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1 **Effect of biochar and redmud amendment combinations on *Salix triandra***
2 **growth, metal(loid) accumulation and oxidative stress response**

3

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16

17 Abstract

18 Remediation of metal(loid) polluted soils is an important area of research nowadays. In particular, one
19 remediation technique is much studied, phytomanagement. Phytomanagement combines amendment
20 application and plant growth in order to reduce the risk posed by contaminants. *Salicaceae* plants
21 showed tolerance towards metal(loid)s and the ability to accumulate high amounts of metal(loid)s in
22 their tissue. Amendments are often applied to counterbalance the reduced soil fertility and high
23 metal(loid) concentrations. Two amendments gathered attention over the last decades, biochar
24 (product of biomass pyrolysis), which can be activated for better effects, and redmud (by-product of
25 alumina production). Those two amendments showed ability to improve soil conditions and thus plant
26 growth, although few studied their combined application. Moreover, since metal(loid)s are known to
27 induce the overproduction of reactive oxygen species, it is important to measure the level of oxidative
28 stress in the plant, to which plants respond using enzymatic and non-enzymatic systems. But no
29 studies evaluate the response of *Salicaceae* plants to metal(loid) stress and amendment application at
30 the biochemical level in a real soil condition. Therefore, a mesocosm study was set up to evaluate the
31 effect of amending a mine soil with redmud combined to diverse biochars on the soil properties and
32 *Salix triandra* growth, metal(loid) accumulation and stress marker levels. Results showed that all
33 amendment combinations improved the soil fertility, reduced metal(loid) mobility and thus
34 ameliorated *Salix triandra* growth, which accumulated metal(loid)s mainly in its roots. Moreover,
35 among the different amendment combinations, *Salix triandra* plants still suffered from oxidative stress
36 when grown on PG soil amended with redmud and chemical activated carbon, showing elevated levels

37 of phenolic compounds and salicinoids and important antioxidant and enzymatic activities. Finally,
38 one treatment showed levels of these stress markers similar or lower than the control, the combination
39 of redmud with steam activated carbon. In conclusion, this treatment seemed a good solution in a
40 phytomanagement strategy using *Salix triandra*, improving soil conditions and plant growth and
41 reducing oxidative stress level in the plant roots.

42

43 Keywords

44 Activated carbon; biochar; metal(loid)s; *Salix triandra*; oxidative stress markers

45

46 Introduction

47 Soil is a geochemical sink for contamination (Kabata-Pendias 2011) and thus, the development of
48 anthropogenic activities, *i.e.* mining, industry, fertilizer and pesticide uses in agriculture, transport...
49 (Panagos et al. 2013, Vamerali et al. 2010), led to an important contamination of the soils worldwide
50 (Panagos et al. 2013). Particularly, metal(loid)s, encountered in more than 50 % of the contaminated
51 soils (Khalid et al. 2016), are of great concern, due to their non-degradability compared to organic
52 pollutants, but also due to their negative effects on the environment and human health. Therefore, the
53 necessity to remediate such contaminated soils has become a priority (Van Ginneken et al. 2007).

54 Among possible remediation techniques, phytomanagement gained attention these last decades over
55 the conventional physical and chemical methods. The goal of phytomanagement is to reduce
56 metal(loid) mobility and thus the risk posed by such pollutants (Dominguez et al. 2008). To
57 accomplish this, phytomanagement involves the manipulation of the soil-plant system and combines
58 plant establishment and amendment application (Tack and Meers 2010). Plant establishment will
59 reduce wind erosion and water leaching risk, thus diminishing spreading of contamination. Moreover,
60 plants will take up contaminants and store them in their roots (phytostabilization) and their aerial parts
61 (phytoextraction).

62 As described in the literature, *Salicaceae* species showed a good potential for phytostabilization, often
63 chosen in the case of elevated and deep contamination, in terms of metal(loid) tolerance (Kuzovkina et
64 al. 2004, Ruttens et al. 2011), growth potential on contaminated soil (Bart et al. 2016, Lebrun et al.
65 2018, Vervaeke et al. 2003) and metal(loid) accumulation (Hartley et al. 2011, Lebrun et al. 2019,
66 Migeon et al. 2009).

67 However, contaminated soils are often characterized by a poor agronomic value (extreme pH, low
68 organic matter and nutrient contents, high metal(loid) concentrations); therefore, amendments often
69 must be applied. Among amendments, biochar has received particular attention in recent years.
70 Biochar is obtained through the pyrolysis of biomass, mostly of vegetal and manure origins, under low
71 oxygen conditions (Wiszniewska et al. 2016). It is characterized by an alkaline pH, a high surface
72 area, a porous structure, a high cation exchange capacity and the presence of many oxygen containing
73 functional groups at its surface (Cantrell et al. 2012, Paz-Ferreiro et al. 2014, Singh et al. 2010). All

74 these properties make biochar a good amendment for metal(loid) contaminated soils that will increase
75 pH, nutrient content and availability, but also and more importantly reduce metal(loid) bioavailability
76 through its sorption capacity (Lima et al. 2018, Meng et al. 2018, Trakal et al. 2017). Such
77 improvements of the soil conditions lead to a better plant growth, demonstrated in many previous
78 studies for diverse plant species: ryegrass (Trakal et al. 2017), maize (Uzoma et al. 2011), tomato
79 (Akthar et al. 2014), and willow (Lebrun et al. 2017, 2018, 2019). Moreover, biochar can also undergo
80 “activation”, *i.e.* a modification of its surface using steam or chemical activations, to further increase
81 its beneficial effects on soil and plants. Such post-activation product is called “activated carbon”.
82 However, biochar showed good potential mainly for cation metal(loid) contaminated soils but it was
83 revealed inefficient or even negative for anions like arsenic (Beesley et al. 2010, 2014). On the
84 contrary, redmud, a by-product of alumina production (Hua et al. 2017), is rich in iron and aluminum
85 oxides and hydroxides that can interact with arsenic and other metal(loid)s (Bertocchi et al. 2006).
86 Redmud is also characterized by a very alkaline pH and a highly corrosive property (Liu et al. 2011).
87 Redmud application to soil can thus increase soil pH and immobilize metal(loid)s (Gautam and
88 Agrawal 2017, Lee et al. 2011), improving consequently plant growth (Gautam and Agrawal 2017,
89 Gray et al. 2006, Castaldi et al. 2009).
90 Furthermore, in addition to hinder plant growth, metal(loid)s also induce an oxidative stress in plants,
91 through the overproduction of reactive oxygen species (ROS) (Ishtiyag et al. 2018). In response to
92 such elevated ROS content, plants can activate their antioxidant system, composed of both enzyme
93 and non-enzyme elements. For instance, flavonoids and phenolic compounds generally increase under
94 stress as they participate in the scavenging of ROS (Jaskulak et al. 2018, Sakihama et al. 2002).
95 Phenolic compounds can also chelate metal(loid)s (Dresler et al. 2017). Finally, enzymes such as
96 superoxide dismutase and peroxidase can scavenge ROS and thus decrease oxidative damage (Wang et
97 al. 2008). The assessment of these different stress markers can thus give an indication of the stress
98 level the plant is under.

99 Although both biochar and redmud have been much studied for their effects on soil properties and
100 plant growth, few studies assessed the effect of their combined application on such parameters.
101 Furthermore, to the best of our knowledge, no studies evaluated the effect of biochar and redmud
102 amendment on *Salix* oxidative stress. Therefore, the goals of this study were to evaluate the effects of
103 amending a former mine technosol contaminated by As and Pb with redmud associated to diverse
104 biochars on: (i) the soil physico-chemical properties, (ii) *Salix triandra* growth and metal(loid)
105 accumulation and (iii) *Salix triandra* oxidative stress status.

106

107 Materials and Methods

108 1. Studied site and amendments

109 This experiment focused on a mine technosol, resulting from the silver-lead extraction on the
110 Pontgibaud mine district (Région Auvergne-Rhone-Alpes, France). All extraction activities stopped in

111 the middle of the nineteenth century but the intense activity generated an important amount of wastes
112 highly contaminated by arsenic ($539 \text{ mg}\cdot\text{kg}^{-1}$) and lead ($11,453 \text{ mg}\cdot\text{kg}^{-1}$) (Cottard 2010). Soil was
113 sampled at one of the four parts of the Pontgibaud mine district: Roure-les-Rosiers.

114 Five amendments were used in this study: a bamboo based biochar (BA) (La Carbonerie), a biochar
115 obtained from bark and sapwood of oak (BS2) (La Carbonerie), a wood activated carbon (steam
116 activation) (EK5) (Jacobi Carbons), a wood activated carbon (chemical activation) (L27) (Jacobi
117 Carbons) and a commercial redmud modified to be less alkaline (R) (Alteo Environment). The
118 amendments were characterized for their pH, electrical conductivity, redox potential, as described in
119 Lebrun et al. (2019) using a multimeter (Mettler-Toledo, Serveur Excellence) and results are presented
120 Table S1.

121

122 2. Substrates preparation

123 In total, six substrates were prepared. The first one was a control (Ctr) prepared by mixing garden soil
124 with perlite (ratio 4:1); the second treatment was the non-amended Pontgibaud technosol (PG); the
125 third substrate was PG amended with 1 % R and 2 % BA (RBA); the fourth one was PG amended with
126 1 % R and 2 % BS2 (RBS2); the fifth treatment was composed of PG amended with 1 % R and 2 %
127 EK5 (REK5) and the sixth one was PG amended with 1 % R and 2 % L27 (RL27). All amendments
128 were added on a w/w basis. The doses at which amendments were applied were chosen based on
129 previous studies (Friesl et al. 2003, Lebrun et al. 2018, Nejad and Jung 2017). Four pots containing 1.5
130 kg of substrate were prepared for each treatment.

