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1 **Biological Denitrification Inhibition (BDI) in the field: a**  
2 **strategy to improve plant nutrition and growth**

3

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17

18 **Keywords:** biological denitrification inhibition, field experiment, nitrate, denitrifiers, lettuce,  
19 rhizosphere, plant traits, productivity.

20

21 **ABSTRACT**

22 Nitrogen is one of the factors limiting in plant growth, is naturally present in soils, and is  
23 mainly assimilated as nitrate and ammonium by plants. However, soil nitrate is also used by  
24 denitrifying bacteria, which reduce it to  $N_2O$  (a greenhouse gas) and  $N_2$ . Therefore, plants  
25 are in direct competition with these bacteria for the assimilation of nitrate. Recently, our  
26 research team has highlighted a strategy developed by some plants consisting of the  
27 production of secondary metabolites (procyanidins) that inhibit the denitrification activity of  
28 microbial communities in soils, referred to as BDI for biological denitrification inhibition  
29 (BDI). This strategy could make nitrate more available in the soil, which may then be used by  
30 plants for their growth. However, the extent to which procyanidins can affect plant growth  
31 and nutrition *via* BDI under field conditions has not yet been investigated. In this study, we  
32 tested the effect of procyanidins exogenously applied in the field on the nutrition and  
33 growth of cos or romaine lettuce crops (*Lactuca sativa*) nutrition and growth. Procyanidins  
34 were added to growing lettuce at 8, 42, 83 and 210 kg ha<sup>-1</sup>. Soil denitrification enzyme  
35 activity (DEA), nitrate concentration, above- and below-ground lettuce traits and the  
36 abundance of total bacteria and denitrifiers were measured in lettuces treated or untreated  
37 with procyanidins. Our results showed that the addition of procyanidins in the field at 210 kg  
38 ha<sup>-1</sup> resulted in: (1) the inhibition of microbial denitrification activity and counter-selection of  
39 denitrifiers in the root-adhering soil of lettuce and (2) an increase in available nitrate and a  
40 significant gain in plant productivity. This study allowed us to propose for the short term the  
41 development of a more environmentally friendly method of sustainable agriculture by  
42 limiting fertilizer inputs, nitrogen losses from the soil, and greenhouse gas emissions while  
43 increasing plant growth and productivity.

44                   **1. INTRODUCTION**

45 Nitrogen is one of the factors limiting plant growth (LeBauer and Treseder, 2008). It is the  
46 most important nutrient used to enhance agricultural yields. Thus, the development of  
47 plants depends on the processes linked to the nitrogen (N) cycle, which orchestrates the  
48 transformation of nitrogen into all its forms including ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ),  
49 the most plant-assimilable forms of nitrogen. The competition between plant roots and  
50 microorganisms for these two N forms is intense (Kuzyakov and Xu, 2013). Microorganisms  
51 often out-compete plant roots, but some plant species are able to bypass this competition  
52 by taking control of the N in their rhizosphere through the production of secondary  
53 metabolites that inhibit N cycle processes (Knops et al., 2002; Chapman et al., 2006;  
54 Subbarao et al., 2013; Bardon et al., 2016). For example, *Brachiaria humidicola*, by exuding  
55 some secondary metabolites (brachialactone), inhibits the nitrification process (Subbarao et  
56 al., 2009).

57 Nitrification is responsible for the conversion of ammonium to nitrate, which is rapidly  
58 converted into  $\text{N}_2\text{O}$  (a greenhouse gas) by denitrification and/or leached in soils (Di and  
59 Cameron, 2002). Denitrification, mostly carried out by bacteria, is the main form of nitrogen  
60 loss in most soils ( Van der Salm et al., 2007; Radersma and Smit, 2011). Denitrification leads  
61 first to nitrate reduction, which is associated with two homologous enzymes, namely,  
62 transmembrane nitrate reductase (Nar) and periplasmic nitrate reductase (Nap), which are  
63 encoded by the *narG* and *napA* genes, respectively (the latter is not present in all  
64 denitrifiers). Depending on the microorganisms, nitrite reduction to nitric oxide (NO)  
65 involves two nitrite reductases, one coupled with copper (encoded by *nirK*) and the other  
66 coupled with cytochrome cd1 (encoded by *nirS*). The transformation of NO into  $\text{N}_2\text{O}$   
67 (dinitrogen monoxide) involves NO reductase, which is a transmembrane enzyme encoded

68 by the *norB* gene (Wallenstein et al., 2006). Finally, N<sub>2</sub>O reductase, which transforms N<sub>2</sub>O  
69 into dinitrogen (N<sub>2</sub>), is a periplasmic enzyme encoded by the *nosZ* gene (Khalil, 2003).  
70 However, this last step leading to the emission of N<sub>2</sub>, which can be fixed by some plants, is  
71 not present in all denitrifiers (Galloway et al., 2004). In European agro-ecosystems, losses  
72 due to N<sub>2</sub>O emissions account for 59% of the N loss of the system (Oenema et al., 2009).  
73 Recently, Bardon et al. (2014) demonstrated that the invasive species *Fallopia spp.* can  
74 inhibit denitrification activity in soils through the release of procyanidins, a phenomenon  
75 termed biological denitrification inhibition (BDI). Procyanidins specifically inhibit membrane-  
76 bound NO<sub>3</sub> reductase, inducing enzymatic conformational changes through membrane  
77 disturbance (Bardon et al., 2016). This strategy leads to a reduction in N<sub>2</sub>O emissions from  
78 soil of up to 95% (Bardon et al., 2014). In addition, it has been shown that the addition of  
79 procyanidins to the soil under experimental conditions in mesocosms leads to a 6-fold  
80 increase in the amount of nitrate compared to untreated soil, which reduces denitrification  
81 activity without affecting either the respiration or mineralization activities of soil microbial  
82 communities (Bardon et al., 2016). This strategy could make nitrate more available in the  
83 soil, which may then be used by plants for their growth.  
84 So far, the extent to which procyanidins can affect plant growth and nutrition *via* BDI under  
85 field conditions has not been investigated. Our hypothesis is that the addition of  
86 procyanidins to cultivated soils under field conditions induces BDI, with an increase in  
87 available nitrate and therefore a gain in productivity. To test our hypothesis, field  
88 experiments were carried out on cos or romaine lettuce crops (*Lactuca sativa*) at the SERAIL  
89 experimental station (Brindas, 69126 Rhône, France). Different concentrations of  
90 procyanidins were tested in the lettuce fields: 0, 8, 42, 83 and 210 kg ha<sup>-1</sup>. Soil denitrification  
91 enzyme activity (DEA), nitrate concentration, above- and below-ground lettuce traits (shoot

92 and root fresh masses, specific leaf area, biomechanical traits of leaves, anthocyanin and  
93 flavonoid contents of leaves and N content of tissues) and the abundance of all bacteria and  
94 denitrifiers were measured in lettuces treated or untreated with procyanidins.

95

## 96 **2. MATERIALS AND METHODS**

97

### 98 *2.1. Plant growth and experimental design*

99 A 202 m<sup>2</sup> field (composed of 24 plots of 1.4 m x 6 m (8.4 m<sup>2</sup>)) located at the SERAIL  
100 experimental station (Brindas, 69126 Rhône, France) (45°43'46.4"N 4°43'37.1"E) was used  
101 for growing romaine lettuces (*Lactuca sativa* var Lotus RZ), following a Fisher system of 4  
102 plots per treatment (Preece, 1990). Lettuce seedlings were planted in three rows spaced  
103 0.45 m apart and each lettuce spaced 0.45 m apart, for a total of 39 lettuces per plot (156  
104 lettuces per treatment). Six treatments were considered: unplanted soil, soil with lettuce  
105 without procyanidin addition, and 4 lettuce crops treated with procyanidins at 8, 42, 83 and  
106 210 kg ha<sup>-1</sup>. These concentrations are based on those used in our previous studies *in vitro*  
107 (Bardon *et al.*, 2014, 2016). Firstly, we considered the dry weight of soil in the field at 10 cm  
108 of depth corresponding to lettuce seedling roots implantation. Secondly, we measured a  
109 surface relative to this weight, and then, we estimated the weight of 10 cm of soil per  
110 hectare. Finally, we calculated how much kg ha<sup>-1</sup> of procyanidins must be added to the plots  
111 to obtain the equivalent of 0.01, 0.05, 0.1 and 0.25 mg of procyanidins per gram of soil.  
112 The entire experiment was watered the first week after planting with 3 mm of water every  
113 day. For the next 10 days, 8 mm of watering was done per day. From then until the end of  
114 the experiment, 12 mm of watering was done every two days. Procyanidins were added 2  
115 weeks after planting (stage 7-9 leaves) on a soil whose nitrate had been brought up to the

116 ZENIT grid standard, i.e. 45 kg-NO<sub>3</sub><sup>-</sup> ha<sup>-1</sup> (Despujols, 1997). The commercial procyanidins  
117 (Laffort TANIN VR GRAPE<sup>®</sup>, Bordeaux, France) were applied in aqueous solution (standard  
118 water) by 2 nozzle spray booms, such as to give 500 L/ha or 0.42 L per plot, between the  
119 rows of lettuce, and the soil was then hoed. The site was watered (with 8 mm) just after the  
120 addition of procyanidins.

