°39′23″, S 20°45′49″ W 40°39′44″ and S 20°45′09″ W 40°39′56″; seeds collecting points are S 20°46′28″ W 40°39′00″ and S 20°46′14″ W 40°39′31″. These areas are characterized by highest percentage of *Avicennia germinans* in Benevente river mangrove forest (Petri et al., 2011). In each sampling point sediments were collected randomly in a 10 m^2 area at the river banks at a depth of 0–10 cm. Sediment were stored in plastic jars, transported to green house and left to dry for a week at air temperature then they were over dried for 48 h at 60 °C in a stove.

2.2. Humic substances extraction and characterization

Sediments were air dried for 48 h in a green house, then crunched in a mortar and ground to pass through a 10-mesh sieve (2 mm). From sediment HS were extracted following the International Humic Substances Society (IHSS) method (Swift, 1996): 10% (w/v) of sediment were mixed with a 0.5 M NaOH solution. The suspension has been shaken for 15 h and separated from humin trough 24 h of settling. The suspension was finally centrifuged at 10000 rpm for 10 min to separate finest clay particles: we avoided the separation onto humic and fulvic acids in order to supply plants with a dissolved HS dose as realistic as possible. In order to separate them from salt, HS were dialyzed by Serva Servapor HMF MWCO 2000 dialysis tubing, against deionized water with 1/10 of volume ratio. After 3 days of dialysis the electric conductivity of the extract was lower than 10 μS/m. The pH of HS solution was adjusted to 7 and dialyzed HS were liofilized by Terroni Enterprise I liofilizer and stored at 4 °C.

The elemental analysis (C, H, N, O, S) was carried out using an elemental analyzer (CHNS-O mod. analyzer Perkin Helmer 2400). All analyses were performed in triplicate.

Infrared spectrum was made by Jasco FT/IR 4100 (Jasco Corp., Tokyo, Japan) equipped with a DR-81 diffuse reflectance accessory (JASCO Corp., Tokyo, Japan). A 1 mg portion of each HS sample was thoroughly mixed with 100 mg of potassium bromide (KBr) (FT-IR grade; Wako Pure Chemical Industries, Ltd., Osaka, Japan) with mortar and pestle, and then placed in an aluminum sample cup. Spectra were collected from 4000 to over 100 scans. The resolution was set to identify the principal bands that contribute to the more complex band resulting from overlapping features. Fourier self-deconvolution (FSD) was performed for the wavenumber region between 4000 and 500 cm^{-1} using the JASCO FT-IR software. Cross polarization NMR spectrum was made by Bruker Biospin Ascend 600 (Bruker Corporation, USA) using 150 MHz frequence and 2 s relaxation delay time; data were processed by Bruker TopSpin® software. NMR samples were prepared dissolving 10 mg of HS in 400 μL of milliQ water.

2.3. Seedlings preparation

A. germinans propagules were rapidly cleaned in 10% alcohol solution and then rinsed 2 times with tap water. They were left on a paper towel some hours until hulls have been broken. Then they have been placed in a tray filled up with clean river sand and irrigated with half strength Hoagland's nutrient solution in field condition. After a week, once cotyledons were fully opened and stem firmly established in sand trough first root, 48 healthy seedlings were chosen for the following experiments. Seedlings were individually placed in Leonard pots settled as follows: 1.2 L of clean river sand connected on the bottom of the plastic bottle with 500 mL of half strength Hoagland nutrient solution (Hoagland and Arnon, 1950). Hoagland solution contained $Ca(NO_3)_2 \cdot 4H_2O$ (25 mM), KNO_3 (25 mM), KH_2PO_4 (0.5 mM), $MgSO_4 \cdot 7H_2O$ (1 mM), H_3BO_3 (2.3 10^{-2} mM), $MnCl_2 \cdot 4H_2O$ (4.6 10^{-3} mM), $CuSO_4 \cdot 5H_2O$ (2.50 10^{-5} mM), $(NH_4)6Mo7O_2 \cdot 4H_2O$ (1.13 10^{-5} mM), $ZnSO_4 \cdot 7H_2O$ (3.82 10^{-4} mM), Fe-EDTA (20 mM). All reagents were analytical grade (SIGMA Aldrich). Each pot was sealed with aluminum foil to avoid any photochemical effect to nutrient solution, algae proliferation and light stress to roots close to the borders of bottle. Seedlings were grown in a greenhouse at a temperature of 27 ± 5 °C, with 11/13 h day/night cycle.

2.4. Plant growth conditions

The first experiment involved 7 days old seedlings and lasted 30 days; the second one started with 90 days old seedling was 45 days long, also including Cd treatments. This setup aimed to find possible different responses of root system in terms of root length and area depending on plant age, developmental step and HS concentration. Cd treatment in the second experiment pointed to understand the effects of HS and Cd interaction in plant development.

Based on HS carbon content, HS were weighted to obtain 2, 4 and 8 mM of C. For the first experiment sixteen one week old seedlings, four seedlings for each treatment, were treated for 30 days with 0 mM (control), 2 mM, 4 mM and 8 mM C L^{-1} concentrations + half strength Hoagland nutrient solution. The 500 mL volume was maintained adding every 2 days new half strength Hoagland solution. The solution was completely replaced every 10 days, including HS.