131

132 3. Plant growth and physiological analysis

133 After mixture preparation, one non-rooted cutting of *Salix triandra* was placed in each pot, making
134 four plant replicates. The cuttings were 20 cm long and obtained on the same tree clone, from 1 year
135 old branches. After buds break, one stem was left to develop and plants were grown for 41 days (16 h
136 of light / 8 h of darkness, $25 \text{ }^{\circ}\text{C}$ / $21 \text{ }^{\circ}\text{C}$ with a photon flux of approximately $800 \text{ mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). At the
137 end of the growing period, plants were harvested and subjected to diverse treatments. Leaves were
138 removed, numbered and scanned in order to determine total leaf area of each plant using Image J
139 software. In addition, the average leaf surface was calculated. Stem lengths were measured. Roots
140 were washed twice with tap water and once with distilled water. One root ramification was sampled
141 for each plant, immediately frozen and stored at $-80 \text{ }^{\circ}\text{C}$ until further analysis. The other part of the root
142 as well as leaves and stems were dried at $60 \text{ }^{\circ}\text{C}$ for 72 h to determine dry weight (DW). Finally, the
143 dried biomass was subjected to acid digestion and ICP-AES analysis (Inductively Coupled Plasma
144 Atomic Emission Spectroscopy; ULTIMA 2, HORIBA, Labcompare, San Francisco, USA) to measure
145 As and Pb concentrations, as described in Bart et al. (2016).

146

147 4. Soil pore water (SPW) sampling and analysis

148 SPWs were sampled at the end of the growing period, just before plant harvest, in each pot using soil
149 moisture samplers (Rhizon®) (model MOM, Rhizosphere Research Produces, Wageningen, The
150 Netherlands) as described in Lebrun et al. (2017). SPW samples were used directly to measure pH,
151 electrical conductivity (EC) and redox potential (Mettler-Toledo, Serveur Excellence). They were then
152 acidified with a few drops of concentrated nitric acid (65%) and analyzed for As and Pb
153 concentrations, using ICP-AES.

154

155 5. Plant non-enzymatic oxidative stress markers

156 The frozen material was used to determine oxidative stress markers. First, total proanthocyanidin and
157 phenolic contents, free radical scavenging and chelating capacity were measured, using the following
158 protocols.

159 *Extraction procedure.* Root extracts were obtained by grinding 3 to 30 mg of lyophilized material in
160 $1 \cdot 10^{-3}$ L of 50 % ethanol (v/v) (HPLC grade, Thermo) followed by an ultra-sonication (60 min, 50 °C,
161 400 W, 45 kHz) (ultrasonic bath USC1200TH, Prolabo). Extracts were then centrifuged 10 min at
162 maximum speed (14 000 x g) and supernatant was recovered. The rest of the root biomass was
163 lyophilized for further analysis.

164 *Quantification of total phenolic content (TPC).* TPC was determined by the Folin-Ciocalteu method.
165 The reagent was prepared by mixing $25 \cdot 10^{-3}$ L of Na_2CO_3 (4 %), $250 \cdot 10^{-6}$ L CuSO_4 (2 %) and $250 \cdot 10^{-6}$
166 L tartrate sodium potassium (2 %). $190 \cdot 10^{-6}$ L of this reagent were mixed with $10 \cdot 10^{-6}$ L of root
167 extract and absorbance at 735 nm was measured after 10 min at room temperature. Gallic acid (Sigma)
168 was used for standard calibration curve and TPC was expressed as milligrams gallic acid equivalent
169 per gram.

170 *Quantification of total proanthocyanidin content.* Total proanthocyanidin content was determined by
171 the aluminum chloride colorimetric method described in Lopez-Contreras et al. (2015).

172 *Determination of free radical scavenging capacity.* Free radical scavenging capacity was evaluated by
173 the DPPH method, assessing the scavenging capacity through hydrogen atom transfer, and the
174 CUPRAC method, evaluating the scavenging capacity through electron transfer. The DPPH method
175 was described by Lopez-Contreras et al. (2015). Briefly, $10 \cdot 10^{-6}$ L of root extract was mixed with
176 $190 \cdot 10^{-6}$ L of DPPH solution ($60 \cdot 10^{-3}$ M prepared in EtOH) and the absorbance at 630 nm was read
177 after 10 min incubation at room temperature. For the CUPRAC method, a solution containing Cu(II)
178 ($10 \cdot 10^{-3}$ M), neocuproine ($7.5 \cdot 10^{-3}$ M) and ammonium acetate buffer (1 M, pH 7) was prepared by
179 adding each component at the same volume. Following, $190 \cdot 10^{-6}$ L of this solution were mixed with
180 $10 \cdot 10^{-6}$ L of root extract and absorbance at 450 nm was read after 10 min incubation at room
181 temperature.

182 *Chelation capacity.* The chelation capacity of the root extracts was determined by the method of Dinis
183 et al. (1994) using ferrous ions. For this, a solution containing FeCl_3 and ferrozine was prepared and
184 $190 \cdot 10^{-6}$ L of this solution were mixed with $10 \cdot 10^{-6}$ L of root extract. After 10 min incubation at room

185 temperature, absorbance at 490 nm was measured. Chelation capacity was calculated as $[(A_0 - A_s)/A_s]$
186 $\times 100$, where A_0 was the absorbance of the control and A_s the absorbance of the extract.

187

188 6. *Salix triandra* root salicinoid contents

189 Salicinoids were quantified by HPLC using HPLC-grade solvents (Sigma Aldrich). Lyophilized
190 material (100 mg) from each sample was homogenized in $500 \cdot 10^{-6}$ L of 75% (v/v) aqueous ethanol
191 using ultra-turrax (T25, Ika) set at 8,000 rpm for 30 seconds and then sonoextracted during 60 minutes
192 with the help an ultrasonic bath USC1200TH (Prolabo) set at an operating frequency of 45 kHz and an
193 extraction temperatures of 50°C. The characteristics of the US bath are: inner dimension of 300×240
194 $\times 200$ mm, electrical power of 400W (*i.e.*, acoustic power of $1W \cdot cm^{-2}$), maximal heating power of
195 400W, variable frequencies, equipped with a digital timer, a frequency and a temperature controller.
196 Following extraction, the extract was centrifuged during 15 min at 3,000 rpm and the supernatant was
197 filtered (0.45 μm ; Merck Millipore) before HPLC analysis. HPLC separation was performed on a
198 Zorbax SB C18-column (Agilent Technology) at 35 °C with a Varian a HPLC system (Agilent
199 Technology) composed of Varian Prostar 230 pump Meta chem Degasit, Varian Prostar 410
200 autosampler and Varian Prostar 335 Photodiode Array Detector (PAD) and driven by Galaxie version
201 1.9.3.2 software (Agilent Technology, Les Ulis, France). Separation was performed using the binary
202 gradient of methanol and water (with 2% tetrahydrofuran; $20 \cdot 10^{-6}$ L $\cdot min^{-1}$) as described by Rubert-
203 Nason et al. (2014). Detection of compounds for quantification was realized DAD (set at 274 nm).
204 Quantification was done based on retention time compare to authentic standards (Sigma Aldrich).
205 Examination of each sample was realized three times.

206

207 7. *Salix triandra* root cell wall analysis

208 The lyophilized root biomass was subjected to Fourier-Transformed Infra-Red analysis using a
209 Nicolet iS10 (Thermo Scientific) (Plateforme des Techniques Analytiques, ICOA, France) in order to
210 assess qualitatively and semi-quantitatively the cell wall components.

211 After experimental analysis, data were normalized using the band at 1670 cm^{-1} (Hano et al. 2006).
212 Next, bands characteristics were measured. For the lignin, two bands were used, the one at 1328 cm^{-1}
213 corresponds to the stretching of the bonds of the syringyl groups present on the aromatic nuclei and is
214 characteristic of the subunit S of the lignin. The band at 1234 cm^{-1} corresponds to stretching the bonds
215 of the guaiacyles groups present on the aromatic nuclei and is characteristic of the subunit G of the
216 lignin (Bykov 2008). Similarly, two bands were used for the cellulose, the band at 897 cm^{-1}
217 corresponds to the stretching of the bonds C-O-C, characteristic of the presence of amorphous
218 cellulose. The band at 1375 cm^{-1} corresponds to the stretching of the bonds C-H and to the vibration of
219 the bonds COO, characteristic of the presence of crystalline cellulose. The ratio of these two bands
220 indicates the crystallinity of the cellulose (Kavkler et al. 2011). Two bands were used for the
221 hemicellulose: the band at 1078 cm^{-1} corresponds to the xyloglucanes and the band at 1089 cm^{-1}

222 corresponds to the xylanes (Scheller and Uluskov 2010). Finally, the band at 1610 cm^{-1} was used for
223 the pectin (Wróbel-Kwiatkowska et al. 2009).

224

225 8. Antioxidant enzyme activities in *Salix triandra* roots

226 *Extraction procedure.* 0.1 g of fresh root biomass was mixed with $1\cdot 10^{-3}$ L of phosphate buffer ($50\cdot 10^{-3}$
227 M, pH 7) containing 1 % polyvinylpyrrolidone (PVP) and $140\cdot 10^{-3}$ M β -mercapto-ethanol, and
228 crushed in a frozen mortar. The solution was then centrifuged ($14\ 000\times g$, 10 min) and the supernatant
229 recovered and stored at $-20\text{ }^{\circ}\text{C}$ until further analysis.