121

## 122 *2.2. Measurement of lettuce traits*

### 123 *2.2.1 Measurement of lettuce mass, specific leaf area (SLA) and flavonoid levels in* 124 *leaves*

125 After 6 weeks of growing, the lettuces were harvested and 12 shoots only per plot were  
126 used to determine the fresh weight of the aerial parts. In addition, 4 whole lettuces per plot  
127 were used to determine their mass and to recover the root system and the three leaves of  
128 the fourth crown for further analysis.

129 Each fresh lettuce was weighed on a balance ( $\pm 0.5$  g). The root system was washed with  
130 distilled water and weighed with a precision balance ( $\pm 0.001$  g), dried at 68°C for 24 h and  
131 weighed again on the same balance in order to determine dry mass.

132 The three leaves from the 4th leaf crown were scanned using Winfolia software (Regent  
133 Instruments Inc., Canada), weighed on a precision balance ( $\pm 0.001$  g), and then used for  
134 biomechanical measurements (see point 2.2). The cut-out parts for biomechanical  
135 measurements were put back with the initial weighed material and finally dried at 68°C for  
136 24 h and weighed again. The SLA was calculated by dividing the surface area by the dry mass  
137 (cm<sup>2</sup> g<sup>-1</sup>). Each week, measurements of flavonoid and anthocyanin levels were made on the  
138 4th leaf of 4 lettuces per plot using Dualex™ (FORCE-A, Orsay, France) technology.

139

140 *2.2.2 Leaf mechanical properties*

141 One piece of tissue was cut from each leaf (on 4 leaves per plot), for biomechanical  
142 measurements. Leaf toughness was measured using 'punch and die' tests, which consist in  
143 punching a hole through the leaf lamina. Tests were performed on a universal testing  
144 machine (Instron 5942, Canton, MA, USA) using a device consisting of a flat-ended cylindrical  
145 steel rod (punch, 2.0 mm diameter) mounted onto the moving head of the testing machine,  
146 and a stationary base with a sharp edged hole with a 0.1 mm clearance according to  
147 Foucreau et al. (2013). The punch moved down at a constant speed of  $10 \text{ mm s}^{-1}$ , without  
148 any friction in the hole. The leaves were positioned to avoid primary and secondary veins.  
149 The force applied to the leaf and the displacement were both recorded simultaneously at 10  
150 Hz. Leaf thickness ( $\pm 0.01 \text{ mm}$ ) was measured with a digital thickness gauge avoiding major  
151 veins. The specific work to punch (called specific toughness,  $\text{J m}^{-3}$ ) was calculated as the area  
152 under the force-displacement curve corrected by the area of the punch and the leaf  
153 thickness (Aranwela et al., 1999).

154

155 *2.3. Plant N content*

156 Total N concentration was measured from 4 leaves and 4 root systems per plot (16 per  
157 treatment), using 1.5 mg of ground root or leaf material enclosed in tin capsules, using a  
158 Flash 1112 Elemental Analyzer (Thermo Scientific, Waltham, MA, USA). Calibration was  
159 performed using aspartic acid (10.52% N) and birch leaf standards (2.12% N; Elemental  
160 Microanalysis, Okehampton, UK) interspersed with the samples and used as quality control.  
161 Leaf and/or root N content represent the percentage of N in leaf and/or root dry mass.

162



163                    *2.4. Denitrification Enzyme Activity (DEA)*

164 Denitrification enzyme activity (DEA) was measured from pooled root-adhering soil (RAS)  
165 retrieved from 4 lettuces per plot and from 4 samples of bulk soil, according to Guyonnet et  
166 al. (2017). First, 5 g of soil was placed in 150 ml airtight plasma-flasks sealed with rubber  
167 stoppers. In each flask, air was removed and replaced with a He/ C<sub>2</sub>H<sub>2</sub> mixture (90: 10, v/v)  
168 to create anoxic conditions and inhibit N<sub>2</sub>O-reductase. A nutritive solution (1 ml) containing  
169 glucose (0.5 mg of C-glucose g<sup>-1</sup> of dried soil), glutamic acid (0.5 mg of C-glutamic acid g<sup>-1</sup> of  
170 dried soil) and potassium nitrate (50 mg of N-KNO<sub>3</sub> g<sup>-1</sup> of dried soil) was added to the soil.  
171 N<sub>2</sub>O levels during incubation at 28°C were measured each hour for 6 h. The slope of the  
172 linear regression (R<sup>2</sup> on average greater than 0.98) was used to estimate DEA as the N<sub>2</sub>O  
173 produced (g<sup>-1</sup>h<sup>-1</sup>). Gases (CO<sub>2</sub> and N<sub>2</sub>O) were measured with a gas chromatograph coupled to  
174 a micro-catharometer detector (µGC-R3000; SRA instruments).

175

176                    *2.5. Nitrate concentrations in soil*

177 NO<sub>3</sub><sup>-</sup> was extracted every week over time (6 extractions per week) from the planted  
178 rhizospheric soils (20 cm from the base of 4 lettuces per plot and pooled) and from the  
179 unplanted soils. Nitrate was extracted from 5 g eq. of dried soil supplemented with 20 ml of  
180 a solution with 0.01 M of CaCl<sub>2</sub> (Houba et al., 2000). Briefly, soil suspensions were shaken at  
181 140 rpm for 2 h at 10°C. The suspension was filtered (0.22 µm) and the NO<sub>3</sub><sup>-</sup> concentration  
182 was quantified using an ionic chromatograph ICS-900 (Thermo Scientific Dionex, Sunnyvale,  
183 California, USA).

184

185                    *2.6. Effect of procyanidins on the NarG of nitrate reducing bacteria*

186 To determine whether procyanidins act specifically on the nitrate reductase (NarG) enzyme  
187 of denitrifiers, we used two nitrate reducers (but not denitrifier) strains: *Escherichia coli*  
188 MG1655 and *Corynebacterium glutamicum* ATCC 13032, and a denitrifying strain:  
189 *Pseudomonas brassicacearum* NFM421. The three strains were grown in 20 ml of Lysogeny  
190 Broth (LB) medium supplemented with KNO<sub>3</sub> (20 mM) and procyanidin at 0.01 mg ml<sup>-1</sup>, or  
191 water as a control. The experiments were performed in plasma flasks sealed with a rubber  
192 stopper. To ensure anaerobic conditions, the air was removed as in denitrification  
193 measurements. Plasma flasks were inoculated with each strain independently in triplicate at  
194 Optical Density (OD) 0.1 and incubated at 28°C for *P. brassicacearum*, 37°C for *E. coli* and  
195 30°C for *C. glutamicum*, with agitation (140 r.p.m.). Growth (OD measurement) and counts  
196 on Petri dishes (LB medium) were done from 1 ml of recovered medium at t<sub>0</sub>, 2, 4, 25 and 30  
197 hours of growth. The count was made using an automatic counter (Scan 1200, Grosseron,  
198 Coueron, France) from the 1 ml of medium recovered each time, and diluted by a factor of  
199 10<sup>4</sup>. Cultured petri dishes were incubated at the same temperature as previously stated for  
200 each strain.

201

## 202 2.7. Quantification of total bacteria and denitrifier abundance

203 Total DNA was extracted from 0.5 g of RAS for three treatments (unplanted soil, soil with  
204 lettuce, soil with lettuce given 210 kg of procyanidins per hectare), in triplicate using the Fast  
205 DNA Spin Kit for Soil (MP), according to the manufacturer's protocol. The amount of DNA  
206 extracted was estimated using a Quant-iT PicoGreen® dsDNA Assay kit. The amounts of  
207 bacteria and denitrifiers were quantified using real-time quantitative PCR (q-PCR) with  
208 primers targeting the 16S rRNA and *nirK/nirS* genes, as described previously by Bru et al.  
209 (2011). For *nirK*, the amplification was performed using the primers nirK876 (5'-

210 ATYGGCGGVCA YGGCGA-3') and nirK1040 (5'-GCCTCGATCAGRTTTRTGGTT-3') (Henry et al.,  
211 2004). The 20 µl final reaction volume contained SYBRgreen PCR Master Mix (QuantiTect  
212 SYBRgreen PCR kit, Qiagen, Courtaboeuf, France), 1 µM of each primer, 400 ng of T4gp32  
213 (MPbiomedicals, Illkwich, France) and 5 ng of extracted DNA. Thermal cycling was as follows:  
214 15min at 95°C; 6 cycles at 95°C for 15s, 63°C for 30s, with a touchdown of -1°C by cycle, 72°C  
215 for 30s; 40 cycles at 95°C for 15s, 58°C for 30s and 72°C for 30s. For *nirS*, the amplification  
216 was performed using the primers nirSCd3aF (5'-AACGYSAAGGARACSGG-3') and nirSR3cd (5'-  
217 GASTTCGGRTGSGTCTTSAYGAA-3') (Throbäck et al., 2004). The 25 µl final reaction volume  
218 contained SYBR green PCR Master Mix (as above), 1 µM of each primer, 400 ng of T4gp32  
219 (MPbiomedicals, Illkwich, France) and 12.5 ng of extracted DNA. Thermal cycling was as  
220 follows: 15min at 95°C; 6 cycles at 95°C for 15s, 59°C for 30s with a touchdown of -1°C by  
221 cycle, 72°C for 30s and 80°C for 30s; 40 cycles at 95°C for 15s, 54°C for 30s, 72°C for 30s and  
222 80°C for 30s). For 16S rRNA, the amplification was performed using the primers 519F (5'-  
223 CAGCMGCCGCGGTAANWC-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (Lane, 1991). The  
224 20 µl final reaction volume contained Mix SYBR® Green Master (Roche Diagnostics,  
225 Penzberg, Germany), 1µM of each primer and 5 ng of extracted DNA. Thermal cycling was as  
226 follows: 10 min at 95°C; 40 cycles at 95°C for 15s, 63°C for 30s and 72°C for 30s; Then 95°C  
227 for 1 s; 65°C for 60 s, 68°C for 20s and a continuous increase to 97°C to determine the  
228 melting point and finally 10 s at 40°C for cooling. The standard curves for *nirK* and *nirS* qPCR  
229 were generated by amplifying 10-fold dilutions ( $10^7 - 10^2$ ) of a linearized plasmid containing  
230 the *nirK* gene of *Sinorhizobium meliloti* 1021 and *nirS* gene of *Pseudomonas stutzeri* Zobell  
231 DNA (GenArt, Invitrogen, Lifetechnologies, Regensburg, Germany). The standard curves for  
232 16S rRNA qPCR were generated by amplifying 10-fold dilutions ( $10^8 - 10^2$ ) of the standard  
233 DNA pQuantAlb16S plasmid (Zouache et al., 2012) for 16S rRNA. Melting curve analysis