For the second experiment 32 three months old seedlings, four seedlings for each treatment, were treated for 45 days as follows: 0 mM C + 0 μM Cd (control), 0 mM C + 50 μM Cd, 2 mM C + 50 μM Cd, 4 mM C + 50 μM Cd, 8 mM C + 50 μM Cd, 2 mM C + 0 μM Cd, 4 mM C + 0 μM Cd, 8 mM C + 0 μM Cd. The nutrient solution volume (500 mL) was maintained by adding new half strength Hoagland solution, with or without 50 μM Cd, every 2 days. The solution was completely replaced every 10 days. pH and EC of the solutions were tested before every use. Cd concentration (50 μM = 5.6 mg L^{-1}) was chosen considering I) the high mortality caused in a preliminary trial by Cd 500 μM (data not shown), II) it represents a realistic concentration in relationship with average sediment contamination and toxicity scale based on the quality guidelines of sediment (SQGs) (Usman et al., 2013). Cd was added to the solution in the form of $CdCl_2$ (SIGMA Aldrich).

For both experiments every 2 days the solution was mixed vigorously to avoid an excessive HS deposition in the bottom and to provide its partial re-oxygenation.

2.5. Plant growth measurement

For both experiments, at the end of the treatments plants were gently removed from the pots and roots were accurately cleaned up with tap water and then with deionized water. Plant roots treated with Cd were also placed 1 min in a HCl 0.1 M solution to clean up root surface from adsorbed Cd ions. Stem length, fresh and dry weight were measured.

Roots pictures for each plant were taken by the camera Nikon Coolpix S7000 fixed to the table by a plastic pole to maintain a steady distance from roots. Images were uploaded into a pc and processed by software Delta T scan (Delta T corporation, UK) to measure total root area, root length and, for the second experiment, leaf area.

At the end of second experiment, before plants harvest, leaf gas exchange parameters, such as the CO_2 assimilation, transpiration rates and stomatal conductance were measured using a portable photosynthesis system (LI 6400 XT, Licor, Nebraska, USA), with a standard 6 cm^2 leaf chamber. The CO_2 concentration in the leaf cuvette was fixed in 400 μmol mol^{-1}. The measurements were taken under 1000 μmol m^{-2} s^{-1} photosynthetic photon flux density. Within the leaf chamber, the average temperature was 27.0 ± 1.3 °C. Gas exchange determinations were performed between 8 and 11 h a.m.

2.6. Cd content measurement

To measure Cd content in roots, stems and leaves samples were separated and dried for 24 h at 60 °C in a stove, then grained in a mortar with pestle; then samples were prepared according to US-EPA method 3501a (Meyer et al., 2016) modified as follows: 250 mg of each samples were mixed with 5 mL of 95% pure HNO_3 (SIGMA) and 2 mL of 35% concentrated H_2O_2 and warmed up to 150 °C in glass tubes until all organic material was digested. Samples were diluted with deionized water in a ratio 1:10 v/v and analyzed in triplicate by Inductively Coupled Plasma Optical Emission Spectrometry, using a Shimadzu ICPE 9000 instrument. After every set of 10 samples, a standard check of 0.20 mg L^{-1} was measured to correct for instrumental instability and matrix effect. A Fluka Multielement Standard solution (10 mg L^{-1}) was used as reference to calibrate the equipment. Instrumental precision for Cd content and Fe, Ca, B, Mn, Mg, K was ascertained using NIST SRM 1573a (Tomato leaves) as Certified Reference Material. Detection limits: Cd 0.0136 mg L^{-1}, Fe 0.004 mg L^{-1}, Ca 0.006 mg L^{-1}, B 0.019 mg L^{-1}, Mn 0.003 mg L^{-1}, Mg 0.002 mg L^{-1}, K 1.98 mg L^{-1}; Average Recovery from CRMs material was 86.37 ± 5.44%.

2.6.1. Analysis of root anatomy

Roots histological samples were obtained as following: for each plant the last 15 mm long of lateral root was taken and dehydrated through an ethanol series; then roots were placed into 2 mL centrifuge tubes in a solution with liquid historesin (Leica®, Germany) and ethanol (1:1 v/v) for 4 h then in pure liquid historesin for 10 days. After historesin completely entered root tissues, samples were placed in a plastic rank with holes of 0.4 mL and immerged in liquid historesin + hardener to allow the sample inclusion. After 2 weeks samples were cross sectioned in 10 μm thick slices with a rotative microtome, placed on a glass strip and stained with 0.05% toluidine blue, pH 4.7 for 3 min (O'Brien et al., 1964). After to have placed the cap on the glass strip, the photomicrographs were obtained using a Nikon Eclipse 50i microscope (Tokyo, Japan). Total diameter and Vascular cylinder diameter measurements were performed using the Nikon NIS-Elements software (Tokyo, Japan).

2.7. Statistical analysis

Shapiro-Wilk test for normality and Levene test for homogeneity of variance were performed. One way ANOVA and Games Howell or Duncan post hoc test, depending on homogeneity of variance, were performed. Pearson two-tail correlation test was performed for selected parameters. All analyses were carried out with IBM SPSS Statistics v24.0.

3. Results

3.1. Chemical spectroscopic characteristics of HS extract

The HS extract had the following elemental composition: C: 25.66%, H: 2.94%, O: 67.58%, N: 3.83%, and S: 0.63%.