230 *Glutathione-S-transferase (GST).* GST activity was measured following the protocol of Mauch and
231 Dudler (1993). Briefly, $30\cdot 10^{-6}$ L of root extract were mixed in a microplate with a volume of CNDB,
232 reduced GSH and buffer solutions corresponding to $1\cdot 10^{-6}$ mole of CNDB, $1\cdot 10^{-6}$ mole of reduced
233 GSH and $100\cdot 10^{-6}$ mole of buffer. Absorbance was read at 340 nm. Activity was calculated using ϵ_{340}
234 $= 9.6\text{ M}^{-3}\cdot\text{cm}^{-1}$.

235 *Peroxidase (POD).* POD activity was measured following the protocol described in Khan et al. (2019).
236 Twenty μL of root extract were mixed with $40\cdot 10^{-6}$ L buffer, $100\cdot 10^{-6}$ L dH_2O , $20\cdot 10^{-6}$ L guaiacol
237 ($100\cdot 10^{-3}$ M) and $20\cdot 10^{-6}$ L H_2O_2 (10 vol.). Absorbance was measured at 470 nm and activity
238 calculated using $\epsilon_{470} = 26.6\text{ M}^{-3}\cdot\text{cm}^{-1}$.

239 *Superoxide dismutase (SOD).* Similarly, SOD activity was measured based on the protocol of Khan et
240 al. (2019). A volume of $30\cdot 10^{-6}$ L extract were mixed with $39\cdot 10^{-6}$ L buffer, $10\cdot 10^{-6}$ L EDTA ($1\cdot 10^{-3}$
241 M), $1\cdot 10^{-6}$ L riboflavin ($0.02\cdot 10^{-3}$ M), $10\cdot 10^{-6}$ L methionine ($130\cdot 10^{-3}$ M) and $20\cdot 10^{-6}$ L NBT ($0.75\cdot 10^{-3}$
242 M). Absorbance was read at 660 nm and SOD activity was calculated using $\epsilon_{660} = 43.6\text{ M}^{-3}\cdot\text{cm}^{-1}$.

243 *Protein quantification.* In order to normalize enzymatic activity values, protein content was quantified
244 using the Bradford method, using BSA as standard.

245

246 9. Statistical analysis

247 Data were analyzed using R software version 3.5.1 (R Development Core Team, 2009). After
248 evaluation of the homogeneity (Shapiro test) and homoscedasticity (Bartlett/Levene tests) of the data,
249 means were compared using Anova (parametric data) or Kruskal (non-parametric data) test, followed
250 by a Tukey post-hoc test. Difference was considered significant when $p < 0.05$.

251 Moreover, a principal component analysis was performed on the plant parameters using the PAST
252 software (Hammer et al. 2001).

253

254 Results

255 1. Amendment characteristics

256 All amendments presented as alkaline pH, except for L27 (chemically activated carbon) that was very
257 acid (Table S1). Similarly, all the amendments were characterized by a high electrical conductivity
258 except for one. Amendment BS2 had an EC of $162\ \mu\text{S}\cdot\text{cm}^{-1}$, while the other amendments were

259 characterized by an EC between $1004 \mu\text{S}\cdot\text{cm}^{-1}$ (redmud) and $2629 \mu\text{S}\cdot\text{cm}^{-1}$ (bamboo biochar) (Table
260 S1). Finally, except for the amendment EK5 that had a negative redox potential (-7 mV) (Table S1),
261 all the other amendments had a positive redox potential, between 83 mV (bamboo biochar) and 525
262 mV (chemically activated carbon) (Table 1).

263

264 2. SPW physico-chemical properties

265 SPWs were sampled at the end of the experiment and analyzed for pH, EC, redox potential and As and
266 Pb concentrations.

267 In the non-contaminated control soil, pH was neutral at 7.1 (Table 1), while the contaminated PG soil
268 was acidic at pH 4.5. Moreover, all amendments significantly increased SPW pH at a similar level
269 than the control.

270 Similarly, EC of the control soil was $1087 \mu\text{S}\cdot\text{cm}^{-1}$ and PG soil had a twice lower EC ($536 \mu\text{S}\cdot\text{cm}^{-1}$)
271 (Table 1) which was significantly increased by all amendments, to levels three times higher than the
272 control and six times higher than PG, on average.

273 On the contrary, redox potential was lower in control (326 mV) than PG (441 mV) and significantly
274 decreased with amendment application compared to PG (Table 1).

275 SPW As concentration was low in all substrates and amended conditions did not differ from control
276 and PG (Table 1) but in RL27 was significantly higher than in RBS2 and REK5.

277 Finally, SPW Pb concentration was high on PG ($13.73 \text{ mg}\cdot\text{L}^{-1}$) and significantly decreased with all
278 amendments, from 82 % to 96 % (Table 1).

279

280 3. *Salix triandra* growth parameters

281 Stem height was low on the non-amended soil PG, corresponding to 7.9 cm and for all amended
282 conditions *Salix triandra* presented a significantly higher stem height, between 25.1 and 30.8 cm
283 (Table S2). No significant difference was observed between amended treatments; however on REK5
284 and RL27, plant stem height was not significantly different than on control.

285 On the control substrate, plants produced 63 leaves on average, much more than on PG (13 leaves).
286 Compared to PG, only RBS2 treatment significantly increased plant leaf number (Table S2).

287 Similarly, leaf area was seven times lower on PG compared to control, such difference was significant.
288 Amendment application significantly increased leaf area compared to PG, to levels that were still
289 significantly lower than the control (Table S2). When looking at the average leaf area, a different trend
290 was observed. Average leaf area was significantly lower on PG (2.43 cm^2) compared to the control
291 (3.84 cm^2) (Table S2). However, only the treatment REK5 had a significant higher average leaf area
292 compared to PG, which was similar to the control (Table S2).

293 Finally, DW production was low on PG, with 17 mg roots, 195 mg leaves and 49 mg stem, which was
294 98 %, 87 % and 95 % lower than the DW produced on the control, respectively (Fig. 1). All
295 amendments significantly and similarly increased organ DW by three fold for leaves, seven fold for

296 stem and 15 fold for roots, on average. However, DW production was still 30 to 50 % lower than the
297 control (Fig. 1).

298

299 4. *Salix triandra* metal(loid) accumulation

300 *Salix triandra* plants accumulated elevated As and Pb concentrations on PG (Fig. 2), with higher
301 concentration in the roots compared to the aerial tissues.

302 Regarding As, all amendments significantly decreased organ As concentrations, except RL27 in leaves
303 and roots. Moreover, As aerial concentration was similar to control in all amended conditions.

304 Regarding Pb, only root concentrations were significantly decreased by amendments.

305

306 5. *Salix triandra* root stress markers

307 Total proanthocyanidin content was lowest on PG and only the condition RBA had a significant higher
308 proanthocyanidin level compared to PG (Fig. 3A).

309 On the contrary, total phenolic content was high on PG compared to the control and a significantly
310 lower content was observed with all amendments, until level similar to the control (Fig. 3B).

311 Antioxidant activity, determined by the CUPRAC and DPPH tests, was high on PG compared to the
312 control and the addition of the amendments significantly lowered antioxidant activity measured at the
313 end of the experiment, compared to PG. The antioxidant activity by electron transfer was significantly
314 lower than PG but at a same level than the control in all cases (Fig. 3C) whereas the antioxidant
315 activity by hydrogen atom transfer was similar than control level for all the amended conditions except
316 RL27 treatment that presented an antioxidant activity higher than control (Fig. 3D). Finally, the
317 antioxidant activity by electron transfer was higher than by hydrogen atom transfer (Fig. 3C and 3D).

318 Additionally, root chelation capacity was assessed and revealed that plants grown on control and PG
319 soils had a similar chelation capacity and only RL27 treatment presented a significantly lower
320 chelation capacity compared to PG (Fig. S1).

321 Finally, three enzyme activities were evaluated: glutathione-S-transferase (GST), peroxidase (POD)
322 and superoxide dismutase (SOD). GST activity did not differentiate between PG and the amended
323 conditions, only RL27 condition presented a significantly higher GST activity than the control
324 condition. A similar pattern was observed for POD, whereas SOD activity did not show any different
325 between treatments (Fig. 4).

326

327 6. *Salix triandra* root salicinoid contents

328 Root extracts were analyzed to measure their contents in salicinoids. The HPLC analysis revealed
329 seven molecules: arbutin, salicin, salicinoside, salicortin, 2'-O-acetylsalicortin, tremuloidin and
330 tremulacin (Fig. S2). All these salicinoids presented a similar trend: a higher content in PG compared
331 to the control, and a lower content with all amendments compared to PG (Fig. 5). In more detail,
332 arbutin content was similar in RBA, RBS2 and RL27, whereas its content in REK5 was significantly

333 lower and similar to the control (Fig. 5A). Salicin content was the lowest on REK5 while RBS2 and
334 RL27 treatments presented significantly higher contents than REK5 (Fig. 5B). Similarly, salidroside
335 content was the lowest in REK5, and significantly lower than in the control and other objects (Fig.
336 5C). Salicartin and 2'-O-acetylsalicortin contents followed the same variations than arbutin (Fig. 5D
337 and 5E). Finally, tremuloidin and tremulacin contents presented similar variations: lowest contents in
338 control and REK5, followed by RBA, then RBS2 and RL27 and finally PG (Fig. 5F and 5G).
339 Globally, control and REK5 conditions presented the lowest salicinoid contents and PG the highest,
340 whereas RBA, RBS2 and RL27 presented similar intermediary contents. Finally, salicinoids were
341 found in different quantities, in the decreasing order: salicin, salidroside, salicortin, 2'-O-
342 acetylsalicortin, arbutin, tremulodin and tremulacin.