234 confirmed the specificity of amplification and amplification efficiencies for 16S rRNA, *nirK*  
235 and *nirS* genes were higher than 90%.

236

### 237 *2.8. Root development of lettuce seedlings on Knop agar*

238 Lettuce seeds (*Lactuca sativa* var Lotus RZ) were disinfected according to Bressan et al.  
239 (2009), then cultured on Petri dishes (12 x 12 cm) with 100 ml of Knop agar (4mM Ca(NO<sub>3</sub>)<sub>2</sub>;  
240 1.8 mM KH<sub>2</sub>PO<sub>4</sub>; 3.4 mM KCl; 1 mM MgSO<sub>4</sub>; 1 μM ZnSO<sub>4</sub>; 14 μM MnCl<sub>2</sub>; 70 μM H<sub>3</sub>BO<sub>3</sub>; 10 nM  
241 CoCl<sub>2</sub>; 0.5 μM CuSO<sub>4</sub>; 0.2 μM Na<sub>2</sub>MoO<sub>4</sub>; 50 μM FeNa-EDTA; Agar concentration: 0.8%)  
242 (Horns Schuh et al., 2002). Commercial procyanidins (Laffort, Bordeaux, France) were added  
243 with a 0.2 μm filter after Knop agar autoclaving. Three conditions were tested, one control  
244 condition without the addition of procyanidins and two conditions with 0.1 and 0.2 mg of  
245 procyanidins per ml of Knop agar, based on Bardon et al. (2016). For each condition, three  
246 Petri dishes containing 9 seeds (3 replicates and 27 pseudoreplicates) were used. Each Petri  
247 dish was grown in phytotron (SANYO, Osaka, Japan) at 22°C, with the day/night period set at  
248 16 h/8 h. After 14 days of growth, the fresh mass of all roots was measured with a precision  
249 balance ± 0.0001g. Root development (root length and root surface) was analysed using  
250 WinRhizo software (Regent Instruments Inc., Canada), then placed in an oven at 68°C for 24  
251 h to obtain the dry mass.

252

### 253 *2.9. Data and statistical analyses*

254 The differences on microbiological and lettuce morphological traits (n ≤ 4) between  
255 treatments was tested non-parametrically with a Wilcoxon test. Linear regression was  
256 performed to test the functional relationship between the procyanidin concentrations and

257 the response variables. The significance of this regression was tested by Bravais-Pearson  
258 test. The difference of lettuce morphological traits ( $n \geq 16$ ) between treatments was  
259 determined using an ANOVA (analysis of variance) followed by a post-hoc Tukey HSD test.  
260 Similarly, the normality (Shapiro test) and the homoscedasticity of variance (Fischer test) of  
261 all the variables were tested. For nitrate concentrations data, we compared the  
262 concentrations between treatments for each date. In addition, the absence of plot effect  
263 was tested on all traits using two-way ANOVA and mixed model. The effect of procyanidins  
264 on lettuce seeds was tested using an two-way ANOVA. All the analyses were done using R  
265 project software (v. 3.5.0).

266

### 267 **3. RESULTS**

268

#### 269 *3.1. Denitrification activity and biological denitrification inhibition*

270 The denitrification activity of microbial communities colonizing the RAS of lettuces treated  
271 with  $210 \text{ kg ha}^{-1}$  of procyanidins was significantly lower ( $p\text{-value} = 0.023$ ) than that of  
272 microbial communities of untreated lettuces. No significant differences in denitrification  
273 activity were observed between the RAS from treated lettuces with  $8, 42$  or  $83 \text{ kg ha}^{-1}$  of  
274 procyanidins and that from untreated lettuces (Fig. 1A). In addition, the BDI at  $210 \text{ kg ha}^{-1}$   
275 was not significantly different from that at other concentrations. The decrease in  
276 denitrification activity at  $210 \text{ kg ha}^{-1}$  of procyanidins represents a BDI of approximately  
277  $27.17\%$  ( $p\text{-value} = 0.023$ ) (Fig. 1B). In addition, untreated lettuce did not induce BDI  
278 compared to unplanted soil as they both presented the same level of denitrification activity.  
279 Moreover, the  $\text{CO}_2$  emissions under these conditions did not differ between treatments

280 (data not shown). Interestingly, the biological denitrification inhibition showed a significant  
281 dose-response relationship to procyanidins (Fig. 1C).

282

### 283 *3.2. Effect of procyanidins on NarG of nitrate-reducing bacteria*

284 As shown in Figure 2, the addition of procyanidins, at 0.01 mg ml<sup>-1</sup>, to *E. coli* and *C.*  
285 *glutamicum* cultures did not affect their growth compared to untreated cultures. However,  
286 *P. brassicacearum* growth was significantly lower (p-value = 0.044 for OD, p-value = 0.037 for  
287 enumeration) after the addition of procyanidins.

288

### 289 *3.3. Abundance of total and denitrifying bacteria*

290 The abundance of denitrifying bacteria was represented by the sum of the copy numbers of  
291 the *nirS* and *nirK* genes (Fig. 3). The results obtained showed significant differences (p-value  
292 = 0.045) among the three treatments (Fig. 3A). The total proportion of denitrifiers from the  
293 RAS of lettuces treated with 210 kg ha<sup>-1</sup> of procyanidins was 4.5 times lower than that from  
294 the RAS of untreated lettuces. However, the total abundance of bacteria, represented by the  
295 copy number of 16S rRNA genes per gram of dry soil, did not show any significant variation  
296 among the different treatments (Fig. 2B). The proportion of denitrifying bacteria (Fig. 3C),  
297 represented by the ratio (*nirK* + *nirS*)/ARNr16S, in the RAS of lettuces treated with  
298 procyanidins was 5-fold lower (p-value = 0.046) than that in the RAS of untreated lettuces.

299

### 300 *3.4. Effect of soil procyanidin addition to soil on nitrate concentrations*

301 In the plant treatments with or without the addition of procyanidins, nitrate levels tended to  
302 decrease over time (Fig 4). This decrease was greater in the planted soils than in the  
303 unplanted soils. The planted soils contained less nitrate than the unplanted soils (p-value =

304 0.034). In addition, there was no difference in nitrate concentration each week between  
305 treatments amended with procyanidins (Fig. 4).

306

### 307 *3.5. Effect of procyanidin addition on lettuce traits*

308 The fresh mass of the shoot and root parts of lettuces tended to increase with the addition  
309 of 0 to 210 kg ha<sup>-1</sup> procyanidins (Figs. 5 A and B). Indeed, the fresh mass of the shoot and  
310 root parts showed a significant dose-response relationship to procyanidins (p-value = 0.003  
311 for the shoot mass and 0.0016 for the root mass) (Fig. 5C and D). The shoot fresh mass of the  
312 lettuces treated with 210 kg ha<sup>-1</sup> procyanidins was significantly (16%) higher per lettuce (on  
313 average 120 g) (p-value = 0.021) than that of untreated lettuces (Fig. 5A). Similarly, the root  
314 fresh mass of lettuces treated with procyanidins at 83 kg ha<sup>-1</sup> and 210 kg ha<sup>-1</sup> was 39% (p-  
315 value = 0.023) and 63% (p-value = 0.002) higher per lettuce, respectively, than the root fresh  
316 mass of untreated lettuces (Fig. 5B). In addition, the root dry mass of lettuces treated with  
317 procyanidins at 83 kg ha<sup>-1</sup> and 210 kg ha<sup>-1</sup> was significantly greater by 52% (p-value = 0.048)  
318 and 97% (p-value = 0.028) per lettuce, respectively, than the root dry mass of untreated  
319 lettuces. Moreover, the anthocyanin and flavonoid levels, as well as the SLA (cm<sup>2</sup> g<sup>-1</sup>), did not  
320 differ between procyanidin- treated and untreated lettuces (Figs. S1 and S2), and the  
321 specific toughness (J m<sup>-3</sup>) did not differ among the three leaves of the 4<sup>th</sup> crown of treated  
322 and untreated lettuces (Fig. 6).