The HS infrared spectroscopy data exhibited a broad band at 3364 cm^{-1}. This band, in HS, has been attributed to O−H stretching of alcohols and/or phenols and N−H stretching of amines and/or amides (Bellamy, 1975). The small band at 2957 cm^{-1} is attributed to C−H stretching of aliphatic groups. The bands at 1595 and 1380 cm^{-1} were COOH groups (Morra et al., 1989; Shin et al., 1999). Other two weak peaks are in 1260 cm^{-1} and in 1240 cm^{-1} which are associated with a mixture of C−O stretching and C−O−H bending vibrations (Shen et al., 2016). The intense peak at 1040 cm^{-1} was vibrations of

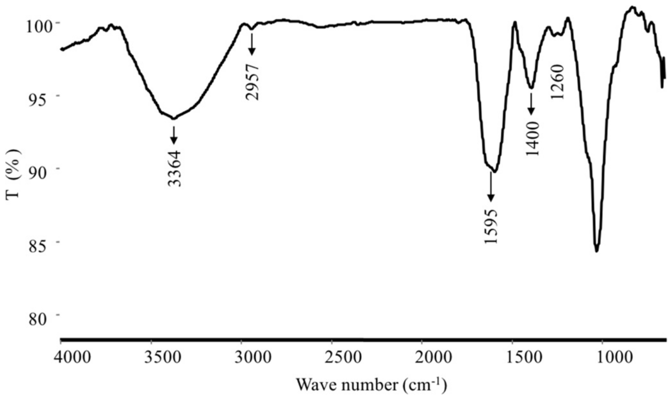

Fig. 1. IR Spectrum of humic substances (HS) extracted from Rio Benevente mangrove forest sediments.

polysaccharides and similar structures (García et al., 2014) (Fig. 1).

CP–MAS ^{13}C NMR (Fig. 2) spectrum was area normalized and integrated in ^{13}C chemical shift intervals, showing the following distribution of C in the HS structure: 29.36% alkyl C (0 ppm–46 ppm), 18.49% methoxyl and N-alkyl C (46 ppm–65 ppm), 25.86% O-alkyl C (65 ppm–90 ppm), 7.75% di-O-alkyl C and some aromatic structures (91 ppm–108 ppm), 13.12% aromatic C and O-aromatic C (109 ppm–160 ppm), 4.04% carboxyl C, amide, and ester C (160 ppm–185 ppm), and 1.38% carbonyl C (185 ppm–220 ppm). The areas relative to these resonance intervals were used to evaluate the percentage of aromaticity and aliphaticity as described in Song et al. (2008): Aromaticity = [(Aromatic C peak 109–160 ppm) × 100] / [Total peak area 0–220 ppm] = 13.12; Aliphaticity = 100 − Aromaticity = 86.88.

The ratio between the hydrophobicity and hydrophilicity (HB/HL) was calculated as described in García et al. (2014): HB/HL = [(0–46 ppm) + (108–160 ppm)] / [(46–108 ppm) + (160–220 ppm)] = 0.81.

3.2. 7 day old seedling experiment

Seedlings do not show any statistically significant differences in overall biomass production, although those treated with 8 mM of C show a difference in stem dry biomass in comparison with 2 mM C. Significant differences were found in root length and area. 4 mM treatment shows the highest significant root area (429.73 mm^2) and length (573.22 mm) while 8 mM shows lowest values in both parameters (Table 1).

3.3. 90 days old seedlings experiment

Leaves fresh and dry biomass did not show differences while leaf area evidenced significant differences: Cd + 4 mM C treatment achieved the highest average leaf area (923.83 mm^2) while Cd treatment shows the lowest value (478.85 mm^2). No differences were encountered in stem fresh and dry biomass among the treatments while plants treated with C 2 mM show the highest and the only statistically different value in stem length (30.87 cm). All treatments show very similar roots fresh and dry biomasses except for the treatment C 2 mM: its value is the highest and statistically different (FW 11.84 g, DW 1.67 g); looking at the absolute values, Cd + C 4 mM treatment produced the lowest fresh and dry root biomass (FW 6.27 g, DW 0.91 g). Although not statistically different, Cd + C 8 mM had the higher root/shoot ratio within Cd treated plants (Table 2).

3.4. Cadmium accumulation

Cd, Cd + C 2 mM and Cd + C 4 mM show similar Cd concentrations in roots tissue while Cd + C 8 mM, accumulated < 50% in comparison with other treatments. Cd concentration in stems and leaves was roughly the 2% of Cd accumulated in roots, always below 10 mg kg^{-1}, without significant differences between groups treated with Cd 50 μM.