343

344 7. *Salix triandra* root cell wall content

345 The total lignin content of the cell wall tended to be higher when plant were grown on the
346 contaminated substrates compared to the control, although it was significant only in the case of RBS2
347 and REK5 treatments (Table S3). In addition, when considering the two lignin types, only the content
348 in lignin S significantly increased in the treatments PG, RBA and RBS2 compared to the control;
349 whereas the content in lignin subunit G increased only in the condition REK5 compared to the control
350 (Table S3). Finally, the ratio lignin G/lignin S did not show variation compared to the control;
351 however this ratio was higher in the REK5 treatment compared to RBA (Table S3).

352 The two cellulose forms, amorphous and crystalline, were not affected by the different treatments
353 compared to the control (Table S3). However, the content in crystalline cellulose was significantly
354 higher in REK5 compared to RBA. Finally, the crystallinity of the cellulose did not show variation
355 between the treatments (Table S3).

356 Similarly to the cellulose content, the content in hemicellulose was not affected by the treatments
357 compared to the control; however the contents in xyloglucanes (XylG) and xylanes (XylA) were again
358 significantly superior in REK5 compared to RBA (Table S3). Finally the ratio XylG/XylA was not
359 affected by the treatments (Table S3).

360 Similarly to the hemicellulose, the content in pectin did not show differences among the treatments
361 (Table S3).

362

363 8. Correlation analysis of the plant parameters

364 Principal component analysis was applied on plant parameters in order to discriminate treatments. The
365 resulting biplot showed that 99.94 % of the variability was explained by F1 axis whereas F2 axis only
366 explained 0.05 % of the variability (Fig. 6). Moreover, the biplot representation showed that three
367 groups could be formed along the F1 axis, which was mainly constrained by Pb root concentrations,
368 and to a lesser extent As root concentrations and Pb leaf concentrations (Fig. S3): PG and control
369 treatments were located at the two extremities, whereas the third group was composed of the amended

370 conditions, located more closely to the control condition. From the second axis (F2), constrained by
371 root As concentrations and to a lesser extent leaf surface area, number of leaves, Pb concentrations in
372 root and stem, GST and POD activities (Fig. S4), three groups could be made among the amended
373 treatments: REK was located below the axis, RL27 at the top, whereas RBA and RBS2 conditions
374 could be grouped together and were located between the other two treatments.

375

376 Discussion

377 1. SPW physico-chemical properties

378 Soil pH is an important parameter to assess as it affects many processes in soil and especially
379 metal(loid) behavior but also nutrient availability. Previous studies showed the potential of biochar
380 and redmud to increase pH of an acidic soil (Lebrun et al. 2017, 2019, Nandillon et al. 2019a, Zhou et
381 al. 2017), mostly explained by their alkalinity (Dai et al. 2018, Moore et al. 2017, Zhou et al. 2017).
382 Indeed, except amendment L27 that was very acidic (pH 1.2) (Table 1), all amendments used were
383 alkaline between pH 8.2 and pH 12.6 (Table 1). Moreover, even though L27 was very acidic, its
384 application together with redmud still led to a SPW pH increase, which was similar to the treatment
385 combining redmud (pH 8.6) and BS2 (pH 8.1). Therefore, redmud seems to be efficient to increase
386 soil pH and counteract L27 acidity.

387 Similarly, SPW EC was increased with all amendments, which is consistent with previous studies and
388 could be related to the high EC of the amendment used (Table 1) (Garau et al. 2014, Lebrun et al.
389 2019, Lee et al. 2009, 2014). However, SPW EC values of the amended substrates were, in most
390 cases, higher than amendment EC values. Thus the increase of SPW EC can be explained by the
391 dissolution of soil and amendments organic matter and other soluble salts into SPW which happened
392 during the entire experiment time course through the interaction between the soil and the amendment
393 (Lebrun et al. 2017, Nandillon et al. 2019b).

394 Contrary to pH and EC, SPW redox potential (Eh) decreased following amendments, which can be
395 related to the low redox potential of most amendments, especially EK5. Moreover, soil Eh is known to
396 behave oppositely to pH, as demonstrated by the highly significant negative correlation between SPW
397 pH and Eh (correlation coefficient $r = -0.99$, $p < 0.001$) (Rinklebe et al. 2016).

398 In previous studies, biochar amendment applied alone to metal(loid)s polluted soils showed generally
399 negative effects on SPW As concentration, *i.e* increase of As mobility, although some studies also
400 showed that biochar had no effect or had a positive effect, *i.e* decrease in SPW As concentration
401 (Beesley et al. 2010, 2014, Lebrun et al. 2017, Nandillon et al. 2019a). Altundogan et al. (2000) and
402 Garau et al. (2011) showed that redmud had an affinity toward As and thus can immobilize it.
403 Therefore, the non-effect of amendment application on SPW As concentration observed here could be
404 due to a compensation of the negative effect of biochar by the positive effect of redmud, leading to a
405 neutralized effect, or to a non-effect of the two amendments. Moreover, As is known to be mobilized
406 with increasing pH, which could have happened here. However, such mobilized As could have been

407 directly sorbed by redmud. Finally, even though SPW As concentrations were not modified, the
408 application of amendment could have modified As speciation, rendering it less toxic.
409 Finally, SPW Pb concentrations were shown greatly decreased by amendments. Indeed, both biochar
410 and redmud can sorb positively charged elements due to their compositions. Biochar surface is
411 negatively charged which allows electrostatic attraction with positively charged ions (Ahmad et al.
412 2016) whereas redmud contains many iron and aluminum oxides than can sequester metal(loid)s
413 (Zhou et al. 2017). However, here the association redmud + biochar did not lead to a better Pb
414 immobilization than the one observed in previous studies with biochar and iron grit (Lebrun et al.
415 2018, 2019). Therefore, other than the sorption on amendment surface, Pb immobilization can be
416 explained by the pH increase induced by amendment, explanation supported by the highly significant
417 negative correlation between SPW pH and SPW Pb concentration ($r = -0.91$, $p < 0.001$). Indeed, the
418 soil pH increase promotes the sorption of metal(loid)s on soil colloids as well as the formation of
419 metal(loid) carbonates and hydroxide precipitates, leading to their immobilization and thus decrease
420 concentration in SPW (Ahmad et al. 2016, Dai et al. 2018, Zhou et al. 2017).

421

422 2. *Salix triandra* growth

423 Compared to the control condition, *Salix triandra* growth parameters were highly decreased on PG,
424 which is one of the negative effects of metal(loid)s (Ali et al. 2006, Fernandez et al. 2013, Chaoui et
425 al. 1997). Additionally, *Salix triandra* growth could have been impaired by the low fertility of the soil,
426 *i.e.* low nutrient availability, low organic matter content and acidic pH, as shown in previous studies
427 (Lebrun et al. 2017, 2018, 2019). Compared to another study, *Salix triandra* presented a higher
428 impairment of leaf DW than *Salix alba* (80 %), *Salix viminalis* (70 %) and *Salix purpurea* (68 %) but a
429 similar decrease of stem and root DW (Lebrun et al. 2017).

430 Amendment application to PG soil increased all *Salix triandra* growth parameters except leaf number,
431 which could be directly related to the amelioration of the soil conditions. Indeed, many studies
432 observed an improvement of plant growth with the amelioration of soil conditions induced by
433 amendment application (Agegnehu et al. 2016, Clemente et al. 2019, Fresno et al. 2017, Mehmood et
434 al. 2018, Zhang et al. 2019).

435

436 3. *Salix triandra* As and Pb accumulation

437 Arsenic and lead plant content were decreased in the amended conditions compared to PG, which
438 could be related to the immobilization in the soil observed following amendment application or a
439 modification of their speciation, which reduced their uptake. Moreover, a lower concentration could
440 also be due to a dilution effect, as organ DWs were higher in the amended treatments.

441 Finally, As and Pb were mainly accumulated in the roots with a low translocation towards upper parts,
442 which is often observed in *Salix* plants (Bart et al. 2016, Lebrun et al. 2017, 2019) and underlines the

443 “trap” function of the roots to protect photosynthetic organs (Drzewiecka et al. 2012) but also the
444 ability of roots to not only absorb metal(loid)s but also absorb them on their surface.

445

446 4. *Salix triandra* biochemical profiles at the end of the experiment

447 Exposure to elevated concentrations of metal(loid)s is known to induce an oxidative stress through the
448 overproduction of ROS (Ahmad et al. 2009, Demirevska-Kepova et al. 2004, Kovacik et al. 2009). In
449 response to such oxidative stress, plants activate their antioxidative system composed of non-
450 enzymatic and enzymatic elements.