323

### 324 *3.6. Nitrogen concentration in plants*

325 Overall, the nitrogen content in the tissues (shoot and root) did not differ between  
326 treatments and was approximatively 3% in shoots and 1.7% in roots (Fig. 7). However, by  
327 relating this percentage of nitrogen in the plant to the mass gain of treated lettuces, we

328 obtain an average gain of 3.6 grams of nitrogen per lettuce treated at a concentration of 210  
329 kg ha<sup>-1</sup>.

330

### 331 *3.7. Effect of procyanidins on root development in vitro*

332 To test whether the addition of procyanidins had a direct impact on root system  
333 development, we measured the length and surface area of fresh lettuce roots in Knop agar  
334 with or without added procyanidins (Fig. 8). The results showed no significant differences in  
335 the fresh mass of young lettuce roots grown in Knop agar with or without added  
336 procyanidins (Fig. 8A). The dry weight was slightly lower at 0.2 mg ml<sup>-1</sup> procyanidins than  
337 under other conditions (Fig. 8B); however, this decrease was not significant. Similarly, no  
338 significant differences were observed in the length (Fig. 8C) and root surface area (Fig. 8D) of  
339 plants grown with or without the addition of procyanidins.

340

## 341 **4. DISCUSSION**

342 In this study, we found that the addition of 210 kg ha<sup>-1</sup> procyanidins to field-grown lettuce  
343 crops lead to a significant gain in productivity, resulting from BDI.

344

### 345 *4.1. The addition of procyanidins under field conditions causes BDI and changes in* 346 *denitrifier abundance*

347 Denitrification is an inducible function that requires favourable conditions to be expressed:  
348 low oxygen concentration, easily available organic compounds as energy sources and the  
349 presence of nitrate. In plant rhizospheres, the roots of growing plants cannot only release  
350 carbon compounds *via* exudation but also modify the soil oxygen partial pressure and the  
351 nitrate concentration (Lecomte et al., 2018; Achouak et al., 2019). In our case, the addition



352 of procyanidins led, as expected, to a significant decrease in the denitrification activity of  
353 lettuce RAS treated with procyanidins at 210 kg ha<sup>-1</sup>, which corresponded to a BDI of  
354 approximately 27.17% (Fig. 1B). Although denitrification enzyme activity did not decrease  
355 under 8, 42 and 83 kg ha<sup>-1</sup> of procyanidin amendment (Fig. 1A), the biological denitrification  
356 inhibition showed a significant dose-response relationship to procyanidins ( $R^2=0.863$ , p-  
357 value = 0.005) (Fig. 1C), which was consistent with the result of a previous study by Bardon  
358 et al. (2014). Indeed, the same concentrations tested *in vitro* on the *P. brassicacearum* strain  
359 induced a significant decrease in DEA (Bardon et al., 2014), which was observed in the field  
360 only at the largest concentration. This result could be explained by the fact that procyanidins  
361 at a low concentration may aggregate with soil particles and become less bioavailable.  
362 However, at 210 kg ha<sup>-1</sup>, the highest procyanidin concentration, procyanidins were more  
363 bioavailable in the soil.

364 Although denitrification by archaea (Shoun et al., 1992) and fungi (Philippot, 2002) cannot  
365 be excluded, procyanidin amendment was demonstrated to have an impact on denitrifying  
366 bacteria, such as the *P. brassicacearum* NFM421 strain. Indeed, Bardon et al. (2016)  
367 demonstrated that procyanidins act specifically by inhibiting membrane-bound NO<sub>3</sub>-  
368 reductase, thereby inducing enzymatic conformational changes through membrane  
369 disturbance. However, some bacteria are not denitrifiers but do possess this enzyme (NarG),  
370 such as *E. coli* (Taniguchi and Itagaki, 1960) and *C. glutamicum* (Nishimura et al., 2007). Our  
371 results showed that the nitrate reductase of these non-denitrifiers was not affected by  
372 procyanidins (Fig. 2). Procyanidins act specifically on the NarG of denitrifiers that contribute  
373 to N<sub>2</sub>O emissions. However, as other nitrate reducers are part of the rhizospheric microbial  
374 community, the *narG* gene cannot be used as marker for denitrifiers. Thus, we chose to  
375 target the *nirK* and *nirS* genes as markers for denitrifiers to assess the impact of procyanidins

376 on denitrifiers. At 210 kg ha<sup>-1</sup> of procyanidins, the proportion of denitrifying bacterial  
377 communities in the lettuce rhizosphere was reduced (Fig. 3A). The loss of the denitrification  
378 function is probably a disadvantage for the denitrifying bacterial community, which becomes  
379 less competitive, thus explaining their counter-selection in the rhizosphere. Denitrifiers can  
380 no longer use nitrate as an electron acceptor, which reduces their multiplication and their  
381 inclusion in the rhizosphere ecological niche, as observed in Dassonville et al. (2011). The  
382 fact that the total bacterial community abundance (Fig. 3B) and CO<sub>2</sub> release from the  
383 microbial community colonizing the RAS of treated lettuces did not change in the presence  
384 of procyanidins suggests that (i) other bacterial communities colonized the niches left vacant  
385 by denitrifying bacteria, as demonstrated by Jones and Hallin, (2010) and (ii) procyanidin  
386 addition did not have an antibacterial effect under field conditions, in contrast to the result  
387 of Mayer et al. (2008) and Lacombe et al. (2012), where procyanidins had antibacterial  
388 properties against some bacterial species.

389

390 *4.2. The addition of procyanidins in the field modifies the soil nitrate concentration*  
391 *and increases lettuce growth.*

392 In *in situ* microcosm experiments, Bardon et al. (2017) observed nitrate conservation in  
393 unplanted soil treated with procyanidins compared to untreated soil. Indeed, BDI, which is  
394 induced by procyanidins, allows up to 6 x greater storage of nitrate in the soil (Bardon et al.,  
395 2017). To limit nitrate leaching and make nitrate available for lettuce crops, we added  
396 procyanidins one week before the period when the lettuces needed the maximum amount  
397 of nitrate for growth (according to the ZENIT grid standard for lettuce). No differences were  
398 observed in soil nitrate levels between lettuces treated or untreated with procyanidins,  
399 regardless of the concentration (Fig. 4), most likely due to nitrate absorption by lettuce

400 crops. The addition of procyanidins at all tested concentrations tended to induce better  
401 lettuce growth as significant dose-response relationship was observed between shoot and  
402 root mass and procyanidins concentrations (Fig. 5). This growth improvement was significant  
403 only at the highest concentration ( $210 \text{ kg ha}^{-1}$ ), with an increase in the shoot of up to 16%  
404 and of the root of up to 63% (Fig. 5). At this concentration, denitrifiers appear to be inhibited  
405 enough to have a visible effect not only on denitrification activity but also on mass gains.  
406 However, at the other concentrations, procyanidins are probably less bioavailable in soil,  
407 explaining why no denitrifier inhibition allowing a decrease in nitrate consumption by  
408 bacteria was observed. Furthermore, the nitrogen levels in the tissues did not differ  
409 between treated and untreated lettuces regardless of the concentration (Fig. 7), which  
410 means that there were no physiological changes in lettuces due to the addition of  
411 procyanidins that allowed greater nitrogen absorption by the lettuces. However, since  
412 lettuces tend to grow better in plots treated with procyanidins, the larger mass implies a  
413 greater nitrogen mass. For example, at  $210 \text{ kg ha}^{-1}$  procyanidins, we obtained up to 3.6 g of  
414 nitrogen on average. Indeed, the procyanidin treatment allowed nitrogen to be preserved in  
415 the form of nitrate *via* biological inhibition of denitrifying bacteria; hence, more nitrate  
416 became available for lettuces, allowing better growth. This finding is consistent with the  
417 greenhouse study performed on several lettuce varieties, where plant nitrogen use  
418 efficiency was not affected by the nitrate concentrations in the soil (Urlić et al., 2017). SLA  
419 trait did not differ between treated and untreated lettuces, regardless of the concentration  
420 (Fig. S1), suggesting that treated lettuces did not change their resource allocation to leaf  
421 structure (Evans and Poorter, 2001). Moreover, the increase in plant growth did not alter the  
422 leaf quality of the lettuce. Indeed, the levels of flavonoids and anthocyanins did not differ  
423 between treated and untreated lettuces (Fig. S2), which explains the absence of stress that

424 would have caused an increase in these levels in treated lettuces, as observed for nitrate-  
425 deficient wine grape and broccoli plants (Cerovic et al., 2008; Tremblay et al., 2008). In  
426 addition, procyanidin amendment did not change the quality of the leaves as no changes in  
427 the applied force to pierce a leaf volume were observed (Fig. 6). This result suggests that the  
428 procyanidin amendment did not alter leaf vulnerability to potential herbivores and pests (  
429 Clissold et al., 2009), and did not induce a gustatory difference for consumers in the chewing  
430 quality of lettuces potentially placed on the market (Lopez-Galvez et al., 1997; Rico et al.,  
431 2007).

