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

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ABSTRACT

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

1. INTRODUCTION

Nitrogen is one of the factors limiting plant growth (LeBauer and Treseder, 2008). It is the most important nutrient used to enhance agricultural yields. Thus, the development of plants depends on the processes linked to the nitrogen (N) cycle, which orchestrates the transformation of nitrogen into all its forms including ammonium (NH_4^+) and nitrate (NO_3^-), the most plant-assimilable forms of nitrogen. The competition between plant roots and microorganisms for these two N forms is intense (Kuzyakov and Xu, 2013). Microorganisms often out-compete plant roots, but some plant species are able to bypass this competition by taking control of the N in their rhizosphere through the production of secondary metabolites that inhibit N cycle processes (Knops et al., 2002; Chapman et al., 2006; Subbarao et al., 2013; Bardon et al., 2016). For example, *Brachiaria humidicola*, by exuding some secondary metabolites (brachialactone), inhibits the nitrification process (Subbarao et al., 2009).

Nitrification is responsible for the conversion of ammonium to nitrate, which is rapidly converted into N_2O (a greenhouse gas) by denitrification and/or leached in soils (Di and Cameron, 2002). Denitrification, mostly carried out by bacteria, is the main form of nitrogen loss in most soils (Van der Salm et al., 2007; Radersma and Smit, 2011). Denitrification leads first to nitrate reduction, which is associated with two homologous enzymes, namely, transmembrane nitrate reductase (Nar) and periplasmic nitrate reductase (Nap), which are encoded by the *narG* and *napA* genes, respectively (the latter is not present in all denitrifiers). Depending on the microorganisms, nitrite reduction to nitric oxide (NO) involves two nitrite reductases, one coupled with copper (encoded by *nirK*) and the other coupled with cytochrome cd1 (encoded by *nirS*). The transformation of NO into N_2O (dinitrogen monoxide) involves NO reductase, which is a transmembrane enzyme encoded

by the *norB* gene (Wallenstein et al., 2006). Finally, N₂O reductase, which transforms N₂O into dinitrogen (N₂), is a periplasmic enzyme encoded by the *nosZ* gene (Khalil, 2003). However, this last step leading to the emission of N₂, which can be fixed by some plants, is not present in all denitrifiers (Galloway et al., 2004). In European agro-ecosystems, losses due to N₂O emissions account for 59% of the N loss of the system (Oenema et al., 2009). Recently, Bardon et al. (2014) demonstrated that the invasive species *Fallopia spp.* can inhibit denitrification activity in soils through the release of procyanidins, a phenomenon termed biological denitrification inhibition (BDI). Procyanidins specifically inhibit membrane-bound NO₃ reductase, inducing enzymatic conformational changes through membrane disturbance (Bardon et al., 2016). This strategy leads to a reduction in N₂O emissions from soil of up to 95% (Bardon et al., 2014). In addition, it has been shown that the addition of procyanidins to the soil under experimental conditions in mesocosms leads to a 6-fold increase in the amount of nitrate compared to untreated soil, which reduces denitrification activity without affecting either the respiration or mineralization activities of soil microbial communities (Bardon et al., 2016). This strategy could make nitrate more available in the soil, which may then be used by plants for their growth.

So far, the extent to which procyanidins can affect plant growth and nutrition *via* BDI under field conditions has not been investigated. Our hypothesis is that the addition of procyanidins to cultivated soils under field conditions induces BDI, with an increase in available nitrate and therefore a gain in productivity. To test our hypothesis, field experiments were carried out on cos or romaine lettuce crops (*Lactuca sativa*) at the SERAIL experimental station (Brindas, 69126 Rhône, France). Different concentrations of procyanidins were tested in the lettuce fields: 0, 8, 42, 83 and 210 kg ha⁻¹. Soil denitrification enzyme activity (DEA), nitrate concentration, above- and below-ground lettuce traits (shoot

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

2. MATERIALS AND METHODS

2.1. Plant growth and experimental design

A 202 m² field (composed of 24 plots of 1.4 m x 6 m (8.4 m²)) located at the SERAIL experimental station (Brindas, 69126 Rhône, France) (45°43'46.4"N 4°43'37.1"E) was used for growing romaine lettuces (*Lactuca sativa* var Lotus RZ), following a Fisher system of 4 plots per treatment (Preece, 1990). Lettuce seedlings were planted in three rows spaced 0.45 m apart and each lettuce spaced 0.45 m apart, for a total of 39 lettuces per plot (156 lettuces per treatment). Six treatments were considered: unplanted soil, soil with lettuce without procyanidin addition, and 4 lettuce crops treated with procyanidins at 8, 42, 83 and 210 kg ha⁻¹. These concentrations are based on those used in our previous studies *in vitro* (Bardon *et al.*, 2014, 2016). Firstly, we considered the dry weight of soil in the field at 10 cm of depth corresponding to lettuce seedling roots implantation. Secondly, we measured a surface relative to this weight, and then, we estimated the weight of 10 cm of soil per hectare. Finally, we calculated how much kg ha⁻¹ of procyanidins must be added to the plots to obtain the equivalent of 0.01, 0.05, 0.1 and 0.25 mg of procyanidins per gram of soil. The entire experiment was watered the first week after planting with 3 mm of water every day. For the next 10 days, 8 mm of watering was done per day. From then until the end of the experiment, 12 mm of watering was done every two days. Procyanidins were added 2 weeks after planting (stage 7-9 leaves) on a soil whose nitrate had been brought up to the

ZENIT grid standard, i.e. 45 kg-NO₃⁻ ha⁻¹ (Despujols, 1997). The commercial procyanidins (Laffort TANIN VR GRAPE[®], Bordeaux, France) were applied in aqueous solution (standard water) by 2 nozzle spray booms, such as to give 500 L/ha or 0.42 L per plot, between the rows of lettuce, and the soil was then hoed. The site was watered (with 8 mm) just after the addition of procyanidins.

