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1 **Biological inhibition of denitrification (BDI) in the field: effect on plant growth in two**
2 **different soils**

3

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18

19 **ABSTRACT**

20 Intensive agriculture uses increasingly large amounts of nitrogen fertilizers to
21 increase yields because nitrogen is one of the limiting factor of plant growth. Plants are in
22 direct competition with denitrifying bacteria for nitrate, but biological denitrification

23 inhibition (BDI) is a strategy developed by some plants in which procyanidins are produced
24 that inhibit denitrification. This phenomenon increases the available nitrate in the soil.
25 Previous results showed that the addition of procyanidins to lettuce fields allowed effective
26 BDI and increased soil nitrate and plant growth. However, agriculture is performed in
27 different soils, and the action of procyanidins could depend on the type of soil. In this study,
28 we tested the effect of procyanidin amendment on lettuce growth in two types of soils:
29 loamy sand and sandy clay loam. Our results show that in both soils, the addition of
30 procyanidins causes inhibition of denitrification, an increase in available nitrate, counter-
31 selection of denitrification communities and a gain in plant mass without modification of the
32 soil structure. This study highlights a sustainable agricultural method effective in a variety of
33 soils.

34

35 **Keywords :** Soil; denitrification; plant growth; soil texture and structure; lettuce

36

37 **1. INTRODUCTION**

38 In Europe, agriculture has expanded significantly over the past fifty years, following the
39 development of phytosanitary products (Tilman et al., 2002) and especially nitrogen (N)
40 fertilizers, allowing an enhance in agricultural yields (Casagrande and Chapelle, 2001) in
41 different soils with variable nitrate and ammonium concentrations. N depletion in soils is
42 partly due to increasingly intense agricultural activity, where many successive crops consume
43 the N initially present in the soil, without giving the ecosystem time to renew itself (Mulvaney
44 et al., 2009). These practices therefore deprive plants and crops of N: the most important but
45 above all the most limiting element for growth (Morot-Gaudry, 1997), which leads farmers to
46 use increasing amounts of N fertilizers (Tilman et al., 2002). These N fertilizers are used for

47 intensive agriculture but are also transferred from the ecosystem or used by soil
48 microorganisms (Kaye and Hart, 1997). Indeed, N in European soils is not entirely consumed
49 by plants and crops but is also subjected to denitrification, with 59% lost as the greenhouse
50 gas N₂O; leached into soils, representing a loss of 20%; and volatilized into NH₃, representing
51 a loss of up to 21% (Oenema et al., 2009). Ecological awareness led to the adoption in 1991
52 of a directive by the European Council to regulate the quantities of N added to the soil (1991:
53 Directive 91/676/EEC; hereafter referred to as NiD). The main purpose of this nitrate
54 directive was to reduce the risk of N leaching, but this does not reduce denitrification losses
55 due to denitrifying bacteria in the soil. Indeed, denitrification by microorganisms i.e. fungi
56 (Philippot, 2002), archaea (Shoun et al., 1992) and bacteria (Lecomte et al., 2018) transforms
57 nitrate into N₂O a greenhouse gas representing a significant loss for the ecosystem (Mosier et
58 al., 1996) and up to N₂ for some bacteria (Kuypers et al., 2018). However, Bardon et al.
59 (2014) describes a phenomenon involving an invasive plant (*Fallopia spp*), which is able to
60 inhibit denitrifying bacteria in the soil *via* exudation at the root level of a flavonoid tannin
61 called procyanidin (Bardon et al., 2016a) and therefore reduce N losses by denitrification.
62 This phenomenon, called biological denitrification inhibition (BDI), therefore reduces the
63 activity and abundance of denitrifying bacteria and increases the nitrate pool in the soil, which
64 can then be a source of N other than fertilizers for crops (Bardon et al., 2016b). Indeed, in
65 Galland et al. 2019, it was demonstrated that in sandy clay loam-textured soil, the addition of
66 procyanidins to the field induced BDI, decreased the abundance of denitrifying communities
67 and allowed nitrate conservation in the soil, which was then used for lettuce farming to
68 increase lettuce growth. These crops, as well as BDI and denitrification, could be soil
69 dependent. Indeed, in France, there is a great diversity of soils (Jamagne, 2011), which differ
70 from each other based on structure, i.e., the spatial arrangement of soil components (Rabot et
71 al., 2018); texture, i.e., the granulometric distribution of soil mineral constituents (Batey and