432 Interestingly, we showed a larger gain in root mass (up to 63%) than in shoot mass (up to  
433 16%) (Fig. 5). This root mass gain is clearly not due to the direct effect of procyanidins on  
434 root development but due to the BDI of denitrifiers and therefore to better availability of  
435 nitrate in the rhizosphere of lettuces treated with procyanidins. Indeed, the absence of  
436 differences between lettuce seedlings grown in sterile medium with and without  
437 procyanidins (Fig. 8) allows us to conclude that procyanidins are not a source of carbon  
438 and/or a phytohormone for root development. As the denitrifier distribution in soil is  
439 heterogeneous, nitrates available for plants due to denitrifier inhibition will present a  
440 heterogeneous spatial distribution in soil. To benefit from this nitrate, plants concentrate  
441 new and more root growth in the nitrate-containing zone, which could explain the gain in  
442 root mass, as already demonstrated for young lettuce plants submitted to a heterogeneous  
443 spatial distribution of nitrate in the root zone (Burns, 1991).

444

445 The procyanidin source used in this study was from the wine industry and is used to mature  
446 wines with a too-low tannin content (Harbertson et al., 2012). The cost of this source of  
447 procyanidin is too high to be economically viable for field applications. We used it in our

448 study to demonstrate, for the first time, that biological denitrification inhibition can occur in  
449 the field and can induce plant growth improvement. In the context of sustainable agriculture  
450 with economic issues, there are other sources of procyanidins that are less expensive and  
451 have a higher concentration of procyanidins, such as mimosa and quebracho bark and grape  
452 seeds (Thompson and Pizzi, 1995; Gabetta et al., 2000; Vivas et al., 2004; Venter et al.,  
453 2012). Future studies using these sources of procyanidins in the field and evaluating the  
454 benefits/costs of such applications are needed.

455

## 456 **5. Conclusions**

457 The addition of procyanidins in the field allows a decrease in denitrification activity, making  
458 nitrates available at ground level, which results in an increase in lettuce yields without  
459 changing their physiology. Based on the results of this study, we propose in the short-term  
460 the development of a more environmentally friendly method of sustainable agriculture-by  
461 limiting fertilizer use and nitrogen losses in the soil while increasing plant growth and  
462 productivity.

463

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472

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631  
632

### 633 **Figure legends:**

634

635 **Figure 1:** Impact of procyanidins applied in the field on microbial denitrification activity.  
636 Effects of different concentrations of procyanidins (0, 8, 42, 83 and 210 kg of procyanidins  
637 per hectare) applied in the field to the soil planted with lettuces on the denitrification

638 activity of microbial communities colonising the root-adhering soil of treated lettuces. n=4  
639 for each treatment. (A) Denitrification Enzyme Activity (DEA,  $\mu\text{g N-N}_2\text{O h}^{-1} \text{g}^{-1} \text{dry soil h}^{-1}$ )  
640 among treatments. (B) Biological Denitrification Inhibition (BDI %). (C) Linear regression of  
641 the functional relationship between the procyanidin concentrations ( $\text{kg.h}^{-1}$ ) and BDI (%).  
642 Vertical bars indicate standard errors. Stars indicate which means differed from the relative  
643 controls (Wilcoxon test;  $\alpha < 0.05$ ).

644

645 **Figure 2:** Effects of procyanidins ( $0.01 \text{ mg ml}^{-1}$ ) on *E. coli* MG1655, *Corynebacterium*  
646 *glutamicum* ATCC 13032 and *Pseudomonas brassicacearum* NFM421 anaerobic growth.  
647 Optical density was measured over time (0, 2, 4, 25 and 30 h). Vertical bars indicate standard  
648 errors. Stars indicate which means differed from the relative controls (Wilcoxon test;  
649  $\alpha < 0.05$ ).

650

651 **Figure 3:** Effects of procyanidins (210 kg of procyanidins per hectare) applied in the field to  
652 the soil planted with lettuces on the abundance of (A) denitrifying bacteria (copy numbers of  
653 *nirK* (grey bar) and *nirS* genes (dark bar)  $\text{g}^{-1}$  of dry soil), (B) whole bacterial community (copy  
654 numbers of 16S rRNA  $\text{g}^{-1}$  of dry soil) and (C) gene abundance ratios of (*nirK*+ *nirS*)/16S rRNA.  
655 n = 3 for each treatment. Vertical bars indicate standard errors. Stars indicate which means  
656 differed from the relative controls (Wilcoxon test;  $\alpha < 0.05$ ).

657

658 **Figure 4:** Effect of procyanidins addition on soil nitrate level. Effects of different  
659 concentrations of procyanidins (0, 8, 42, 83 and 210 kg of procyanidins per hectare) applied  
660 in the field to the soil planted with lettuces on the nitrate concentration ( $\mu\text{g N-NO}_3 \text{g}^{-1} \text{dry}$   
661 soil) measured every week (weeks 1 to 6) from the root-adhering soil of lettuce.

662 Procyanidins were applied to soil planted on week 2. N = 4 for each treatment. Vertical bars  
663 indicate standard errors. Stars indicate which means differed from the relative controls  
664 (Wilcoxon test;  $\alpha < 0.05$ ).

665

666 **Figure 5:** Effects of different concentrations of procyanidins (0, 8, 42, 83 and 210 kg of  
667 procyanidins per hectare) applied in the field to the soil planted with lettuces on the fresh  
668 mass of (A) shoots (g) and (B) roots (g). Linear regression of the functional relationship  
669 between procyanidins concentrations and shoot (C) and root (D) fresh mass (g). n=16 for  
670 root system and n= 64 for shoots, for each treatment. Vertical bars indicate standard errors.  
671 Stars indicate which means differed from the relative controls (Tukey test;  $\alpha < 0.05$ ).

672

673 **Figure 6:** Effects of different concentrations of procyanidins (0, 8, 42, 83 and 210 kg of  
674 procyanidins per hectare) applied in the field to the soil planted with lettuces on specific  
675 toughness ( $J m^{-3}$ ). n = 16 for each treatment. Vertical bars indicate standard errors. No  
676 significant effect (Tukey's test;  $\alpha < 0.05$ ).

677

678 **Figure 7:** Nitrogen concentration in plants treated with procyanidins. Effects of different  
679 concentrations of procyanidins (0, 8, 42, 83 and 210 kg of procyanidins per hectare) applied  
680 in the field to the soil planted with lettuces on (A) leaf and (B) root N content (% of dry  
681 mass). n=4 for each treatment. Vertical bars indicate standard errors. No significant effect  
682 (Wilcoxon test;  $\alpha < 0.05$ ).

683

684 **Figure 8:** Effect of procyanidins on root development *in vitro*. Effects of different  
685 concentrations of procyanidins (0; 0.1 and 0.2 mg procyanidins per ml agar) amended to

686 lettuce seedling growing on Knop agar on: (A) Root fresh mass (mg), (B) Root dry mass (mg),  
687 (C) Root length (cm) and (D) Root surface area (cm<sup>2</sup>). n = 3 replicates for each treatment and  
688 each replicate is the average of 9 seedlings. Vertical bars indicate standard errors. No  
689 significant effect (two-way ANOVA;  $\alpha < 0.05$ ).

690

691 **Figure S1:** Effects of different concentrations of procyanidins (0, 8, 42, 83 and 210 kg of  
692 procyanidins per hectare) applied in the field to the soil planted with lettuces on the Specific  
693 Leaf Area (SLA) (cm<sup>2</sup> g<sup>-1</sup>) of the 3 leaves of the 4th leaf crown (cm<sup>2</sup> g<sup>-1</sup>). n=16 for each  
694 treatment. Vertical bars indicate standard errors. Stars indicate which means differed with  
695 the relative controls (Tukey test;  $\alpha < 0.05$ ).

696

697 **Figure S2:** Effects of different concentrations of procyanidins (0, 8, 42, 83 and 210 kg of  
698 procyanidins per hectare) applied in the field to the soil planted with lettuces on the  
699 chlorophyll level ( $\mu\text{g cm}^2$ , black bar) and the anthocyanin level ( $\mu\text{g cm}^2$ , grey bar). n=16 for  
700 each treatment. Vertical bars indicate standard errors. Stars indicate which means differed  
701 with the relative controls (Tukey test;  $\alpha < 0.05$ ).