2.2. Measurement of lettuce traits

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

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

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

The three leaves from the 4th leaf crown were scanned using Winfolia software (Regent Instruments Inc., Canada), weighed on a precision balance (± 0.001 g), and then used for biomechanical measurements (see point 2.2). The cut-out parts for biomechanical measurements were put back with the initial weighed material and finally dried at 68°C for 24 h and weighed again. The SLA was calculated by dividing the surface area by the dry mass (cm² g⁻¹). Each week, measurements of flavonoid and anthocyanin levels were made on the 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 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, 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

2.4. Denitrification Enzyme Activity (DEA)

Denitrification enzyme activity (DEA) was measured from pooled root-adhering soil (RAS) retrieved from 4 lettuces per plot and from 4 samples of bulk soil, according to Guyonnet et al. (2017). First, 5 g of soil was placed in 150 ml airtight plasma-flasks sealed with rubber stoppers. In each flask, air was removed and replaced with a He/ C₂H₂ mixture (90: 10, v/v) to create anoxic conditions and inhibit N₂O-reductase. A nutritive solution (1 ml) containing glucose (0.5 mg of C-glucose g⁻¹ of dried soil), glutamic acid (0.5 mg of C-glutamic acid g⁻¹ of dried soil) and potassium nitrate (50 mg of N-KNO₃ g⁻¹ of dried soil) was added to the soil. N₂O levels during incubation at 28°C were measured each hour for 6 h. The slope of the linear regression (R² on average greater than 0.98) was used to estimate DEA as the N₂O produced (g⁻¹h⁻¹). Gases (CO₂ and N₂O) were measured with a gas chromatograph coupled to a micro-catharometer detector (μGC-R3000; SRA instruments).

2.5. Nitrate concentrations in soil

NO₃⁻ was extracted every week over time (6 extractions per week) from the planted rhizospheric soils (20 cm from the base of 4 lettuces per plot and pooled) and from the unplanted soils. Nitrate was extracted from 5 g eq. of dried soil supplemented with 20 ml of a solution with 0.01 M of CaCl₂ (Houba et al., 2000). Briefly, soil suspensions were shaken at 140 rpm for 2 h at 10°C. The suspension was filtered (0.22 μm) and the NO₃⁻ concentration was quantified using an ionic chromatograph ICS-900 (Thermo Scientific Dionex, Sunnyvale, California, USA).

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

To determine whether procyanidins act specifically on the nitrate reductase (NarG) enzyme of denitrifiers, we used two nitrate reducers (but not denitrifier) strains: *Escherichia coli* MG1655 and *Corynebacterium glutamicum* ATCC 13032, and a denitrifying strain: *Pseudomonas brassicacearum* NFM421. The three strains were grown in 20 ml of Lysogeny Broth (LB) medium supplemented with KNO₃ (20 mM) and procyanidin at 0.01 mg ml⁻¹, or water as a control. The experiments were performed in plasma flasks sealed with a rubber stopper. To ensure anaerobic conditions, the air was removed as in denitrification measurements. Plasma flasks were inoculated with each strain independently in triplicate at Optical Density (OD) 0.1 and incubated at 28°C for *P. brassicacearum*, 37°C for *E. coli* and 30°C for *C. glutamicum*, with agitation (140 r.p.m.). Growth (OD measurement) and counts on Petri dishes (LB medium) were done from 1 ml of recovered medium at t₀, 2, 4, 25 and 30 hours of growth. The count was made using an automatic counter (Scan 1200, Grosseron, Coueron, France) from the 1 ml of medium recovered each time, and diluted by a factor of 10⁴. Cultured petri dishes were incubated at the same temperature as previously stated for each strain.

2.7. Quantification of total bacteria and denitrifier abundance

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

210 ATYGGCGGVCA YGGCGA-3') and nirK1040 (5'-GCCTCGATCAGRTTGTGGTT-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 CAGCMGCCGCGGTAA-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

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

2.8. Root development of lettuce seedlings on Knop agar

Lettuce seeds (*Lactuca sativa* var Lotus RZ) were disinfected according to Bressan et al. (2009), then cultured on Petri dishes (12 x 12 cm) with 100 ml of Knop agar (4mM Ca(NO₃)₂; 1.8 mM KH₂PO₄; 3.4 mM KCl; 1 mM MgSO₄; 1 µM ZnSO₄; 14 µM MnCl₂; 70 µM H₃BO₃; 10 nM CoCl₂; 0.5 µM CuSO₄; 0.2 µM Na₂MoO₄; 50 µM FeNa-EDTA; Agar concentration: 0.8%) (Hornschuh et al., 2002). Commercial procyanidins (Laffort, Bordeaux, France) were added with a 0.2 µm filter after Knop agar autoclaving. Three conditions were tested, one control condition without the addition of procyanidins and two conditions with 0.1 and 0.2 mg of procyanidins per ml of Knop agar, based on Bardon et al. (2016). For each condition, three Petri dishes containing 9 seeds (3 replicates and 27 pseudoreplicates) were used. Each Petri dish was grown in phytotron (SANYO, Osaka, Japan) at 22°C, with the day/night period set at 16 h/8 h. After 14 days of growth, the fresh mass of all roots was measured with a precision balance ± 0.0001g. Root development (root length and root surface) was analysed using WinRhizo software (Regent Instruments Inc., Canada), then placed in an oven at 68°C for 24 h to obtain the dry mass.