451 Total phenolic compounds (TPC) content is a highly sensitive stress marker that generally increases in
452 response to stress (Jaskulak et al. 2018). Indeed, plants submitted to oxidative stress promote phenolic
453 production that participates in the scavenging of ROS (Kovacik and Klejduš 2008). The high level of
454 TPC in plants grown on non-amended PG soil reflected the important oxidative stress encountered by
455 these plants. On the contrary, TPCs in plants grown on amended PG were low and similar to the
456 control, revealing that plants did not suffer from oxidative stress when grown on amended PG.
457 Comforting this fact, antioxidant activity was increased in PG compared to control, showing that an
458 important free radical scavenging activity occurred in roots of *Salix triandra* grown on PG (Ali et al.
459 2006). Moreover, by comparing the two tests used to assess antioxidant activity, CUPRAC and DPPH,
460 it can be seen that the scavenging activity occurred mainly through electron transfer even in non-
461 stressed conditions. Furthermore, even though amendments decreased oxidative stress, antioxidant
462 activity through hydrogen atom transfer was higher in RL27 compared to the other amended
463 treatments, showing a slightly higher oxidative stress in this condition, which can be related to the
464 lower chelation capacity of the roots in such condition.

465 Moreover, in response to stress, plants can also activate enzymes that will detoxify free radicals (Bai et
466 al. 2009). Enzyme activities differed depending on treatment and enzyme type. However, in general,
467 PG plants showed an elevated GST and SOD activities whereas RL27 plants presented high activities
468 of GST and POD. The other amended treatments showed similar activities to the control. Elevated
469 enzyme activity is a marker of enhanced ROS production (Goswami and Das 2016) and related
470 scavenging. Indeed, GST, POD and POD have a role in metal(loid) and ROS detoxification (Tamas et
471 al. 2008). SOD is a metalloprotein catalyzing the dismutation of superoxide to H₂O₂ (Goswami and
472 Das 2016) and its elevated level might protect plants from oxidative damage (Gao et al. 2010, Wang et
473 al. 2008). Similarly, POD is one of the principal enzymes involved in the elimination of ROS
474 (Goswami and Das 2016). These first results showed that on Pontgibaud soil, plants greatly suffered
475 from oxidative stress. This stress was suppressed by the addition of amendment combinations, except
476 for one treatment (RL27) that only reduced oxidative stress but in which oxidative stress was still
477 higher than the control condition.

478 Salicinoid contents were also greatly increased when grown on the contaminated PG soil. Such
479 observation was commonly observed when plants are under stress. Indeed, although their study did not

480 focus on the response to metal(loid) stress, previous studies showed that *Salicaceae* species increased
481 the synthesis of secondary metabolites, such as salicin, arbutin and other phenolic glycosides, when
482 exposed to water, Ag and herbivory stresses (Boeckler et al. 2011, Cheynier et al. 2013, Popovic et al.
483 2016, Zhang et al. 2018). Such observations underlined the importance of salicinoid compounds in the
484 defense towards the high metal(loid) (As and Pb) concentrations encountered on Pontgibaud.
485 Moreover, such elevated salicinoid contents were reduced when amendments were added, especially
486 for one treatment (REK5) that presented similar or lower levels of salicinoids than in control. These
487 results again testified that adding amendments reduced the stress plants were under, which could be
488 related to the reduced acidity and metal(loid) mobility induced by amendments.

489 Finally, the content of the cell wall of *Salix triandra* root did not show great modification in response
490 to metal(loid) stress and amendment application. The most important response was an increase in
491 lignin, especially in the case of the amended conditions. Lignin has the effect to enhance the rigidity of
492 the cell wall and is an important barrier against plant stress. Indeed, the stress induced by biotic or
493 abiotic factors to the plant is often accompanied by an increase in ROS content but also in lignin
494 content (Liu et al. 2018). Lignin contains an elevated number of functional groups that can bind
495 metal(loid)s and thus prevent their entry in the cytoplasm and thus their translocation towards upper
496 parts (Liu et al. 2018). Therefore, the increase in lignin observed under the contaminated treatments
497 can be a direct response of the presence of metal(loid)s in the soil and their entry into the roots.

498

499 Conclusion

500 A mesocosm study was set up in order to evaluate the effect of diverse amendment combinations on
501 soil properties and *Salix triandra* growth, metal(loid) accumulation and oxidative stress level, and thus
502 their potential in phytomanagement.

503 The results showed that on Pontgibaud soil, plants greatly suffered from stress, as shown by their
504 reduced growth and high stress markers. All the combinations of redmud with carbon-based material
505 improved soil conditions, by reducing soil acidity and immobilizing Pb. However, none of the
506 treatments were able to immobilize As. The combination redmud + bamboo biochar was the one
507 showing both higher soil acidity reduction and Pb immobilization, compared to the other treatments.
508 Such ameliorations led to a better plant growth. In general, plant growth and metal(loid) accumulation
509 patterns did not discriminate amended conditions. However, biochemical analysis of the root material
510 showed that among the diverse treatments, plants grown on RL27 amendment still presented high
511 enzymatic and non-enzymatic anti-oxidative compounds, whereas in the other conditions, stress
512 marker levels were reduced. Moreover, salicinoid contents were the lowest with REK5 amendments.
513 Finally, when taking all of plant parameters together, REK5 seems to be the best amendment, showing
514 clearly a better growth than on PG and stress marker levels similar or lower than on control.

515 In conclusion, in a phytomanagement strategy, the combination of neutralized redmud associated to
516 stream activated carbon could be applied on Pontgibaud soil. This combination will reduce soil acidity

517 and immobilize Pb, and thus ameliorate *Salix triandra* growth. Moreover, it will reduce, and almost
518 erase, the oxidative stress the plant suffers on the contaminated soil, probably by reducing the
519 generation of reactive oxygen species and thus decreasing the need to activate the antioxidative
520 system, and thus the energy cost of the plant.

521

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525

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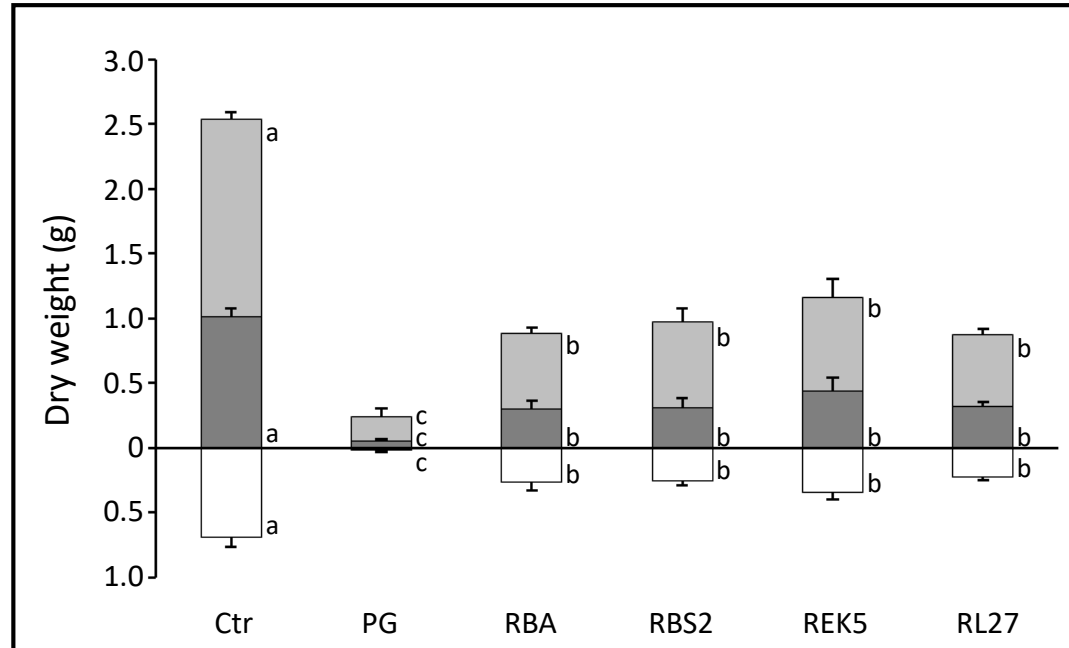


Fig. 1: Leaf (light grey), stem (dark grey) and root (white) dry weight (g) of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Different letters indicate significant difference ($p < 0.05$) ($n = 4$).