702

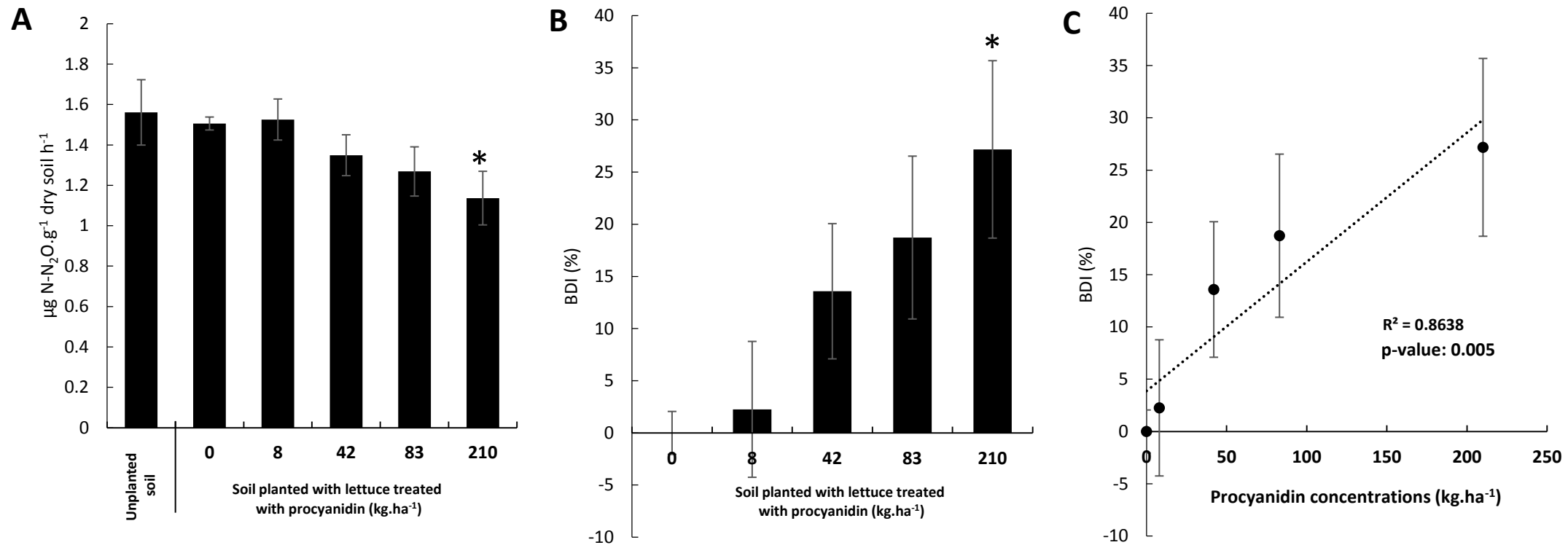


Figure 1

Figure 2

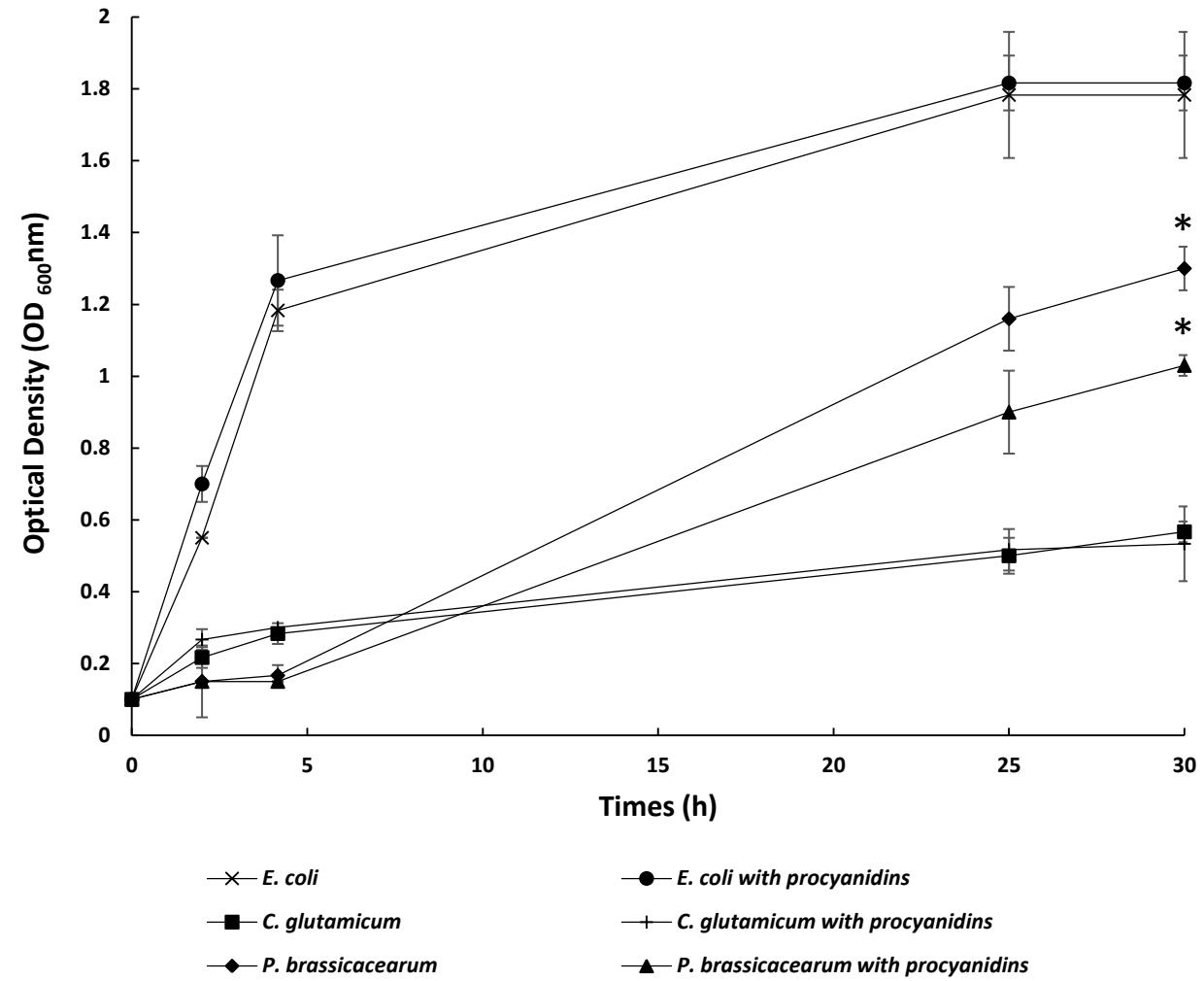


Figure 2

Figure 3 revised

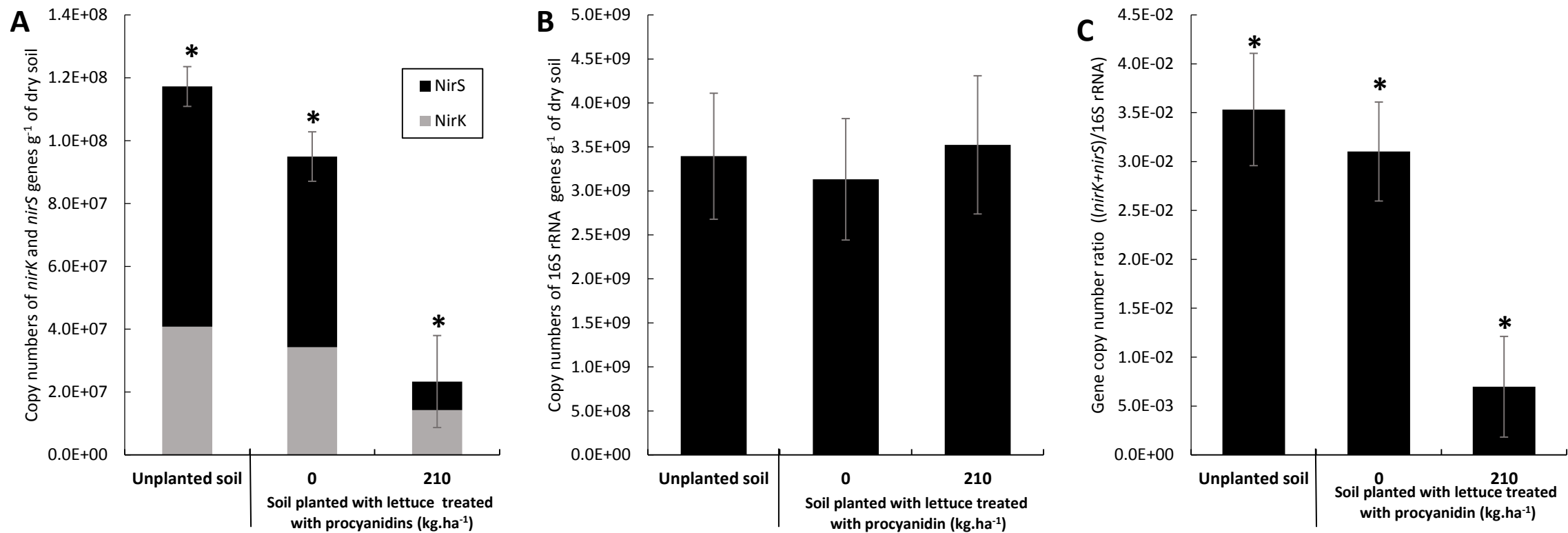


Figure 3