2.9. Data and statistical analyses

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

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

3. RESULTS

3.1. Denitrification activity and biological denitrification inhibition

The denitrification activity of microbial communities colonizing the RAS of lettuces treated with 210 kg ha⁻¹ of procyanidins was significantly lower (p-value = 0.023) than that of microbial communities of untreated lettuces. No significant differences in denitrification activity were observed between the RAS from treated lettuces with 8, 42 or 83 kg ha⁻¹ of procyanidins and that from untreated lettuces (Fig. 1A). In addition, the BDI at 210 kg ha⁻¹ was not significantly different from that at other concentrations. The decrease in denitrification activity at 210 kg ha⁻¹ of procyanidins represents a BDI of approximately 27.17% (p-value = 0.023) (Fig. 1B). In addition, untreated lettuce did not induce BDI compared to unplanted soil as they both presented the same level of denitrification activity. Moreover, the CO₂ emissions under these conditions did not differ between treatments

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

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

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

3.3. Abundance of total and denitrifying bacteria

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

3.4. Effect of soil procyanidin addition to soil on nitrate concentrations

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

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

3.5. Effect of procyanidin addition on lettuce traits

The fresh mass of the shoot and root parts of lettuces tended to increase with the addition of 0 to 210 kg ha⁻¹ procyanidins (Figs. 5 A and B). Indeed, the fresh mass of the shoot and root parts showed a significant dose-response relationship to procyanidins (p-value = 0.003 for the shoot mass and 0.0016 for the root mass) (Fig. 5C and D). The shoot fresh mass of the lettuces treated with 210 kg ha⁻¹ procyanidins was significantly (16%) higher per lettuce (on average 120 g) (p-value = 0.021) than that of untreated lettuces (Fig. 5A). Similarly, the root fresh mass of lettuces treated with procyanidins at 83 kg ha⁻¹ and 210 kg ha⁻¹ was 39% (p-value = 0.023) and 63% (p-value = 0.002) higher per lettuce, respectively, than the root fresh mass of untreated lettuces (Fig. 5B). In addition, the root dry mass of lettuces treated with procyanidins at 83 kg ha⁻¹ and 210 kg ha⁻¹ was significantly greater by 52% (p-value = 0.048) and 97% (p-value = 0.028) per lettuce, respectively, than the root dry mass of untreated lettuces. Moreover, the anthocyanin and flavonoid levels, as well as the SLA (cm² g⁻¹), did not differ between procyanidin- treated and untreated lettuces (Figs. S1 and S2), and the specific toughness (J m⁻³) did not differ among the three leaves of the 4th crown of treated and untreated lettuces (Fig. 6).

3.6. Nitrogen concentration in plants

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

obtain an average gain of 3.6 grams of nitrogen per lettuce treated at a concentration of 210 kg ha⁻¹.

3.7. Effect of procyanidins on root development in vitro

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

4. DISCUSSION

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

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

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

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

Although denitrification by archaea (Shoun et al., 1992) and fungi (Philippot, 2002) cannot be excluded, procyanidin amendment was demonstrated to have an impact on denitrifying bacteria, such as the *P. brassicacearum* NFM421 strain. Indeed, Bardon et al. (2016) demonstrated that procyanidins act specifically by inhibiting membrane-bound NO₃⁻ reductase, thereby inducing enzymatic conformational changes through membrane disturbance. However, some bacteria are not denitrifiers but do possess this enzyme (NarG), such as *E. coli* (Taniguchi and Itagaki, 1960) and *C. glutamicum* (Nishimura et al., 2007). Our results showed that the nitrate reductase of these non-denitrifiers was not affected by procyanidins (Fig. 2). Procyanidins act specifically on the NarG of denitrifiers that contribute to N₂O emissions. However, as other nitrate reducers are part of the rhizospheric microbial community, the *narG* gene cannot be used as marker for denitrifiers. Thus, we chose to target the *nirK* and *nirS* genes as markers for denitrifiers to assess the impact of procyanidins

on denitrifiers. At 210 kg ha⁻¹ of procyanidins, the proportion of denitrifying bacterial communities in the lettuce rhizosphere was reduced (Fig. 3A). The loss of the denitrification function is probably a disadvantage for the denitrifying bacterial community, which becomes less competitive, thus explaining their counter-selection in the rhizosphere. Denitrifiers can no longer use nitrate as an electron acceptor, which reduces their multiplication and their inclusion in the rhizosphere ecological niche, as observed in Dassonville et al. (2011). The fact that the total bacterial community abundance (Fig. 3B) and CO₂ release from the microbial community colonizing the RAS of treated lettuces did not change in the presence of procyanidins suggests that (i) other bacterial communities colonized the niches left vacant by denitrifying bacteria, as demonstrated by Jones and Hallin, (2010) and (ii) procyanidin addition did not have an antibacterial effect under field conditions, in contrast to the result of Mayer et al. (2008) and Lacombe et al. (2012), where procyanidins had antibacterial properties against some bacterial species.

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

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

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

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

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

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

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

5. Conclusions

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

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Figure legends:

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

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

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

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

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

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

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

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

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

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

686 lettuces 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 (cm²). 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) (cm² g⁻¹) of the 3 leaves of the 4th leaf crown (cm² g⁻¹). 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 (µg cm², black bar) and the anthocyanin level (µg cm², 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 revised 2