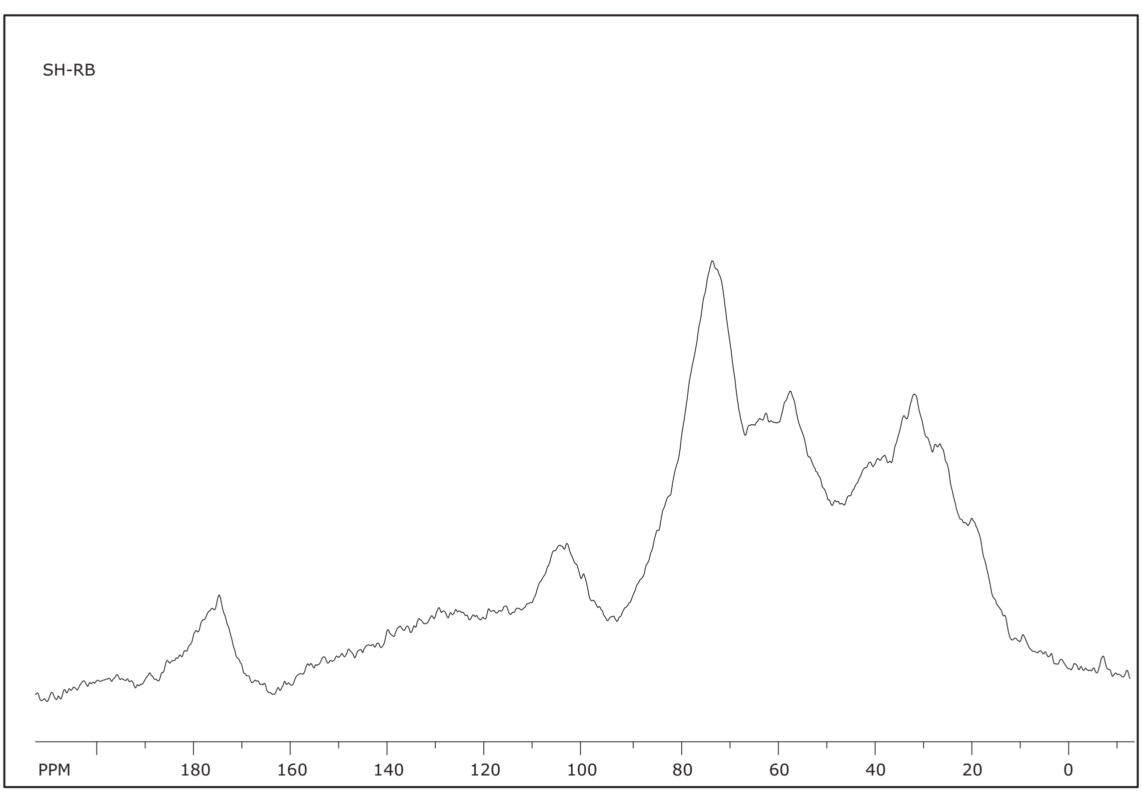

Fig. 2. ^{13}C NMR spectrum of Humic Substances extracted from Rio Benevente mangrove forest sediments.

Table 1
Biometric parameters of week old *Avicennia germinans* seedlings after 30 days of treatment. C = Carbon in Humic Substances; FW = Fresh Weight; DW = Dry Weight. Values are reported as means ± Standard Error ($n = 4$). Different letters mean statistical differences among the treatments ($p < 0.05$).

Treatments	Stems FW	Stems DW	Roots FW	Roots DW	Roots area	Roots length	Leaf FW	Leaf DW
	g	g	g	g	mm^2	mm	g	g
Control	0.95 ± 0.15^{a}	0.21 ± 0.03^{a}	1.20 ± 0.24^{a}	0.23 ± 0.06^{a}	259.14 ± 12.29^{c}	350.35 ± 10.67^{c}	1.07 ± 0.21^{a}	0.27 ± 0.05^{a}
C 2 mM	1.28 ± 0.18^{a}	0.28 ± 0.04^{a}	1.97 ± 0.23^{a}	0.28 ± 0.03^{a}	324.07 ± 3.65^{b}	426.77 ± 3.61^{b}	1.37 ± 0.19^{a}	0.34 ± 0.04^{a}
C 4 mM	1.14 ± 0.22^{a}	0.25 ± 0.05^{a}	1.65 ± 0.36^{a}	0.25 ± 0.06^{a}	429.73 ± 9.37^{a}	573.22 ± 27.13^{a}	1.18 ± 0.24^{a}	0.31 ± 0.07^{a}
C 8 mM	0.76 ± 0.11^{a}	0.17 ± 0.03^{a}	1.51 ± 0.27^{a}	0.21 ± 0.03^{a}	173.92 ± 5.93^{cd}	392.31 ± 8.27^{d}	0.90 ± 0.12^{a}	0.23 ± 0.03^{a}

Table 2
Three months old *Avicennia germinans* seedlings treated for 45 days with Cd 50 μM and/or three different C concentrations. C = Carbon in Humic substances; FW = Fresh Weight; DW = Dry Weight; Shoot DW = Leaves DW + Stems DW. Values are reported as means coupled ± Standard Error ($n = 4$). Different letters indicate statistical differences among the treatments ($p < 0.05$).