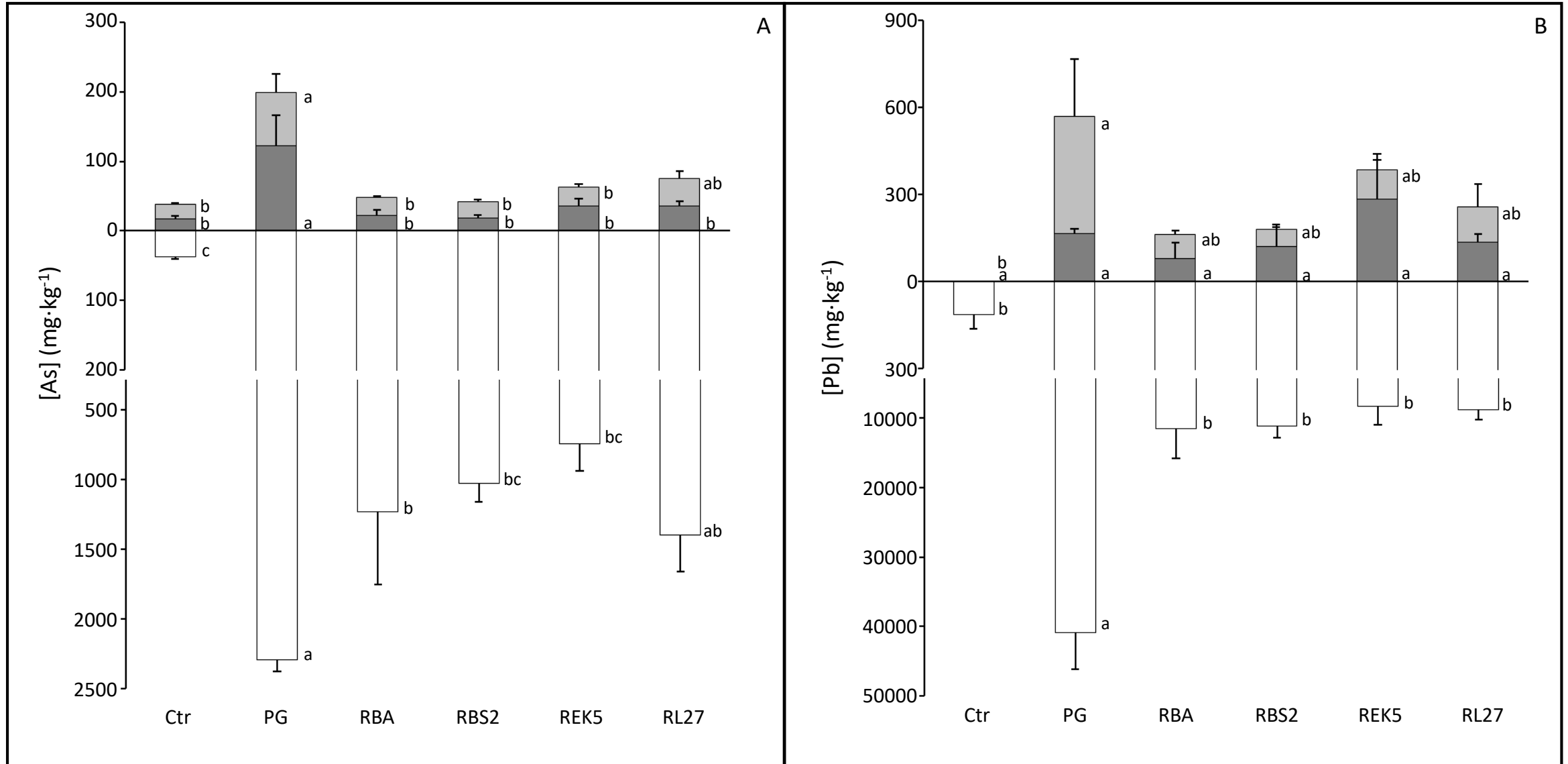


Fig. 2: Leaf (light grey), stem (dark grey) and root (white) As (A) and Pb (B) concentrations (mg·kg⁻¹) of *Salix triandra* plant grown for 41 days on the different substrates. Ctrl = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Different letters indicate significant difference ($p < 0.05$) ($n = 4$).

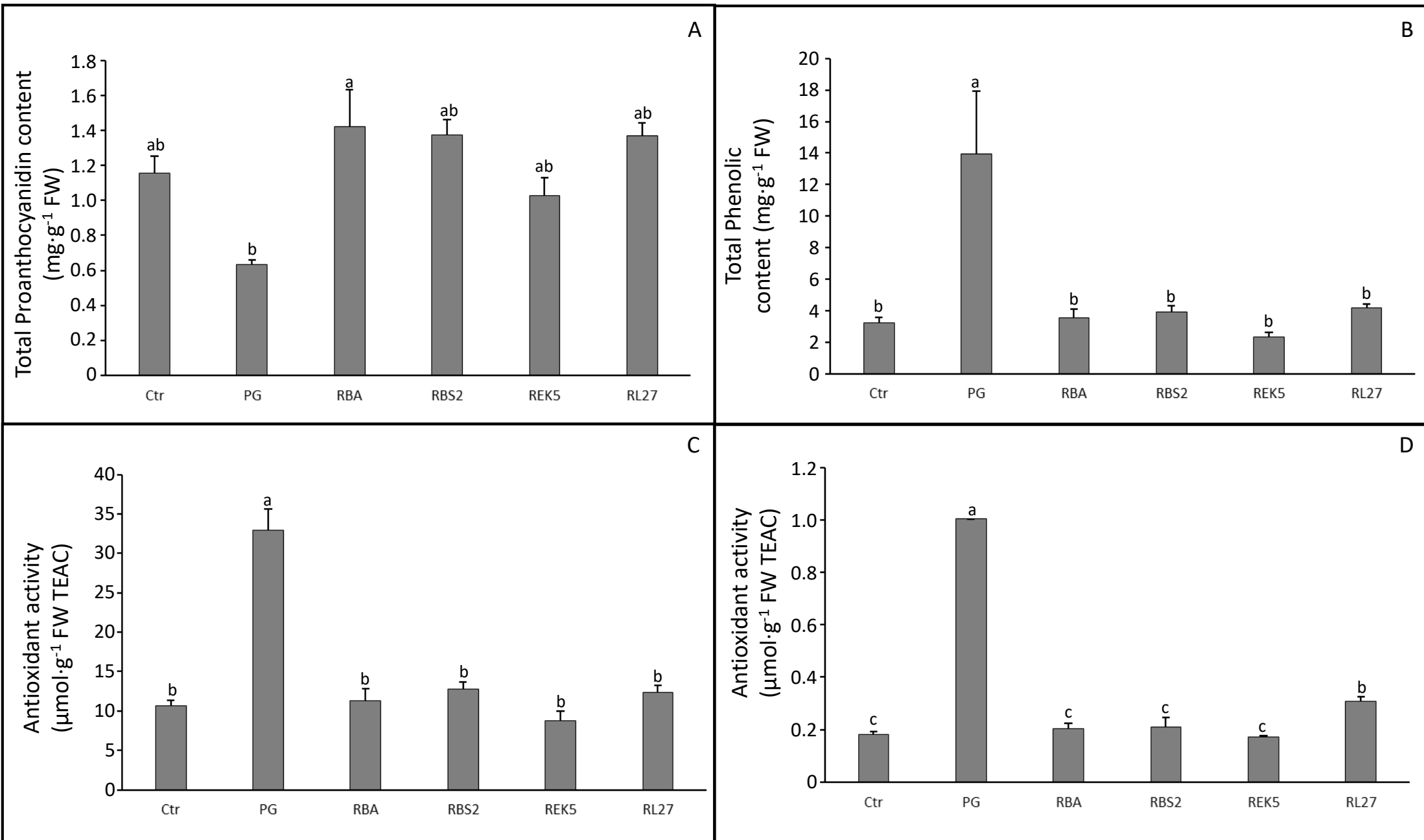
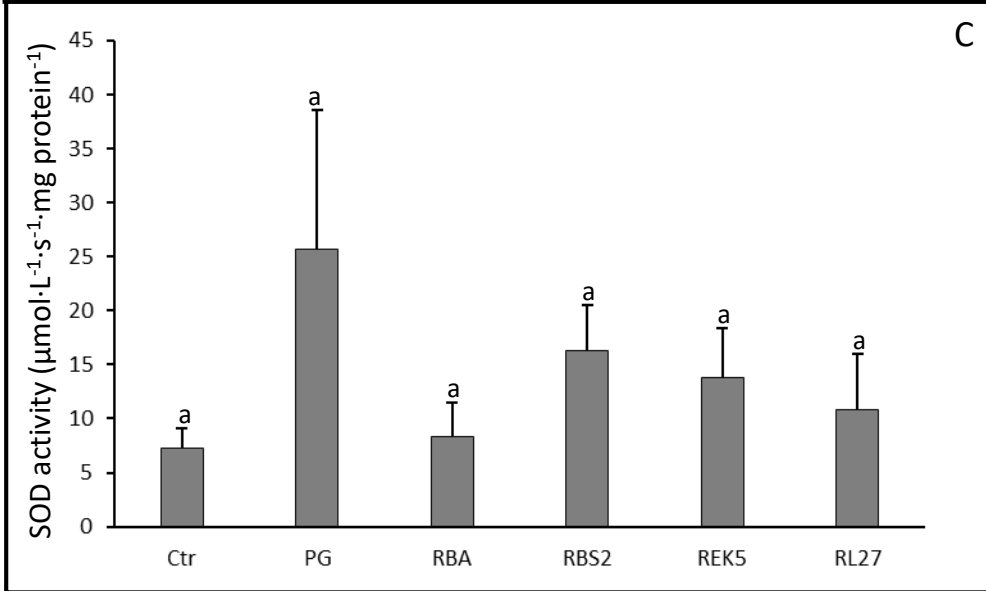
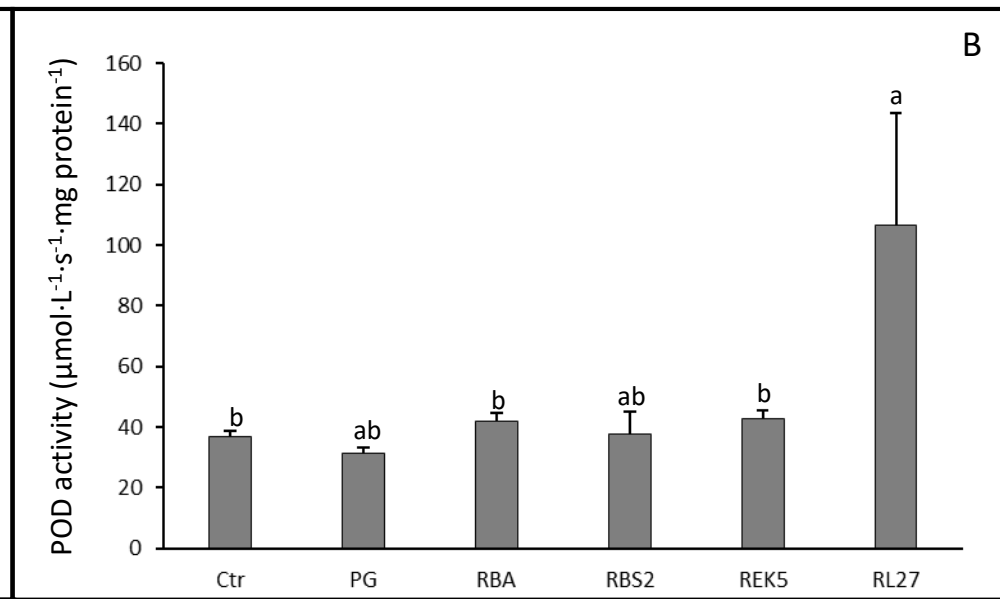
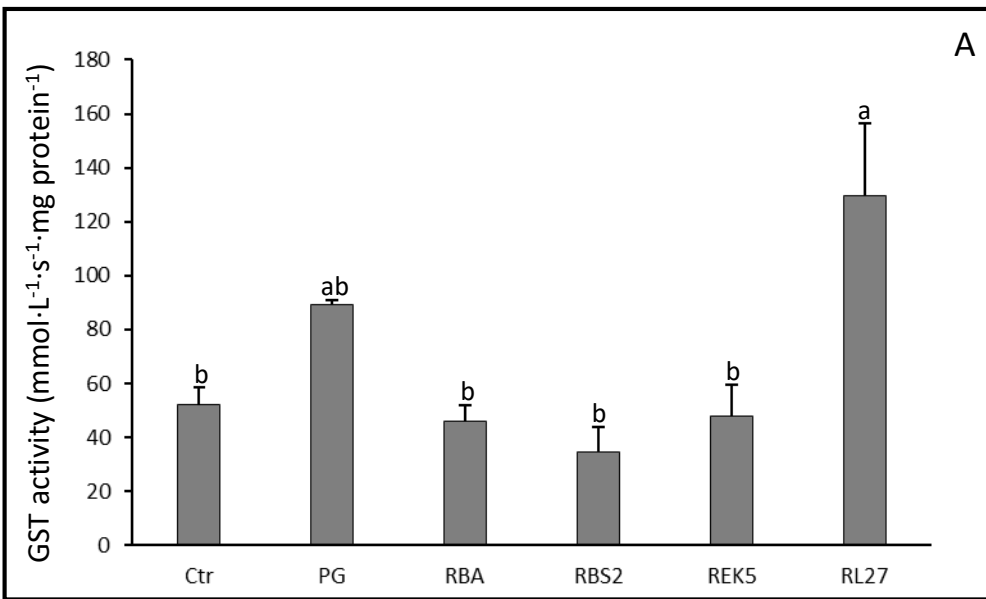