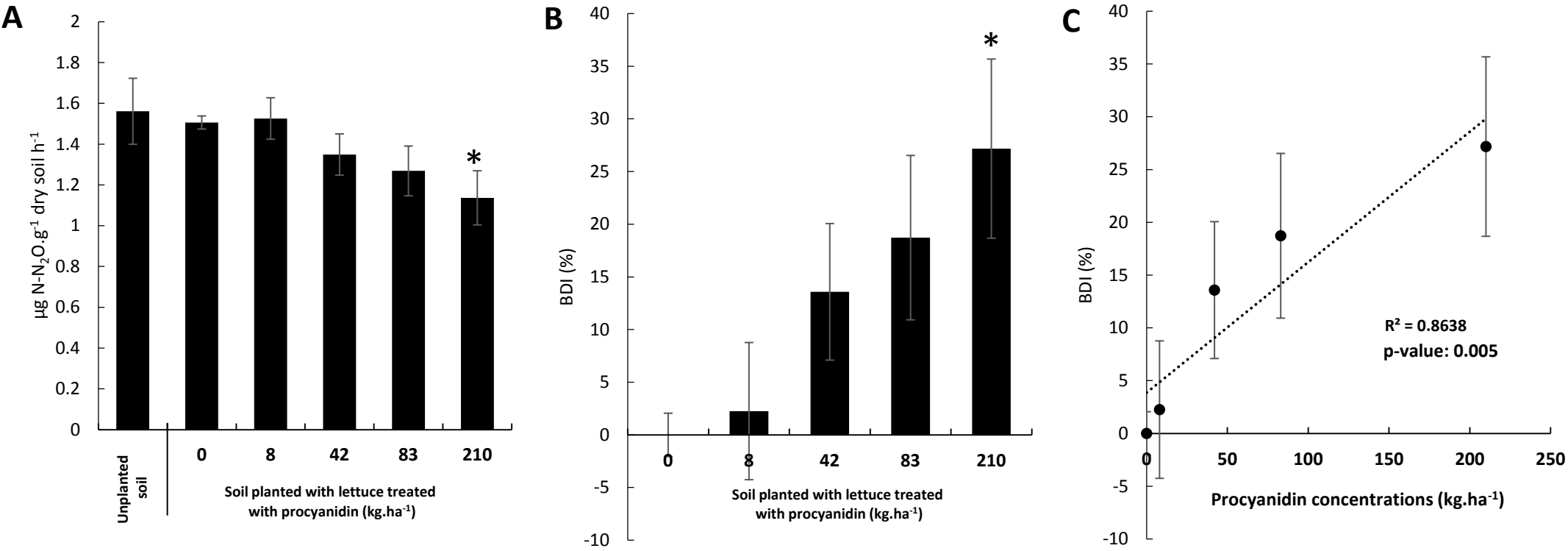


Figure 1

Figure 2

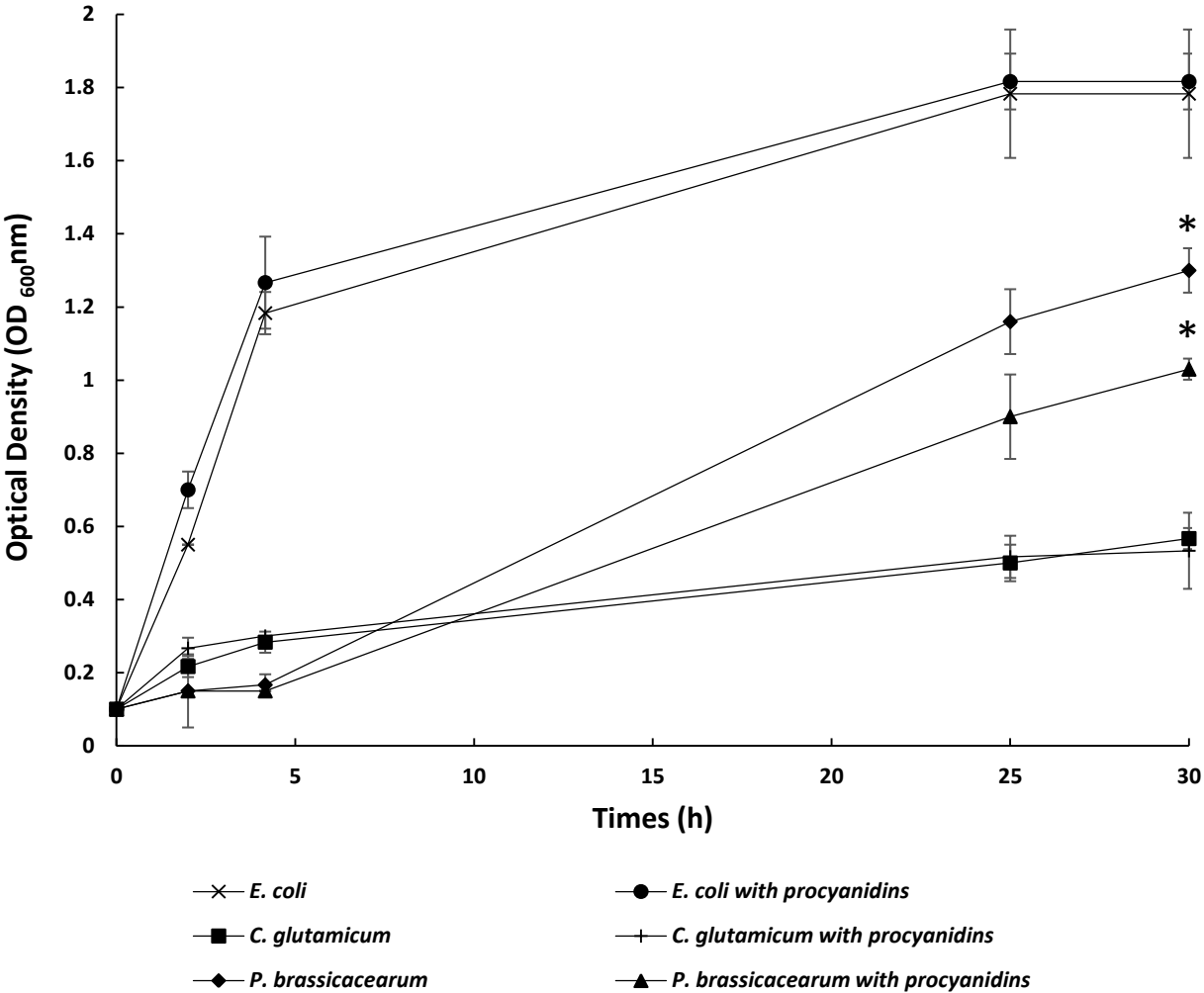


Figure 2

Figure 3 revised