Treatments	Stem length	Stem FW	Stem DW	Leaves FW	Leaves DW	Leaves area	Root FW	Root DW	Root DW/shoot DW
	cm	g	g	g	g	mm^2	g	g	
Control	26.37 ± 0.99^{ab}	2.48 ± 0.24^{a}	0.66 ± 0.05^{a}	2.93 ± 0.35^{a}	0.90 ± 0.10^{a}	667.20 ± 5.32^{c}	7.38 ± 0.38^{b}	1.08 ± 0.53^{ab}	0.62 ± 0.11^{ab}
Cd	25.25 ± 1.30^{b}	2.87 ± 0.25^{a}	0.88 ± 0.10^{a}	3.44 ± 0.32^{a}	0.91 ± 0.05^{a}	478.85 ± 20.55^{e}	6.60 ± 0.45^{b}	1.01 ± 0.68^{b}	0.57 ± 0.06^{ab}
Cd + C 2 mM	25.00 ± 2.18^{b}	3.00 ± 0.48^{a}	0.91 ± 0.16^{a}	3.23 ± 0.56^{a}	0.94 ± 0.16^{a}	770.99 ± 30.56^{b}	7.35 ± 1.00^{b}	1.05 ± 0.14^{ab}	0.60 ± 0.06^{ab}
Cd + C 4 mM	26.25 ± 1.18^{ab}	3.14 ± 0.29^{a}	0.95 ± 0.09^{a}	3.15 ± 0.45^{a}	0.88 ± 0.09^{a}	923.83 ± 12.94^{a}	6.27 ± 0.96^{b}	0.91 ± 0.13^{b}	0.49 ± 0.04^{b}
Cd + C 8 mM	25.00 ± 1.08^{b}	2.96 ± 0.32^{a}	0.84 ± 0.10^{a}	3.10 ± 0.54^{a}	0.85 ± 0.09^{a}	833.44 ± 6.20^{e}	8.42 ± 0.62^{ab}	1.19 ± 0.10^{ab}	0.71 ± 0.03^{ab}
C 2 mM	30.87 ± 1.46^{a}	3.81 ± 0.42^{a}	0.98 ± 0.12^{a}	3.55 ± 0.55^{a}	1.18 ± 0.17^{a}	589.96 ± 19.74^{d}	11.84 ± 0.29^{a}	1.67 ± 0.18^{a}	0.80 ± 0.13^{a}
C 4 mM	30.05 ± 2.26^{ab}	3.71 ± 0.67^{a}	0.95 ± 0.21^{a}	3.40 ± 0.88^{a}	1.16 ± 0.25^{a}	856.72 ± 7.81^{e}	10.06 ± 2.82^{ab}	1.41 ± 0.43^{ab}	0.63 ± 0.08^{ab}
C 8 mM	29.50 ± 2.25^{ab}	3.42 ± 0.59^{a}	1.36 ± 0.49^{a}	3.10 ± 0.49^{a}	0.97 ± 0.23^{a}	610.26 ± 5.67^{d}	8.72 ± 1.14^{ab}	1.24 ± 0.16^{ab}	0.63 ± 0.13^{ab}

Cd traces were detected in control and groups treated only with humic substances probably due to unavoidable sand and humic substances contamination (Table 3).

Cd and/or HS treatment did not significantly alter B, Ca, Fe, Mg and Mn uptake and translocation as demonstrated by elements leaves contents (Supplemental material). K leaf content resulted significantly higher in HS treated plants compared to controls with or without Cd in the substrate.

3.5. Gas exchange

Significantly highest values in Carbon fixation (16.01 μM CO_2 m^{-2} s^{-1}), stomatal conductance (0.16 Mol H_2O m^{-2} s^{-1}) and transpiration (3.45 mM H_2O m^{-2} s^{-1}) were found in C 2 mM treatment, while the lowest were in Cd + C 2 mM, 5.69 μM CO_2 m^{-2} s^{-1}, 0.09 Mol H_2O m^{-2} s^{-1}, 1.03 mM H_2O m^{-2} s^{-1}) although there are not significant differences among plants treated with C and Cd. In general, all plants treated only with HS show values higher than Cd + HS with significant differences (Table 3).

3.6. Root anatomy and development

The root diameter/vascular cylinder diameter ratio increases linearly in Cd presence with humic substances concentration, increasing from control to Cd + C 8 mM. In absence of Cd, HS treatment induced no significant differences compared to control (Table 3; Fig. 5). There are significant differences among control and Cd, Cd + C and C treated plants in terms of root length and area (Figs. 3, 4). Apart from Cd + 4 mM C, all Cd-treated plants evidenced shorted roots compared to HS-treated plants without Cd. Cd and Cd + C 8 mM showed the lowest values for both root length and area (Fig. 3). Root area follows the same pattern, with highest values for plants treated with 2 and 4 mM C HS without Cd and significantly lower values for Control and Cd staying below 500 mm^2. (Fig. 4).

Blue toluidine coloration did not evidence any enhanced Casparian strip and exodermis lignification (green color) and or suberification (blue color) among the treatments in absorbing roots. (Fig. 5).

4. Discussion

IR spectrum of HS extracted from mangrove sediments present some

Table 3
Three months old *Avicennia germinans* seedlings treated for 45 days with Cd 50 μM and/or three different C concentrations. $C_{\varnothing}/VC_{\varnothing}$: Cortex diameter and Vascular Cylinder diameter ratio; root section taken at 1.5 ± 0.2 cm from a lateral root apex. Cd Roots, Cd Stems and Cd Leaves: Cadmium concentration. Photosynthesis: Carbon fixation rate. St. conductance: H_2O leaving stomatal chambers; Transpiration: H_2O loss rate. C: Carbon in Humic Substances.TF = Translocation Factor [Cd stem]/[Cd roots]. Cd concentration in the solution is 50 μM. Values are reported as means coupled ± Standard Error ($n = 4$). Different letters indicate statistical difference among the treatments ($p < 0.05$).