Fig. 3: Root total proanthocyanidin (A) and phenolic contents (B) (mg·g⁻¹ FW) and root antioxidant activity (μmol·g⁻¹ FW TEAC) in terms of electron transfer (CUPRAC test) (C) and hydrogen atom transfer (DPPH test) (D) of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Different letters indicate significant difference ($p < 0.05$) ($n = 4$).



C Fig. 4: Enzyme activities (glutathione-S-transferase (GST) ($\text{mmol}\cdot\text{L}^{-1}\cdot\text{s}^{-1}\cdot\text{mg protein}^{-1}$) (A), peroxidase (POD) ($\mu\text{mol}\cdot\text{L}^{-1}\cdot\text{s}^{-1}\cdot\text{mg protein}^{-1}$) (B) and superoxide dismutase (SOD) ($\mu\text{mol}\cdot\text{L}^{-1}\cdot\text{s}^{-1}\cdot\text{mg protein}^{-1}$) (C)) in roots of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Different letters indicate significant difference ($p < 0.05$) ($n = 4$).

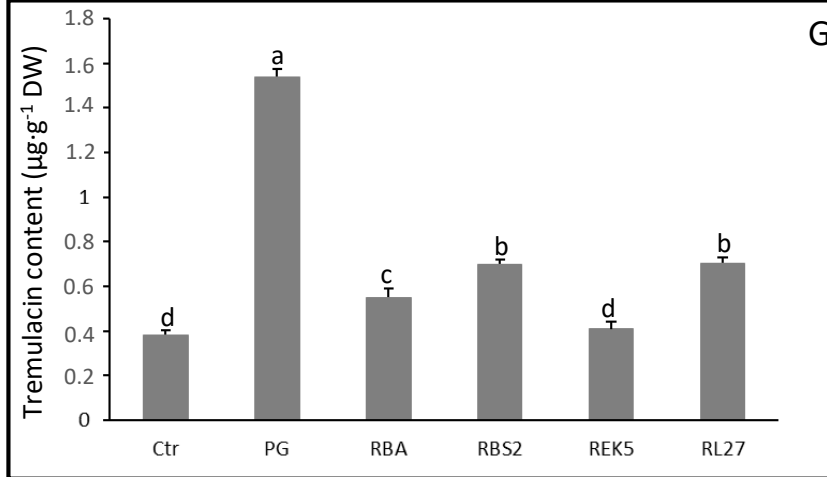
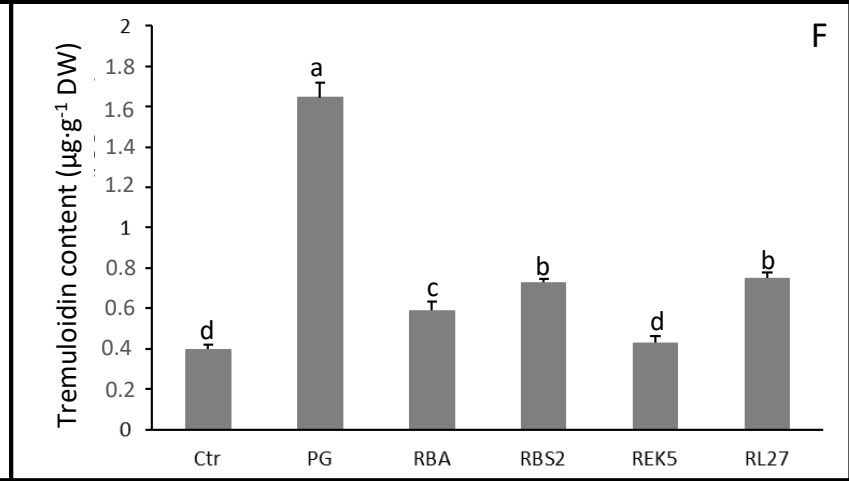
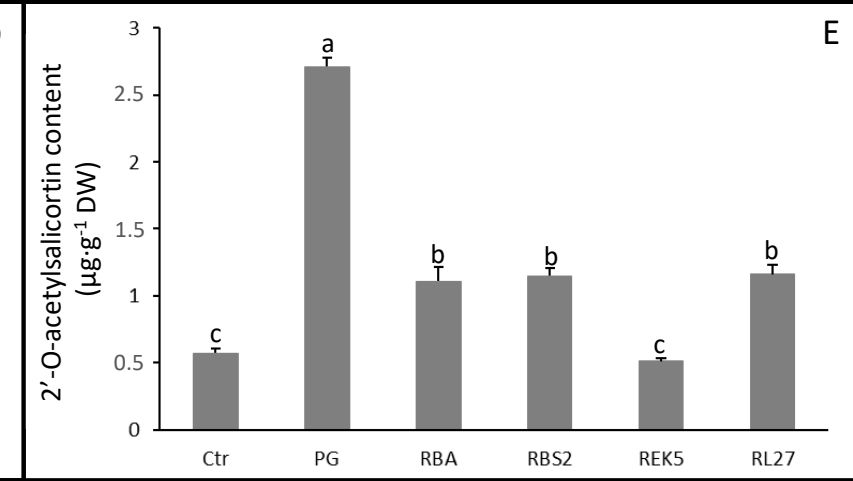
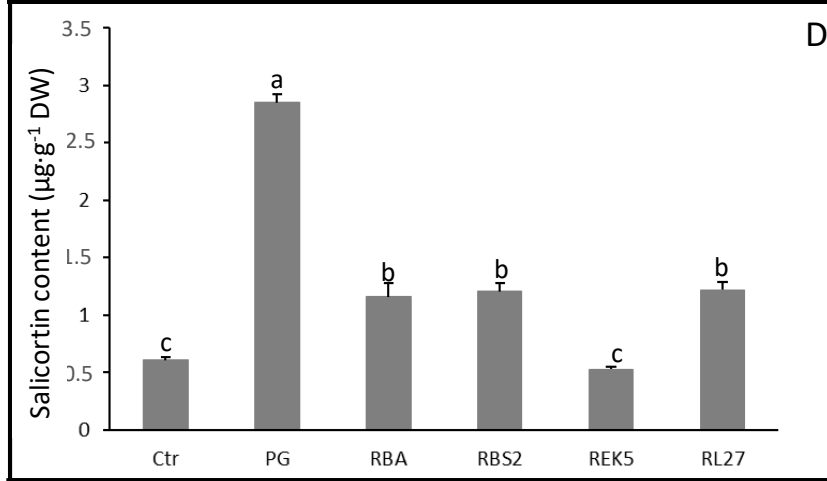
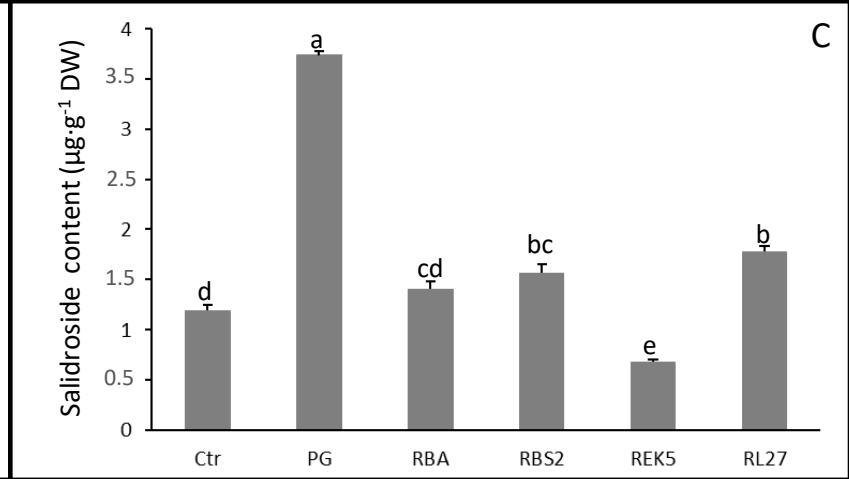
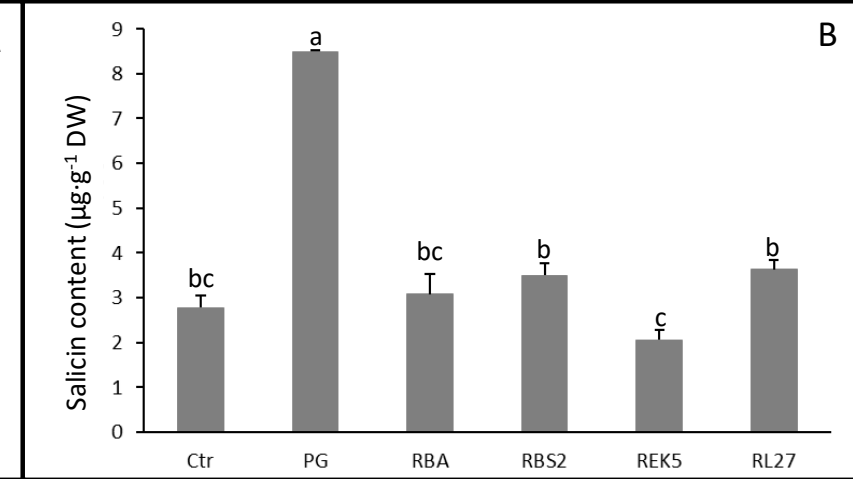
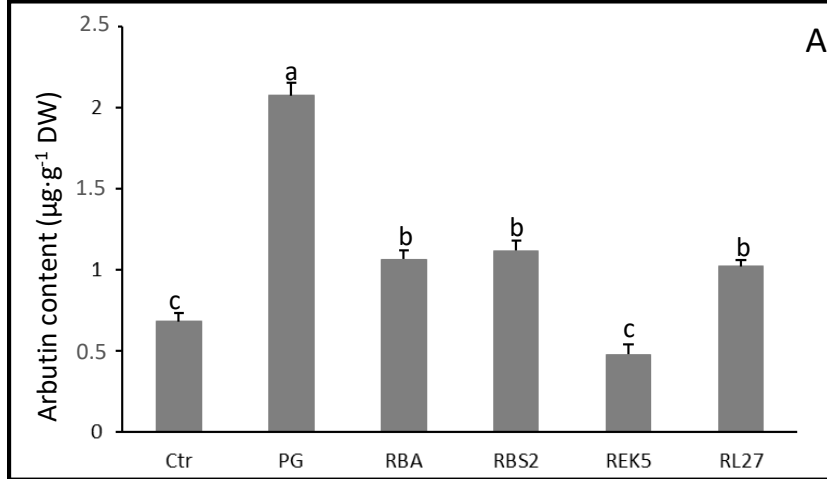