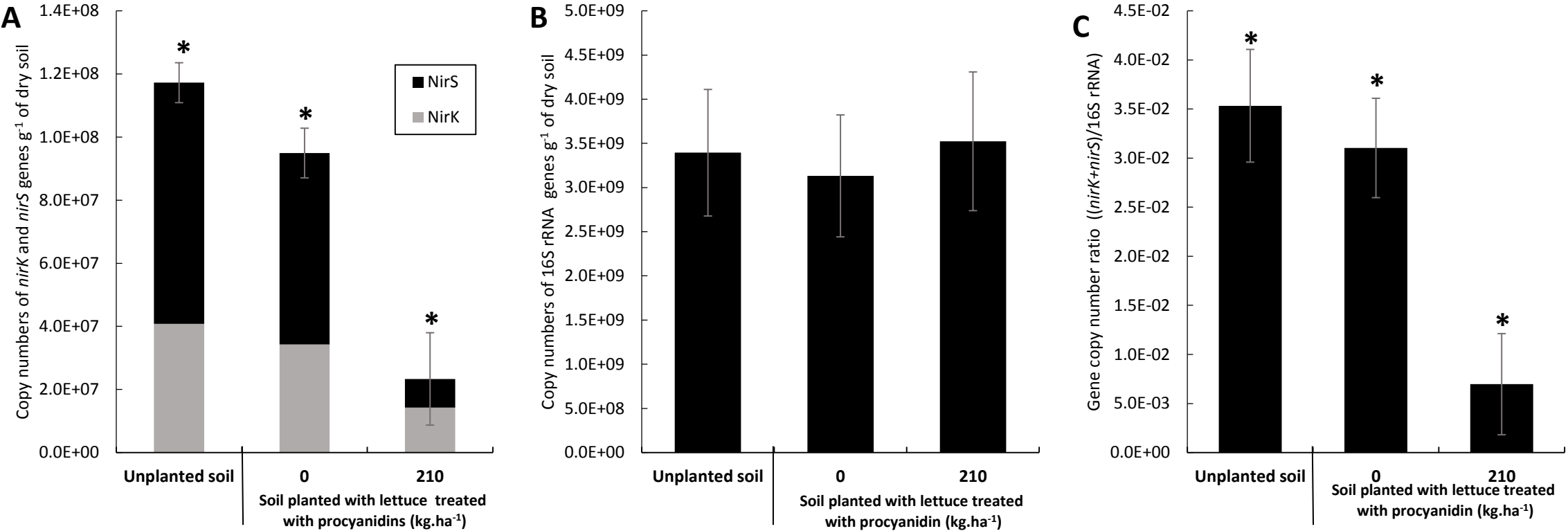


Figure 3

Figure 4 revised

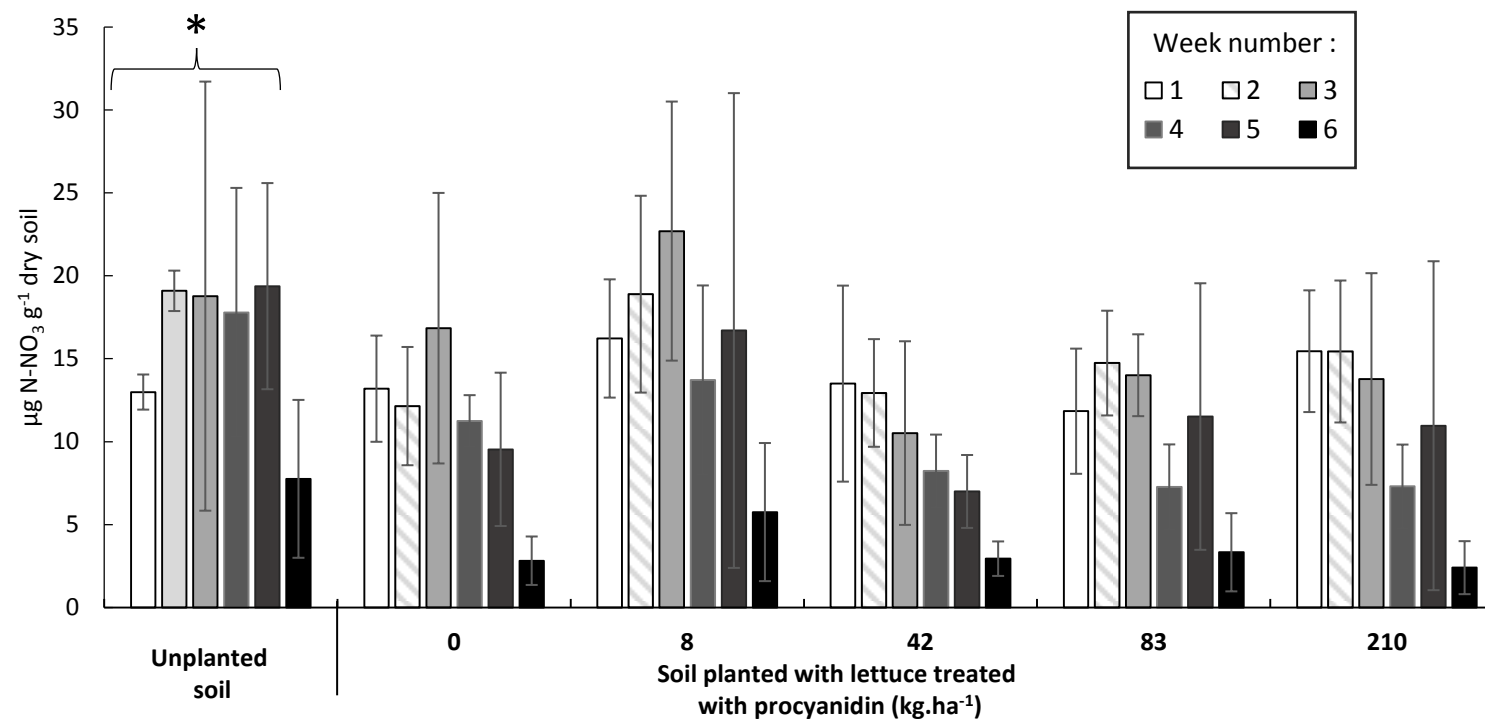