Treatments	$C_{\varnothing}/VC_{\varnothing}$	Cd Roots	Cd Steams	Cd Leaves	TF	Photosynthesis	St. conductance	Transpiration
		mg kg^{-1}	mg kg^{-1}	mg kg^{-1}		μM CO_2 m^{-2} s^{-1}	Mol H_2O m^{-2} s^{-1}	mmol H_2O m^{-2} s^{-1}
Control	3.51 ± 0.12^{c}	0.10 ± 0.0^{c}	0.30 ± 0.13^{b}	1.39 ± 0.23^{b}	–	13.69 ± 2.58^{ab}	0.24 ± 0.04^{ab}	2.67 ± 0.38^{ab}
Cd	3.74 ± 0.17^{bc}	459.00 ± 27.19^{a}	5.87 ± 1.17^{a}	5.83 ± 0.68^{a}	0.01 ± 0.00	8.75 ± 2.09^{bc}	0.14 ± 0.04^{b}	1.60 ± 0.42^{bc}
Cd + C 2 mM	3.84 ± 0.12^{bc}	467.25 ± 133.86^{a}	7.42 ± 1.39^{a}	6.11 ± 1.24^{a}	0.02 ± 0.00	5.69 ± 1.14^{c}	0.09 ± 0.01^{b}	1.03 ± 0.14^{c}
Cd + C 4 mM	4.42 ± 0.12^{ab}	418.75 ± 128.79^{a}	9.5 ± 3.34^{a}	4.97 ± 0.91^{a}	0.06 ± 0.05	6.30 ± 1.23^{c}	0.11 ± 0.03^{b}	1.24 ± 0.29^{c}
Cd + C 8 mM	4.68 ± 0.22^{a}	180.00 ± 51.03^{b}	6.82 ± 1.92^{a}	5.03 ± 0.27^{a}	0.04 ± 0.01	7.63 ± 2.57^{bc}	0.11 ± 0.03^{b}	1.25 ± 0.36^{c}
C 2 mM	3.56 ± 0.18^{c}	0.75 ± 0.27^{c}	0.59 ± 0.20^{b}	1.8 ± 0.08^{b}	–	16.01 ± 2.17^{a}	0.36 ± 0.08^{a}	3.45 ± 0.64^{a}
C 4 mM	3.25 ± 0.19^{c}	1.56 ± 0.46^{c}	1.51 ± 0.32^{b}	1.32 ± 0.49^{b}	–	10.00 ± 1.96^{abc}	0.17 ± 0.04^{b}	1.76 ± 0.36^{bc}
C 8 mM	3.21 ± 0.18^{c}	1.17 ± 0.35^{c}	0.69 ± 0.29^{b}	2.35 ± 0.66^{b}	–	11.12 ± 3.14^{abc}	0.24 ± 0.06^{ab}	2.24 ± 0.49^{abc}

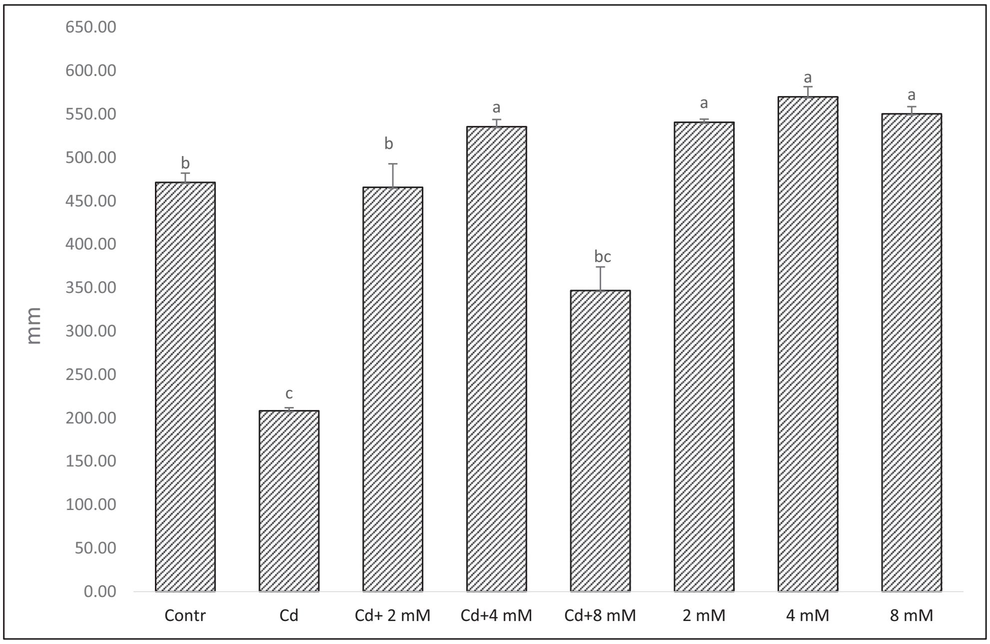

Fig. 3. Root length of three months old *Avicennia germinans* seedlings treated for 45 days with Cd 50 μM and/or three different C concentrations. 2, 4 and 8 mM represent carbon concentration of C contained in humic matters. Error bars represent Standard Error. Different letters indicate significant differences ($p < 0.05$).

of the typical absorption bands, such as the C═O stretching of COOH and the C─H stretching of methyl and methylene groups, which have been previously reported in HS obtained from river sediments by Giovanela et al. (2010). However, the ^{13}C NMR spectrum of studied HS shows a low content of aromatic compounds in comparison with HS derived from Vermicompost and terrestrial soil (Sohn and Weese, 1986; García et al., 2014), while presents characteristics of both estuarine and marine sediments: the low content in aliphatic compounds in region 0–50 ppm is typical of lignin rich HS but on the other side the spectrum shows aromatic contents in the region 110–160 ppm below 15% like poor lignin HS (Cameron and Sohn, 1992). Dobbss et al. (2010) reported that the hydrophobic structures present in HA stimulate root system growth in *Zea mays*, and *Lycopersicon esculentum*. Although Canellas et al. (2012) showed that the loss of hydrophobic structures in HA impairs its biological activity, our HS, poor in hydrophobic structures, significantly stimulated *A. germinans* root growth. Probably the high HB/HL ratio (0.81) has a role in eliciting plant responses, not depending on absolute hydrophobic structure content: Aguiar et al. (2012) observed in maize that the higher is HB/HL ratio of HS, the greater was root growth.