72 McKenzie, 2006); and the bacterial community (Nannipieri et al., 2003; Fierer and Jackson,
73 2006; Schreiter et al., 2014). These differences in soils play a role in the quality of crops but
74 also in denitrifying bacterial communities. These bacteria may differ according to the soil and
75 not have the same diversity and/or abundance, which may then change the use of N in the soil
76 and its future in the soils (Cavigelli and Robertson, 2000). In addition, the distribution of
77 porosities based on soil structure can change the water content and oxygen availability
78 (Gliński et al., 2004), which play a role in denitrification, which is an anaerobic process
79 (Kraft et al., 2011). Thus, differences in soil based on texture, structure and bacterial
80 communities, among other parameters, can modify denitrification and the effect of
81 procyanidins on denitrification activity, which, similar to other organic molecules, exhibit
82 variable behaviours and affinities depending on the soil with which they are in contact
83 (Lützow et al., 2006). In this study, we aimed to test the effect of two different soils that
84 theoretically do not have the same texture, structure and bacterial community on the action of
85 procyanidins and consequently on BDI and traits related to lettuce growth. The assumption is
86 that procyanidin amendment of any soil can induce BDI, which allows an increase in nitrate
87 in the soil and thus an increase in the traits of lettuce. To test these hypotheses, two fields
88 were used: one at the SERAIL Experimental Station (Brindas, 69126 Rhône, France), with a
89 sandy clay loam soil texture, and an agricultural field in Manziat (01570 Ain, France), where
90 the soil has a loamy sand texture. During the summer of 2018, the same variety of Batavia
91 lettuce was planted in both fields in parallel, and the planted and unplanted soils were
92 amended with procyanidins at three concentrations (0, 87 and 210 kg ha⁻¹). Soil denitrification
93 enzyme activity (DEA), soil nitrate concentration, shoot and root morphological traits (fresh
94 masses of aerial and root parts, biomechanical characteristics of leaves and the N content of
95 tissues), the abundance of total bacteria and the denitrifying community, and the porosities of
96 the soils used were measured in lettuce and soil with or without procyanidin amendment.

97

98 **2. MATERIALS AND METHODS**

99 **2.1. Plant growth and experimental design**

100 Lettuce (*Lactuca sativa* var Loubresac, Earl LIMOUSIN) was grown in two fields at the same
101 time in July 2018 following a Fisher system with 4 plots per treatment (Preece, 1990); the
102 fields were located in an agricultural field in Manziat (01570 Ain, France) (46°22' 2.28 "N,
103 4°54' 3.671 "E) with loamy sand soil (sand: 82.5%, silt: 8.9% and clay: 8.5%; pH : 7.2; cation
104 exchange capacity (CEC): 60 meq kg⁻¹; organic carbon: 9.9 g kg⁻¹) and at the SERAIL
105 Experimental Station (Brindas, 69126 Rhône, France) (45°43'46.4 "N, 4°43'37.1 "E) with a
106 sandy clay loam soil (sand: 68.7%, silt: 21.9% and clay: 9.5%; pH : 7.1; CEC: 58 meq kg⁻¹;
107 organic carbon: 6.9 g kg⁻¹). In both fields, the experimental design was the same: lettuce was
108 planted in 4 parallel rows of 20 lettuces in 6 m x 1.4 m plots (8.4 m²), with an unplanted area
109 of 2 m x 1.4 m (2.8 m²) for measuring the corresponding characteristics of soil without plants.
110 For soils with or without lettuce, 3 treatments were applied: unamended without procyanidins
111 and amended with procyanidins at the two concentrations found to be most effective in
112 Galland et al. (2019): 87 kg ha⁻¹ and 210 kg ha⁻¹. The two experiments were watered the first
113 week after planting with 3 mm of water every day. For the next 10 days, 8 mm of water was
114 applied per day. From then until the end of the experiment, 12 mm of water was applied every
115 two days. Procyanidins were added 2 weeks after planting (stage with 7-9 leaves) to soil
116 whose nitrate had been brought to the ZENIT grid standard, i.e., 40 kg NO₃⁻ ha⁻¹ (Despujols,
117 1997). Commercial procyanidins (Laffort TANIN VR GRAPE ®112, Bordeaux, France)
118 were applied in aqueous solution (standard water) by 2 nozzle spray booms to provide 500 L
119 ha⁻¹ or 0.42 L per plot, between the rows of lettuce, and the soil was then hoed. Each site was
120 binned and slightly watered (with 8 mm) just after the addition of procyanidins.

121

122 **2.2. Denitrification Enzyme Activity (DEA)**

123 Briefly, as described by Galland et al. (2019), DEA was measured for both fields from 5 g dry
124 equivalent of pooled root-adhering soil (RAS) retrieved from 4 lettuces per plot and 4 samples
125 of bulk soil from unplanted plots with or without amendment, the day of the final harvest.
126 BDI (%) was measured by calculating the percentage of denitrification activity inhibition
127 recorded by DEA compared to the corresponding unamended control.