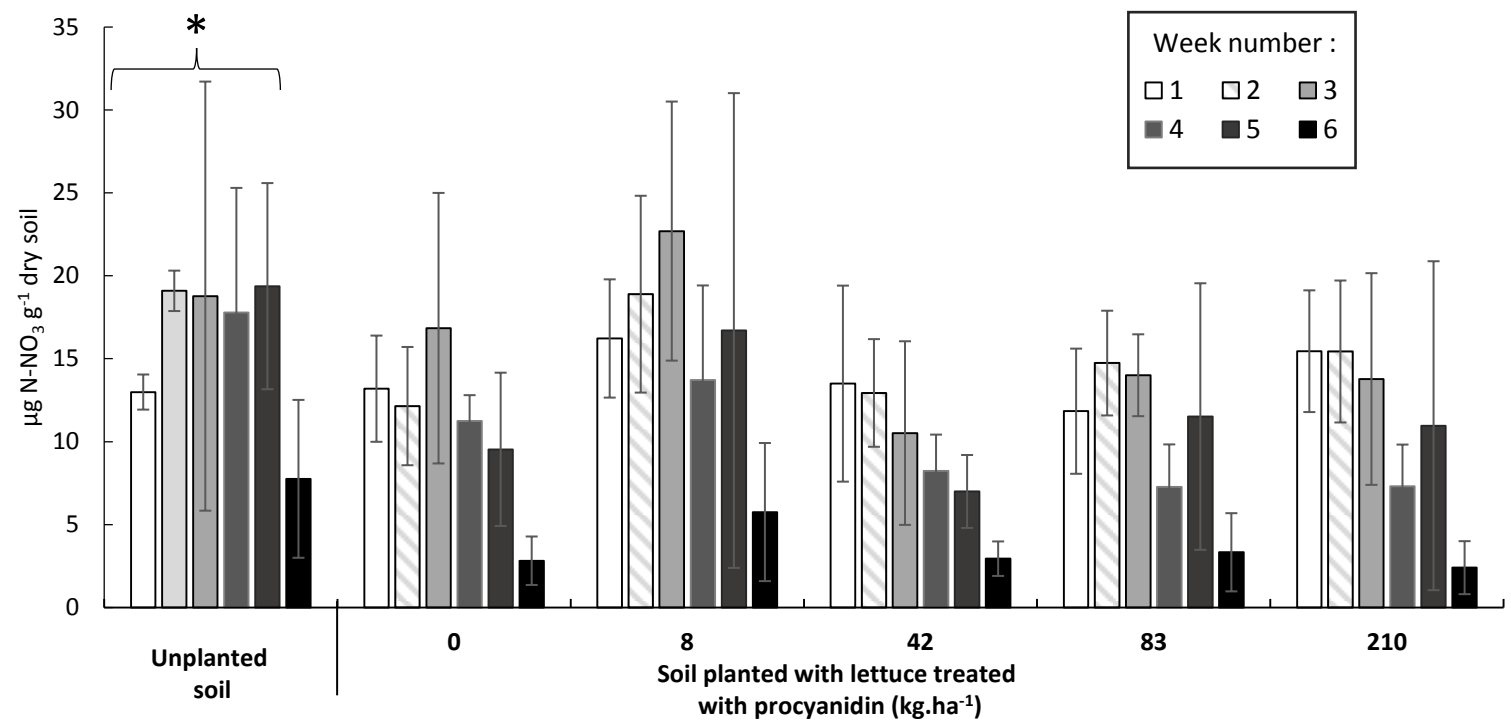


Figure 4

Figure 5 revised 2

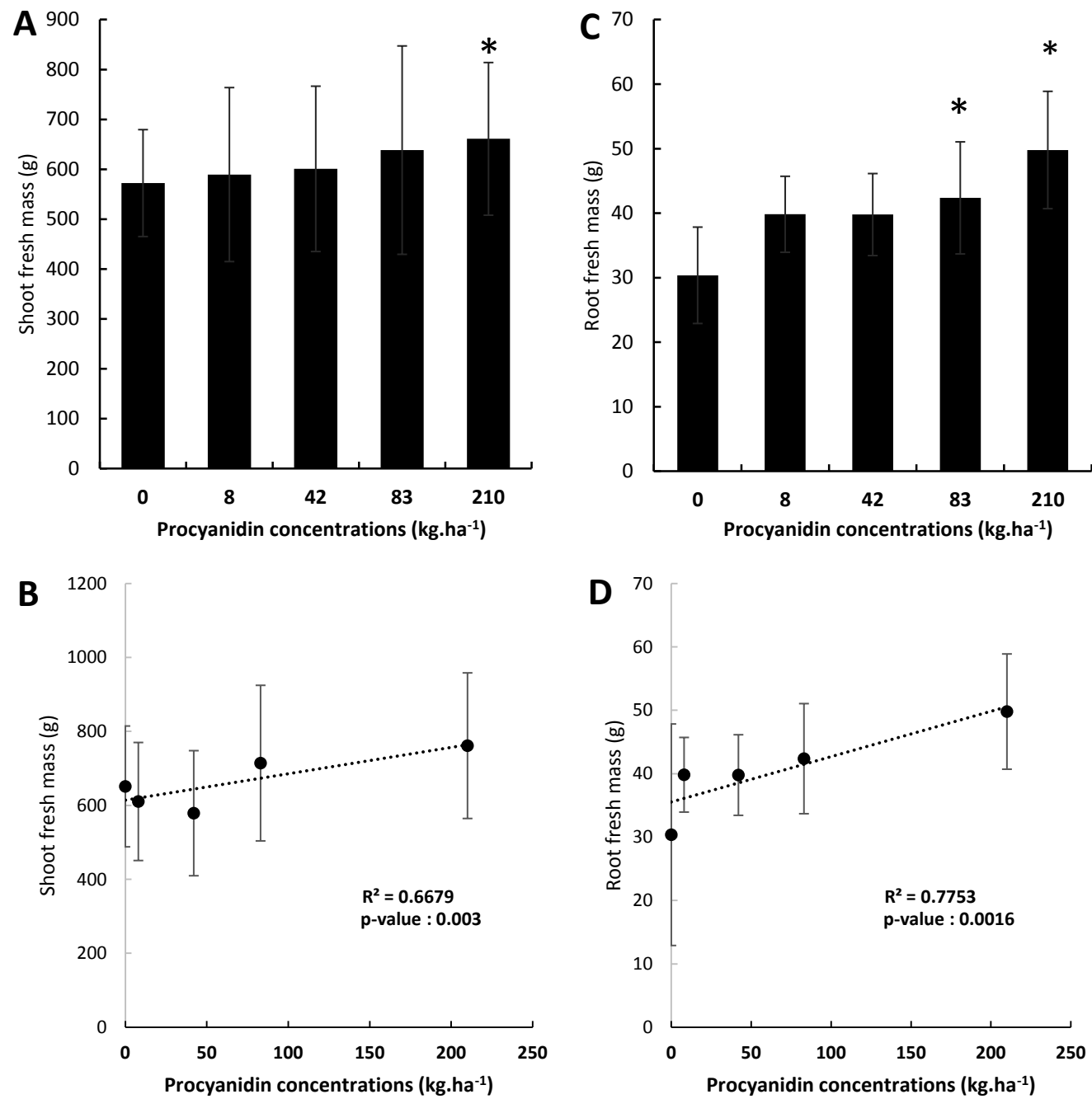


Figure 5

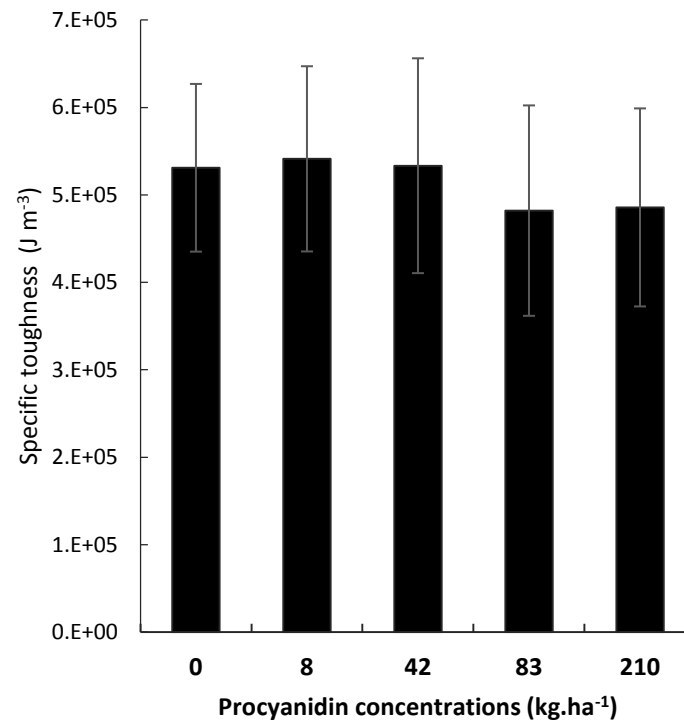


Figure 6

Figure 7