HS concentration of 4 mM C induced the highest values in terms of root area and length both for 7 days old seedlings and 90 days old

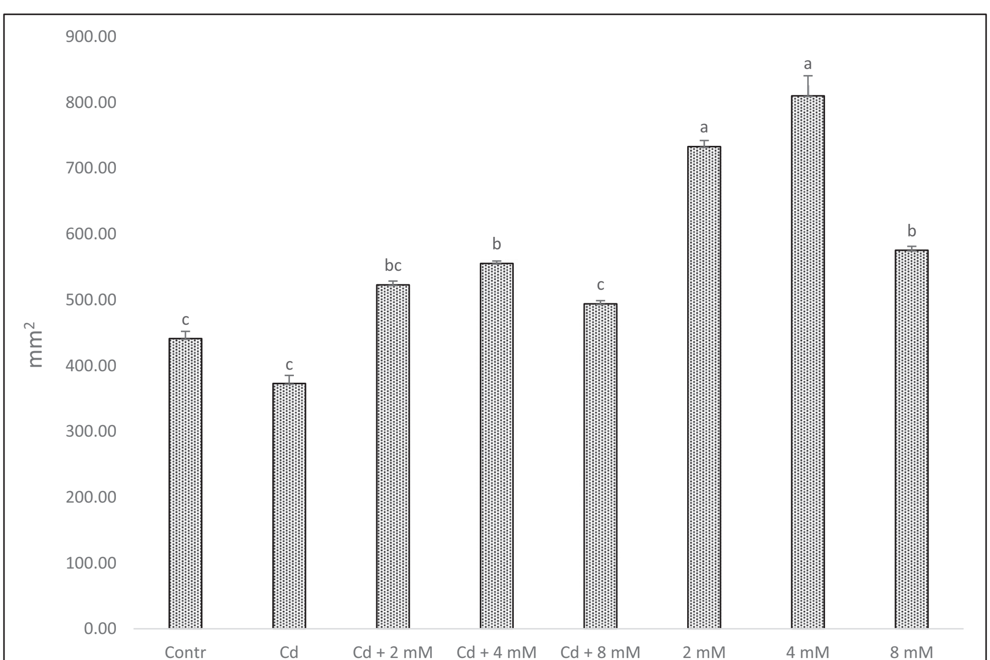

Fig. 4. Root area of three months old *Avicennia germinans* seedlings treated for 45 days with Cd 50 μM and/or three different C concentrations. 2, 4 and 8 mM represent carbon concentration of C contained in humic matters. Error bars represent Standard Error. Different letters mean indicate differences among the treatments ($p < 0.05$).

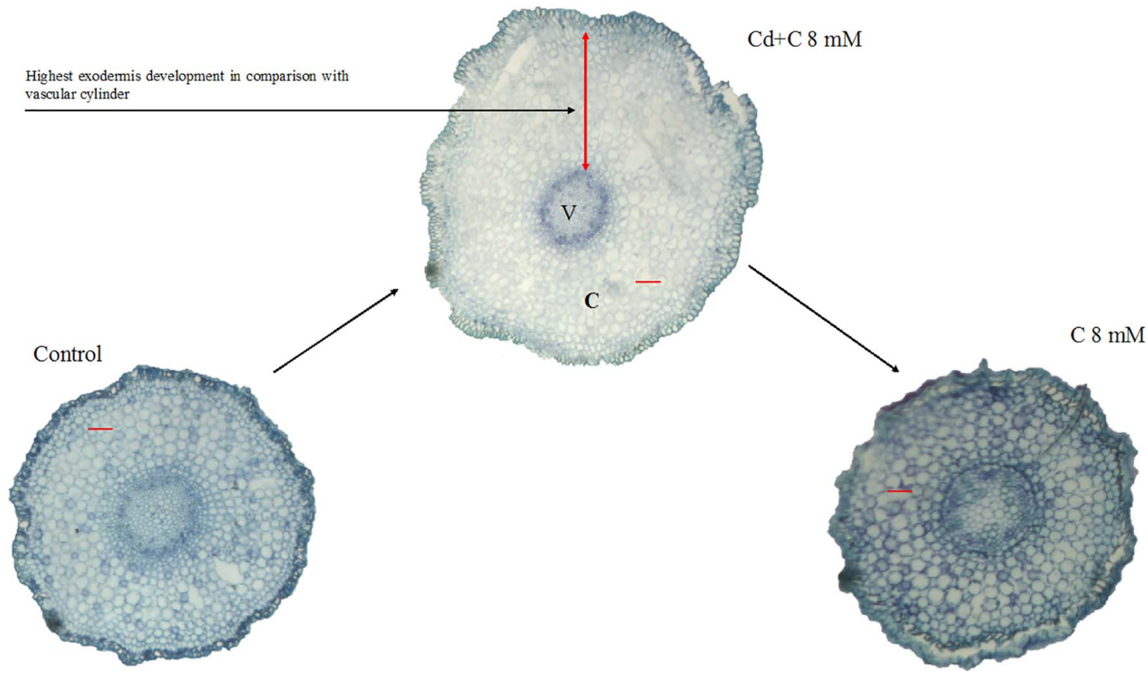

Fig. 5. *Avicenna germinans* fine roots cross sections kept 1.5 cm far from tip, among 3 of 8 treatments. C = cortex; *V* = Vascular cylinder. Red bars = 175 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

plants, significantly different to control plants. Among Cd treated plants the same concentration induced significant differences in root length and area compared to control. Plant responses to HS are known to change as a function of their concentration, the source of the substance, the conditions of the trial, the plant species and its age. It has been also reported that different organs of intact plants respond to humic substances to varying extents (Rose et al., 2014). In our case of study, results indicate that optimal HS concentration for enhanced root growth is 4 mM C irrespective of plant developmental stage and of the presence of Cd.