128

129 **2.3. Quantification of total bacterial and denitrifier abundance**

130 Total DNA was extracted from 0.5 g of RAS from three treatments (unplanted soil, soil with
131 lettuce, and soil with lettuce amended with only the higher concentration of 210 kg of
132 procyanidins per hectare) in triplicate according to the manufacturer's protocol with a Fast
133 DNA Spin Kit for Soil (MP Biomedical, Solon, OH, USA). The amount of DNA extracted
134 was then estimated using a Quant-iT PicoGreen® dsDNA Assay kit (Molecular Probes,
135 USA). Total bacterial and denitrifier abundances were quantified by qPCR using primers
136 targeting the 16S rRNA and *nirK/nirS* genes, respectively, as described previously by Galland
137 et al. (2019).

138

139 **2.4. Nitrate concentrations in soil**

140 Briefly, NO₃⁻ was extracted one week before procyanidin application (T0) from the planted
141 rhizospheric soils (20 cm from the base of 4 lettuces per plot and pooled) and on the day of
142 harvest (TF) from the RAS soils and from the unplanted soils for both fields. Nitrate was
143 extracted from 5 g eq. of dried soil according to Galland et al. (2019).

144

145 **2.5. Measurement of lettuce mass**

146 After 5 weeks of growth in the Manziat field and 6 weeks of growth in the SERAIL field, 12
147 lettuces per plot or 48 lettuces per treatment were harvested and weighed to determine the
148 fresh mass of the aerial parts. Among these 12 lettuces, 4 root systems were harvested (or 16
149 per treatment) to determine the mass of the root parts. Each fresh lettuce was weighed on a
150 balance (± 0.5 g). The root system was washed with distilled water and weighed with a
151 precision balance (± 0.001 g), dried at 68°C for 24 h and weighed again on the same balance
152 in order to determine dry mass.

153

154 **2.6. Leaf mechanical properties**

155 One piece of tissue was cut from each leaf (4 leaves per plot for a total of 16 leaves per
156 treatment in each field) for biomechanical measurements. Specific leaf toughness was
157 measured in accordance with Galland et al. (2019).

158

159 **2.7. Plant N content**

160 As described in Galland et al. (2019), the total N concentration was measured using an
161 elemental analyser (FlashEA 1112, Thermo Scientific, Waltham, USA) from 4 leaves and 4
162 root systems per plot (16 per treatment in each field) using 2 mg of ground root or leaf. The
163 leaf and/or root N contents was expressed in percentage of N in the leaf and/or root dry mass.

164

165 **2.8. Soil porosity measurement**

166 For each treatment, to determine volumetric soil water moisture after capillary rise and bulk
167 density, four undisturbed soil samples were collected using a stainless steel cylinder of 100

168 cm³ (5.6 cm diameter, 4.05 cm height) with bevelled edges to ease insertion from unplanted
169 soil or rhizospheric soil. The core samples were capped at both ends with two plastic lids to
170 minimize soil and evaporative loss until analysis. After removing the two plastic lids, the core
171 samples were placed on a geotextile. This geotextile had the ability to let water but not soil
172 particles pass through. The geotextile was placed on a PVC rigid support with large mesh.
173 The geotextile was 3 cm from the bottom of large plastic bowls. Water was added to the
174 plastic bowls until the level of water obtained was 2 mm higher than the bottom of the base of
175 the core samples. The bowls were closed to prevent evaporation during the time of the
176 experiment (approximately 34 h), and volumetric soil moisture after capillary rise and bulk
177 density were then determined in the 100 cm³ cylindrical soil cores (V) by successive weighing
178 of the wet and 105°C-oven-dried soil cores. The volumetric soil water content after water
179 capillary rise represents the water retained in the aggregates by capillary forces and the
180 ‘immobile’ water, which moves slowly through the network of micropores (Chen et al., 1993)
181 and fills capillary pores occurring within soil aggregates. The micropores between particles
182 and microaggregates belonging to the elementary fabric units are referred to as capillary
183 porosity in this paper. Capillary porosity, expressed in cm³ cm⁻³, was estimated by subtracting
184 the 105 °C-oven-dried soil weight from the wet soil weight and normalizing the difference to
185 the 105 °C-oven-dried soil weight and the volume of the cylindrical soil cores (V) thereafter.
186 The bulk density of each soil core was determined as the ratio between the total 105 °C-oven-
187 dried soil weight and the soil cylinder volume V. The total porosity was calculated from the
188 bulk density and solid density thereafter (assuming that the solid density was 2.65 g cm⁻³).
189 Macropores between the units of fabric, referred to as macroporosity and expressed in
190 cm³.cm⁻³, represent the rapid water transport and aeration of the soil. Macroporosity was
191 calculated as the difference between total porosity and capillary porosity.