Figure 4

Figure 5 revised 2

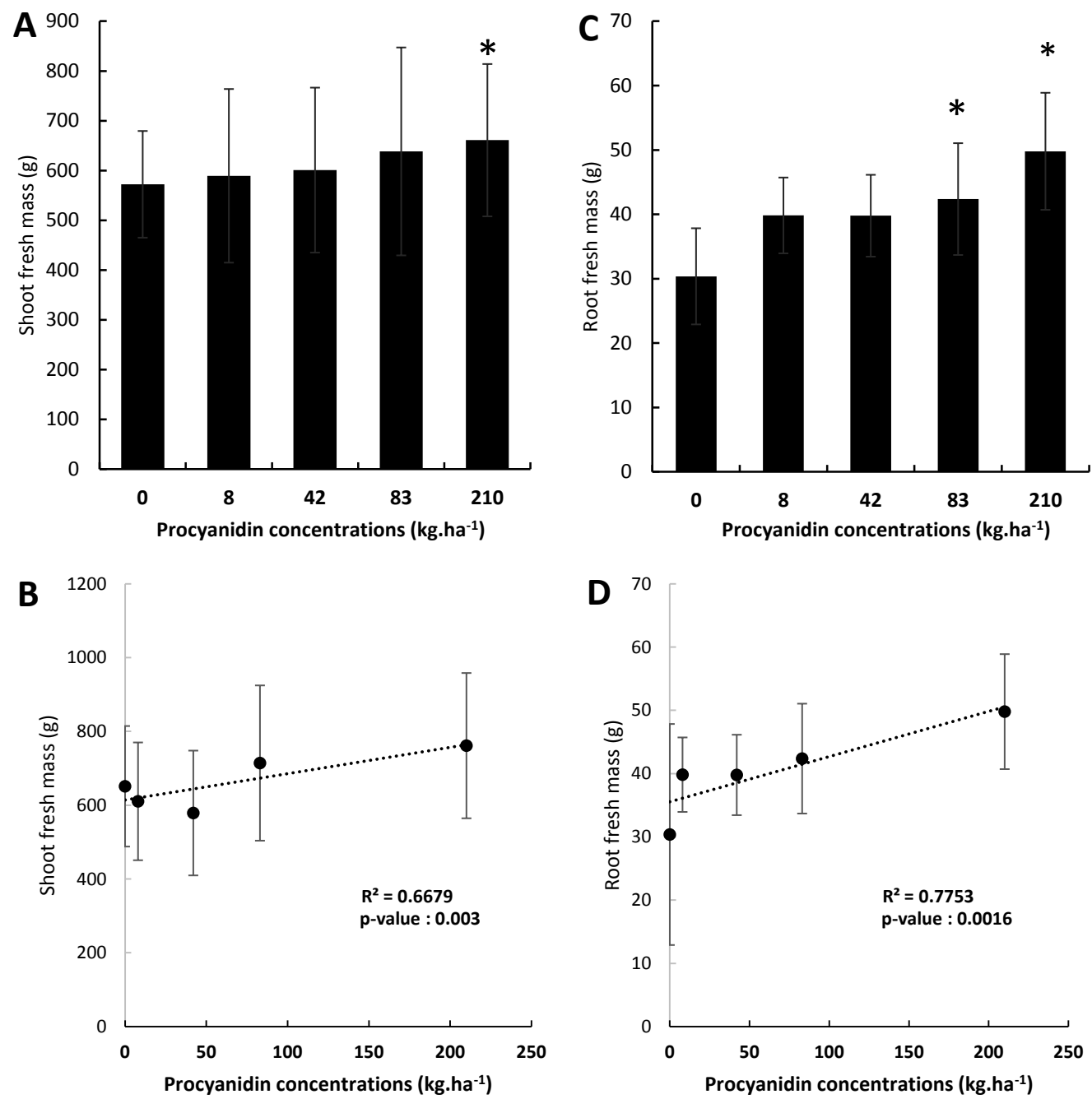


Figure 5

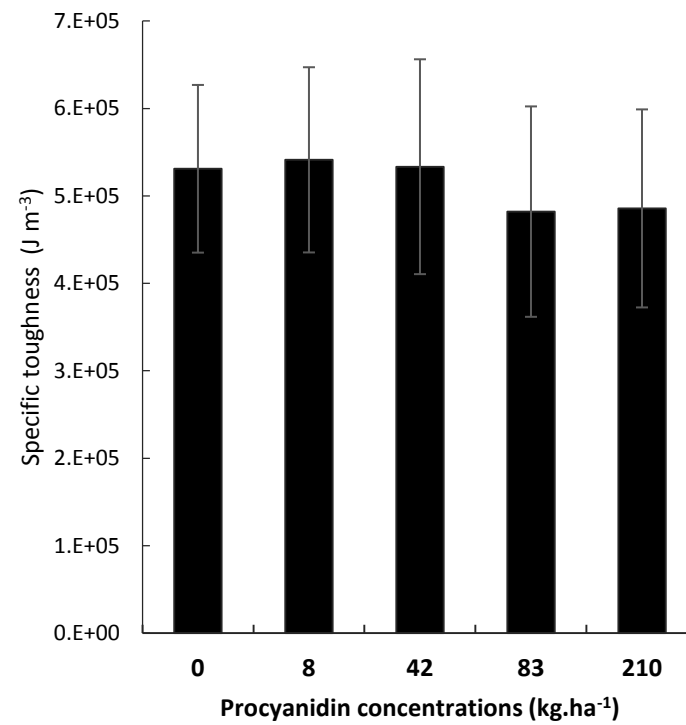