Morphological changes in response to humic substances have been long reported (for a review Nardi et al., 2009), mainly on root apparatus growth and differentiation (e.g. Nardi et al., 2000; Canellas et al., 2002). Root anatomy is also known to be sensitive to physical and chemical conditions, like Cd presence in the medium, although relatively little information is available about Cd-induced changes in the development of cells and tissues localized within the central part of roots. This topic requires more attention, especially considering the importance of xylem loading in regulating Cd fluxes to the shoot (Lux et al., 2011). In species that tolerate waterlogged and hypoxic conditions the root cortex and the presence of aerenchyma is related to the amount of oxygen available in the medium. In *Avicennia shaueriana* Souza et al. (2015) found an inverse correlation between oxygen concentration in sediments and cortex/vascular cylinder (C/*V*) ratio, cortex thickness and aerenchyma development in root samples collected in field, irrespective of different heavy metal concentrations in the medium. Our results evidence a direct correlation between C/V ratio and HS concentration in Cd treated plant roots (Pearson correlation coefficient 0.803; $p < 0.01$); while no correlation between C/V ratio and HS has been found in plants without Cd treatment. Plant growth conditions in our experiment allow ruling out the influence of substrate oxygen concentration in C/V ratio. This suggests that the increase of cortex thickness depends on humic and organic matter concentration and it is triggered by the presence of Cd in the substrate. Such morphological change can be one of the anatomical adaptive changes to reduce metal in roots, which is enhanced by the HS biological activity. This is also in line with Souza et al. (2015) results where highest C/V values where found in the sites with higher organic matter, and thus HS, concentration.

Cd content in roots treated with increasing HS concentration resulted not significantly different compared to control (450 mg kg^{-1}) except for plants treated with 8 mM C which accumulated only 180 mg kg^{-1} Cd. HS effects on plant metal uptake and accumulation comprise direct and indirect effects. HS may, for example, chelate a cation, thus changing its concentration in growth solution, while in the case of a direct effect, the humic material may influence the permeability of plant membranes or interfere with the active ion uptake carriers and mechanism (Nardi et al., 2009). Our results evidence that concentrations lower than 8 mM C induce no difference on root Cd accumulation. For 8 mM C HS, direct effects on plant anatomy, in terms of reduced root length and area coupled with higher root fresh and dry weigh, due to increased root diameter with increased cortex and C/V ratio might be responsible for lower root Cd accumulation. However indirect effects cannot be excluded: increase in HA content in sediment was reported to decreased Cd bioavailability and uptake in *Elodea nuttallii* (Wang et al., 2010).

Cd translocation to stems and leaves was apparently not affected by humic substances nor by Cd concentration in root tissues, with a translocation factor (TF) lower than 0.1 in all treatments. These data are in agreement with common mangrove behavior, that is blocking and accumulate trace elements in root system (MacFarlane et al., 2007) as demonstrated in other lab trials (González-Mendoza et al., 2007). On the other side, although not significantly different, TF in plants treated with Cd + 4 mM HS is six times the value of plants treated only with Cd, confirming the attitude of humic substances in enhancing Cd translocation from roots to shoots (Pinto et al., 2004).

5. Conclusions

Our results suggest that, for *Avicennia* spp., there is an optimal dose to maximize root length and area (4 mM C) and it exerts the same effect in both very early stage of root development and in an already established root system. There is a different optimal dose to maximize root cortex development (8 mM) but it seems to be effective only in presence of an abiotic stress like heavy metals. Furthermore, differently from that happens in other mangrove species, *Avicennia germinans* roots seems not to enhance the Casparian strip and epidermis lignification and suberification neither in presence of pollutants nor under humic substances treatment. This demonstrates that organic matter/humic substances seems to have a specific role in those anatomical changes, likely triggered by heavy metals pollution.

Humic matters extracted from mangrove sediments show an effective biostimulant activity, enhancing root growth and root area, even in the presence of Cd pollution. Although mangrove sediments might already contain high concentration of natural organic matter, purified HS could be added at controlled concentration in seedlings or adult plants rhizosphere area to exploit this improved plant growth and induce specific morphological changes, such as root cortex development.

Furthermore, in case of re-planting in mangrove forests degraded areas, humic substances pre-treatment helps a quicker and higher roots development that favorite the mud establishment. The present data can be useful in any program of degraded mangrove forest restauration and/or ex-situ phytoremediation of dredged sediments, where HS can be added depending on the sediment volume, its characteristics, and heavy metals concentration.

Finally, the different effects of HS on root system depending on their concentrations might allow to design different strategies to achieve different targets: I) phytoextraction, favoring root length and area development in mangrove species, like *Avicennia* spp., able to translocate metals more efficiently than other mangroves species. II) phytostabilization favoring root aerenchyma and cortex development to block metals in roots tissues and/or changing rizosphere chemical properties trough oxygen supply.

In any case, a pilot scale experiment, possibly coupled with genes expression analysis, is recommended to confirm these conclusions.

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