Fig. 5: Salicinoid (arbutin (A), salicin (B), salidroside (C), salicortin (D), 2' oxo-acetylsalicortin (E), tremuloidin (F), tremulacin (G)) contents ($\mu\text{g}\cdot\text{g}^{-1}\text{ DW}$ (dry weight)) in roots of *Salix triandra* plant grown for 41 days on the different substrates. Ctrl = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Different letters indicate significant difference ($p < 0.05$) ($n = 4$).

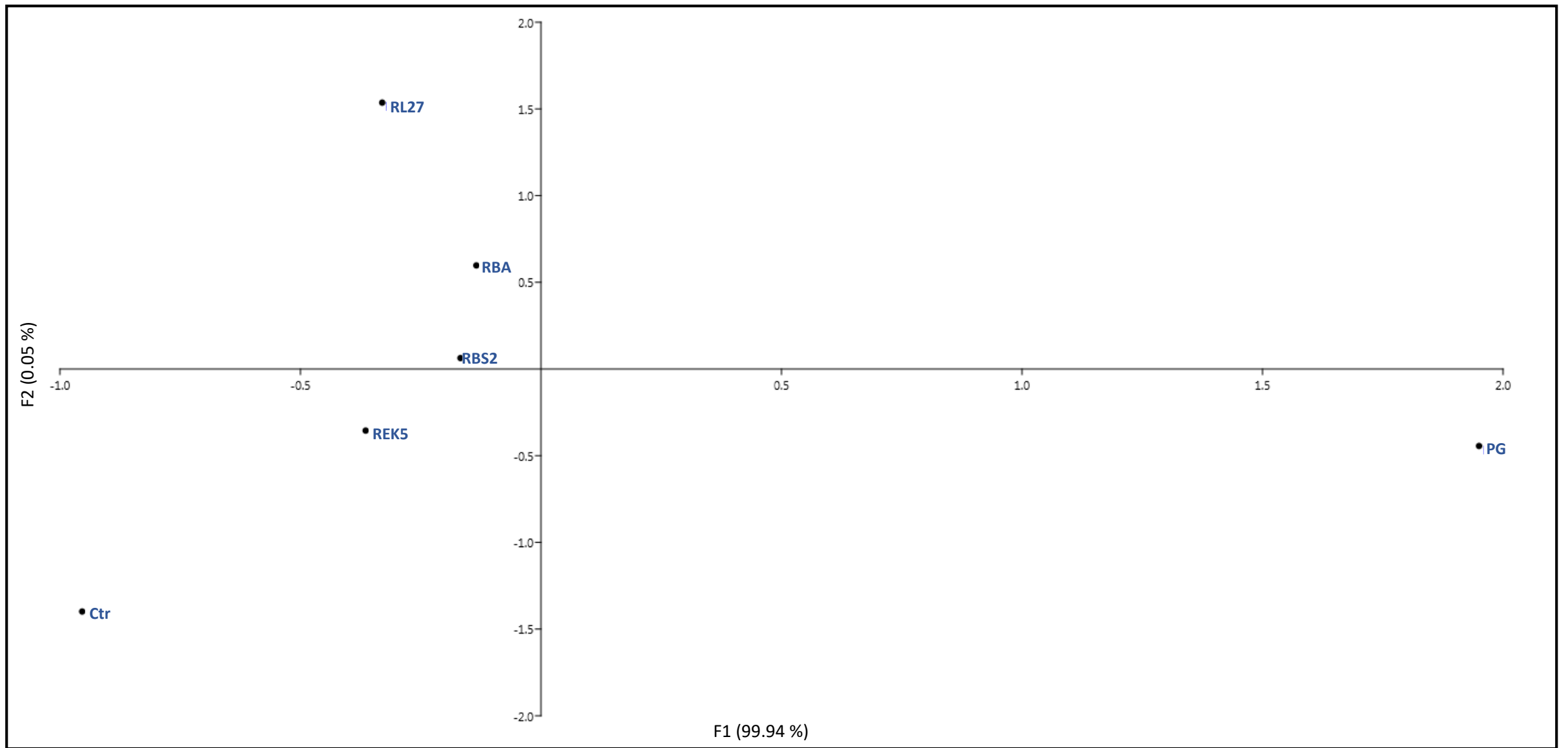


Fig. 6: Principal component analysis of the parameters measured in *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon.

Table 1: Soil pore water physico-chemical properties (pH, electrical conductivity (EC) ($\mu\text{S}\cdot\text{cm}^{-1}$), redox potential (mV), As and Pb concentrations ($\text{mg}\cdot\text{L}^{-1}$)) determined after 41 days of *Salix triandra* growth on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Different letters indicate significant difference ($p < 0.05$) ($n = 4$).

	pH	EC ($\mu\text{S}\cdot\text{cm}^{-1}$)	Redox potential (mV)	[As] ($\text{mg}\cdot\text{L}^{-1}$)	[Pb] ($\text{mg}\cdot\text{L}^{-1}$)
Ctr	7.1 \pm 0.1 ab	1087 \pm 96 b	326 \pm 6 bc	0.14 \pm 0.01 ab	0.15 \pm 0.02 c
PG	4.5 \pm 0.2 c	536 \pm 29 c	441 \pm 10 a	0.12 \pm 0.00 ab	13.73 \pm 0.83 a
RBA	7.1 \pm 0.2 ab	3785 \pm 386 a	329 \pm 5 bc	0.12 \pm 0.01 ab	1.50 \pm 0.20 bc
RBS2	6.7 \pm 0.0 a	3746 \pm 452 a	347 \pm 2 b	0.11 \pm 0.00 b	1.79 \pm 0.12 b
REK5	7.4 \pm 0.1 b	3359 \pm 235 a	315 \pm 3 c	0.11 \pm 0.00 b	2.43 \pm 0.25 b
RL27	6.7 \pm 0.2 a	2675 \pm 334 a	344 \pm 7 b	0.16 \pm 0.02 a	1.08 \pm 0.12 bc