Figure 6

Figure 7

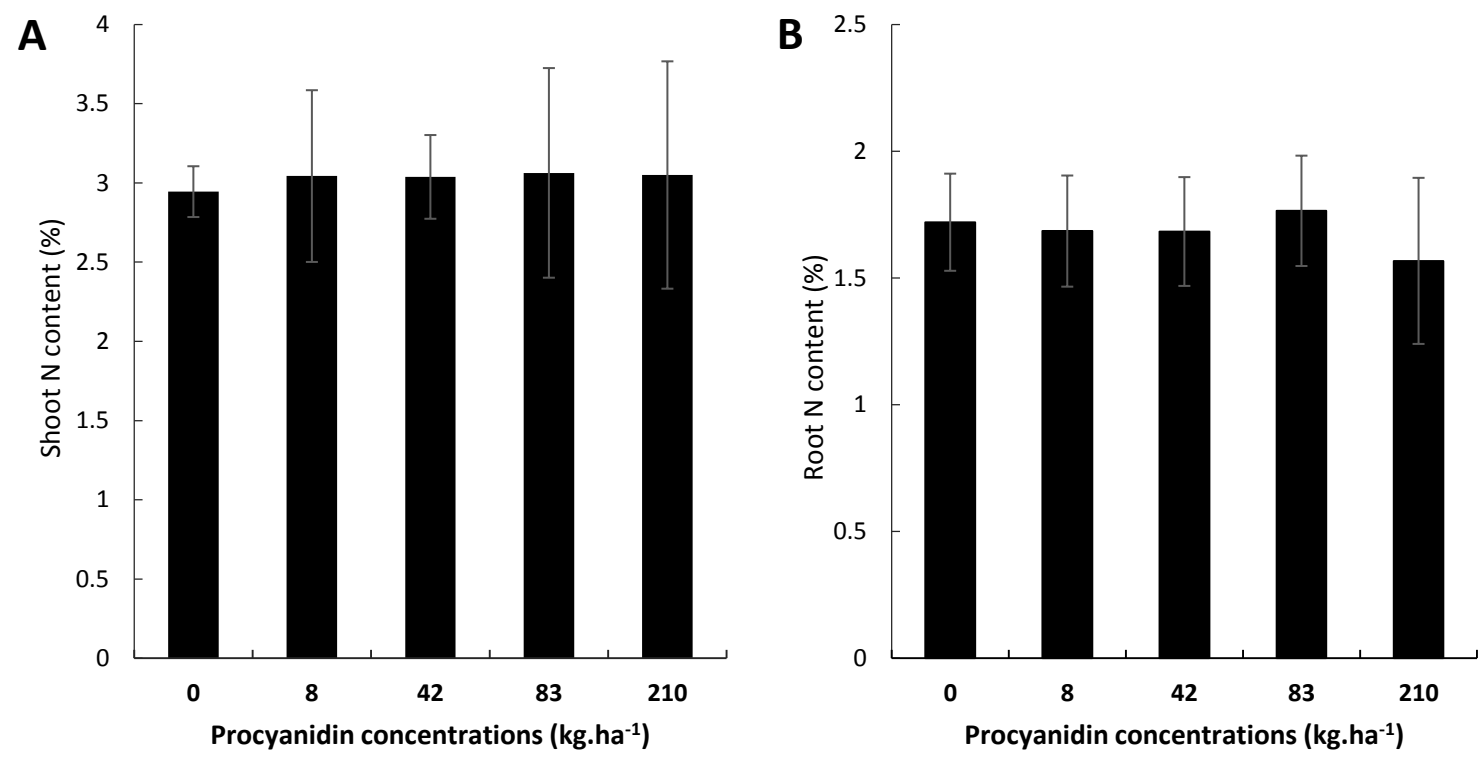


Figure 7

Figure 8 revised 2

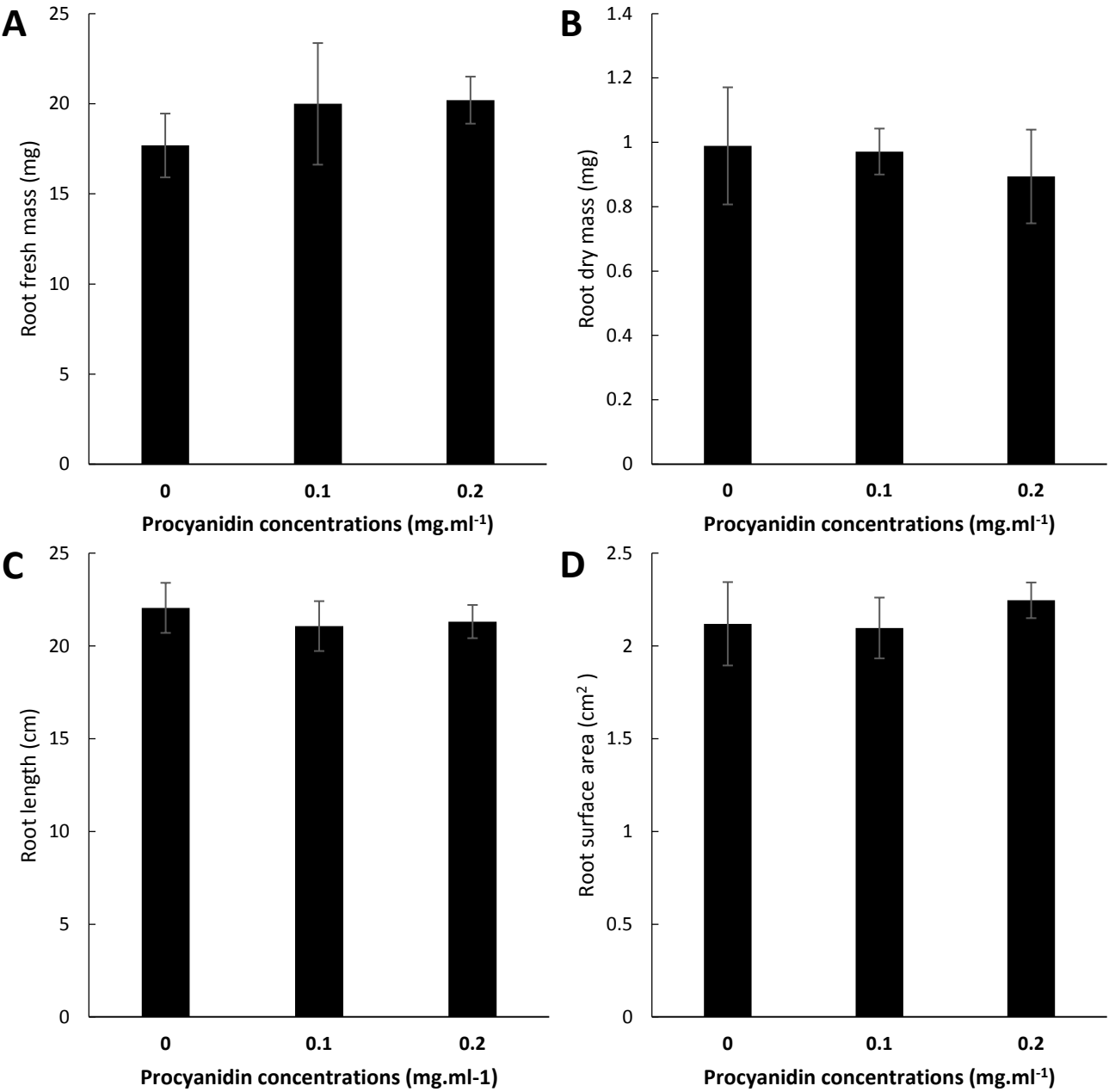