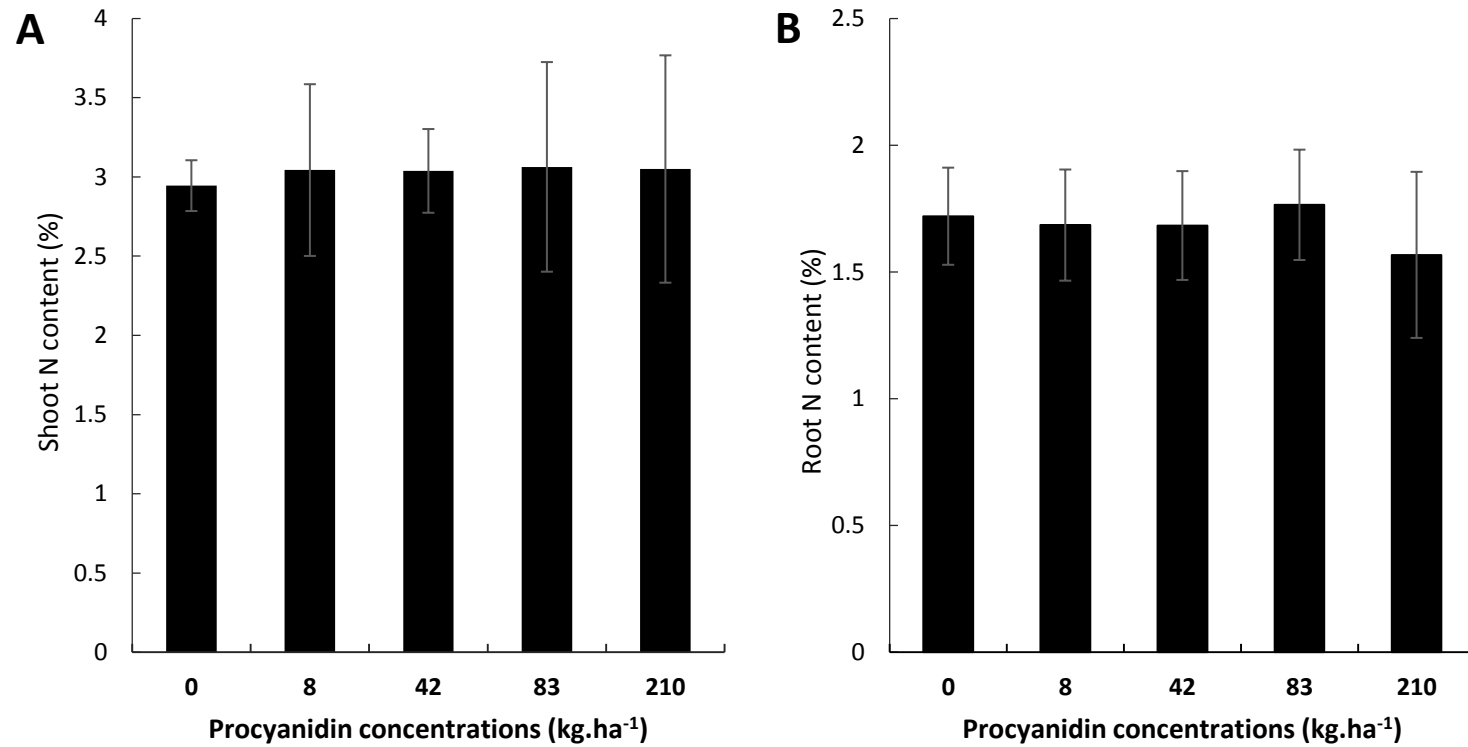


Figure 7

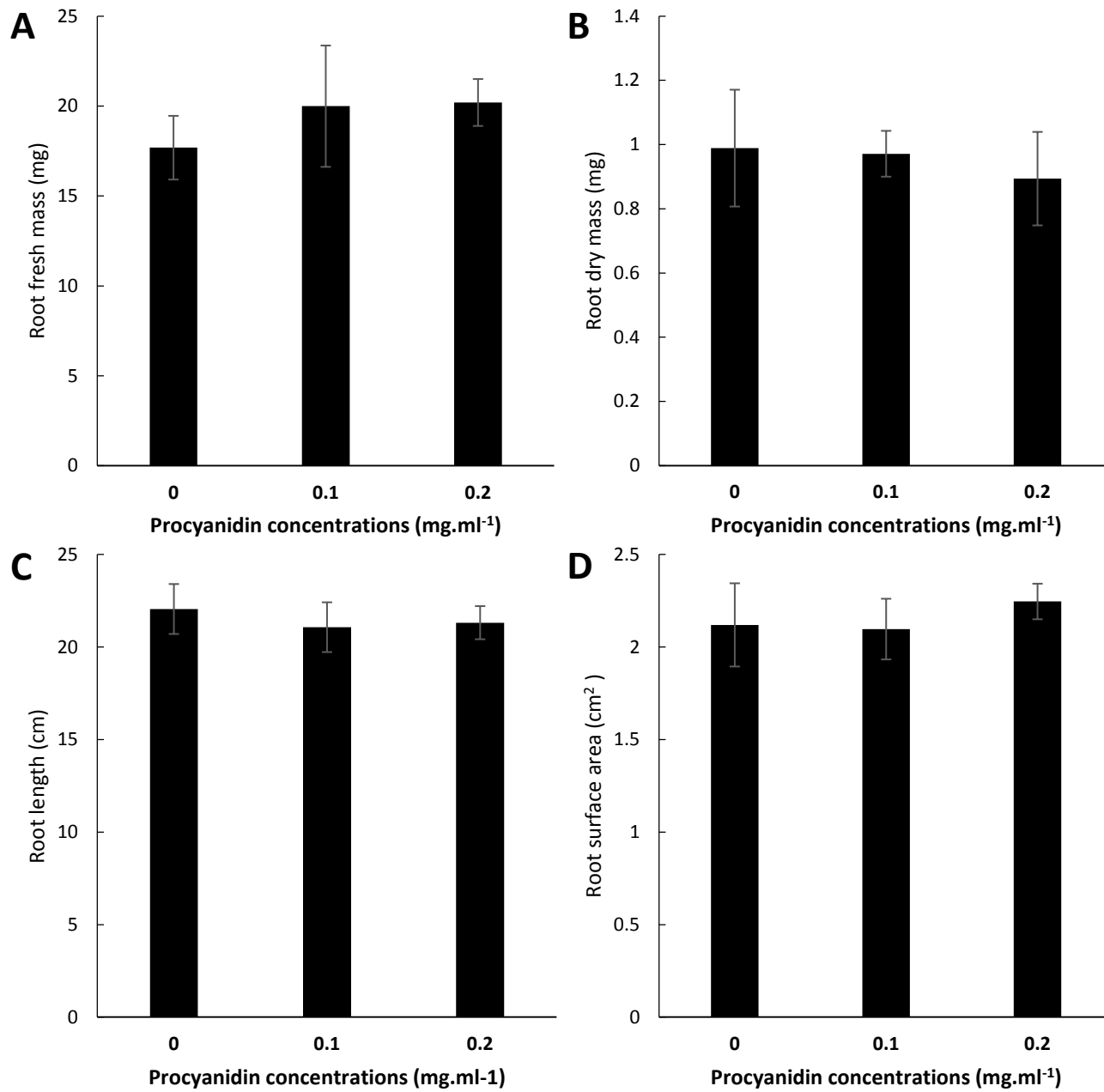
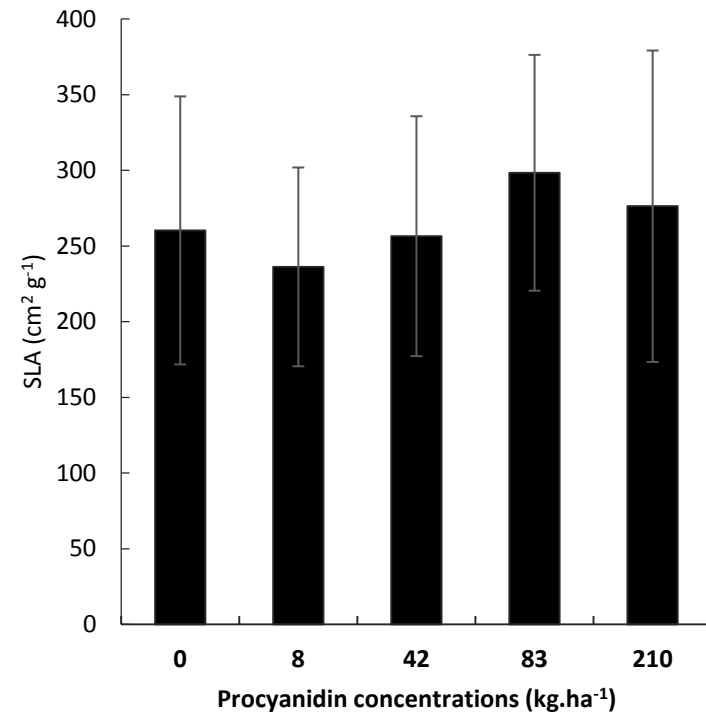


Figure 8



**Figure S1**

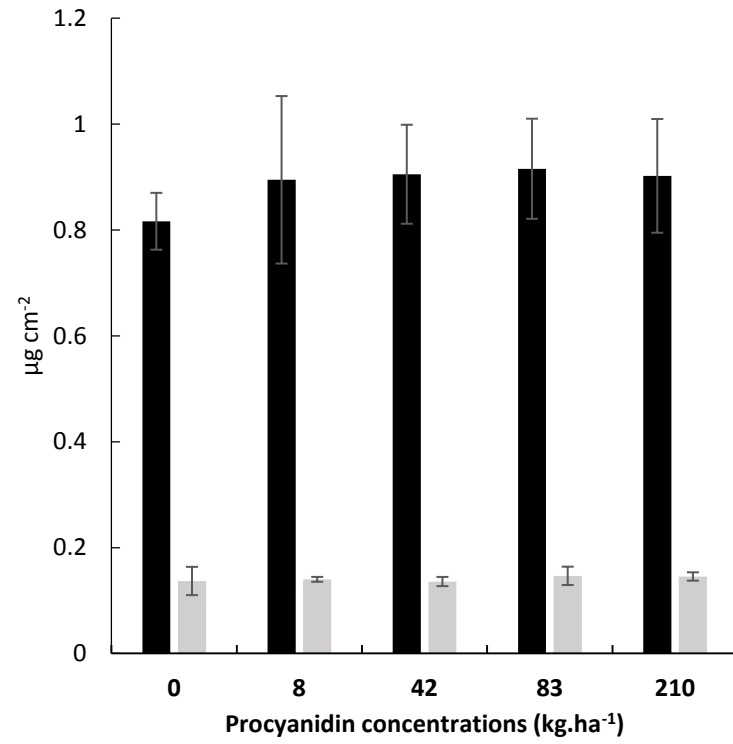


Figure S2