Figure 8

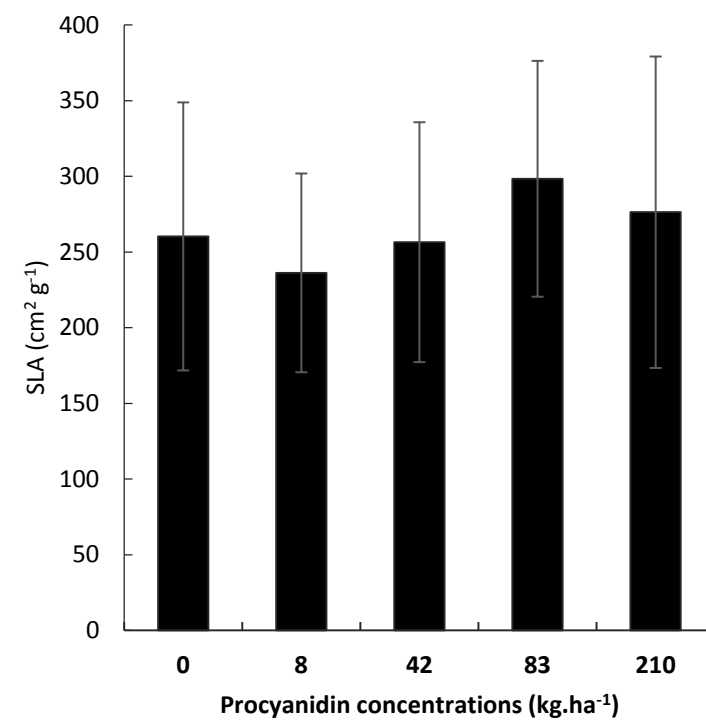


Figure S1

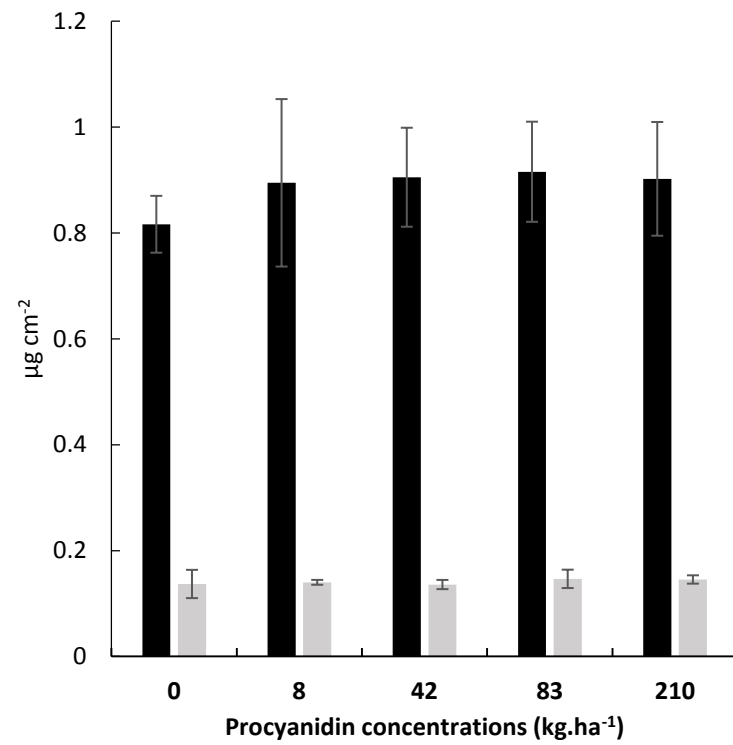


Figure S2