192

193 **2.9. Data and statistical analyses**

194 The difference in lettuce morphological traits ($n > 48$) between treatments was determined
195 using analysis of variance (ANOVA) followed by a post hoc (Tukey's HSD) test. Similarly,
196 the normality (Shapiro test) and homoscedasticity of the variance (Fischer test) of all the
197 variables were tested. The significance of microbiological traits, nitrate concentration, soil
198 measurement and N level in plant ($n > 3$) between treatments was tested non-parametrically
199 with a Kruskal-Wallis test followed by a post hoc Nemenyi test. The same statistical tests
200 were used to measure the differences between equivalent treatments and between the two
201 fields, and the plot effect was tested and discarded for all traits (two-way ANOVA and mixed
202 model). All the analyses were performed using R project software (v. 3.5.0).

203

204 **3. RESULTS**

205 **3.1. Biological denitrification inhibition**

206 As shown in Figure 1, in soil from the Manziat field with 87 kg ha^{-1} procyanidin amendment,
207 a BDI of approximately 31% (p-value: 0.014) was observed compared to the unplanted soil
208 without procyanidin addition. However, no significant BDI was observed in response to the
209 210 kg ha^{-1} procyanidin amendment. In the soil planted with lettuce, no significant BDI with
210 the 87 kg ha^{-1} procyanidin amendment compared to the planted soil without procyanidin
211 amendment was observed, whereas BDI (p-value: 0.03) of approximately 31% was observed
212 for the highest concentration (210 kg ha^{-1}). In the unplanted soil in the SERAIL field, there
213 was BDI in the plots amended with 87 and 210 kg ha^{-1} procyanidins of 24% and 32%,
214 respectively (p-value: 0.048), compared with the unamended soil. In addition, in planted soils,
215 there was BDI of 27% (p-value: 0.047) relative to unamended planted soil for the 87 kg ha^{-1}

216 procyanidin-amended lettuce and soils and BDI of 33% (p-value: 0.04) for the 210 kg ha⁻¹
217 procyanidin-amended lettuce and plots. When comparing both fields, we noticed that in the
218 unplanted and planted soil, BDI was induced for each tested concentration and was more
219 important at the highest concentration, except for the BDI observed in unplanted and amended
220 soil with procyanidin addition of 87 kg ha⁻¹, where the BDI was larger and different from than
221 in planted soil planted with the same procyanidin concentration.

222

223 **3.2. Abundance of total and denitrifying bacterial communities**

224 In the Manziat and SERAIL fields, the total bacterial communities measured by the 16S
225 rRNA gene (Fig. 2A) did not differ statistically between treatments, although there was a
226 tendency at the SERAIL field for the total bacterial abundance to be reduced in the presence
227 of lettuce plants. In the Manziat field, the abundance of the *nirS* gene (Fig. 2B) was identical
228 in the unamended soil and in the RAS of unamended lettuce and amended lettuce at 210 kg
229 ha⁻¹ procyanidins. However, the abundance of the *nirS* gene was lower in lettuce amended
230 with 210 kg ha⁻¹ procyanidins than in unamended lettuce. In the SERAIL field (Fig. 2B), there
231 were generally fewer bacteria harbouring the *nirS* gene than in the Manziat field, although the
232 abundance of the community with the *nirS* gene in the RAS soil of unamended lettuces was
233 identical to that in the unamended and planted soil. The abundance of *nirS* genes was higher
234 in the unamended soil than in the RAS of lettuce amended with procyanidins at 210 kg ha⁻¹
235 (p-value: 0.018). In Manziat, the abundance of bacteria harbouring the *nirK* gene (Fig. 2C)
236 was identical between unplanted soil and unamended planted soil but lower in planted soils
237 amended with procyanidins (p-value: 0.013). At SERAIL, the abundance of the *nirK* gene
238 (Fig. 2C) was higher in unplanted soil (p-value: 0.0229) than in planted soil with or without
239 procyanidins. The amendment of procyanidins slightly reduced the abundance of bacteria in
240 the RAS of lettuce but not significantly compared to the RAS of unamended lettuce.

241

242 **3.3. Effect of procyanidin addition to soil on nitrate concentrations**

243 In the Manziat field on date T0 (Fig. 3A), nitrate concentrations were equivalent for unplanted
244 soils and similar for planted soils. In addition, the nitrate concentrations in planted soils were
245 significantly lower than those in unplanted soils. On the TF date, overall, the nitrate
246 concentrations of the planted soils were significantly lower than those of the unplanted soils.
247 However, at 87 kg ha⁻¹, the nitrate concentration in unplanted soil was increased by 113 % (p-
248 value: 0.05) compared to that in unplanted and unamended soil. Moreover, the nitrate
249 concentration was not significantly different (59 % higher in unplanted and amended soil at
250 210 kg ha⁻¹). In all treatments, there was a decrease in the nitrate concentration between T0
251 and TF, except for soils unplanted and amended with 87 or 210 kg ha⁻¹ procyanidins. In the
252 SERAIL field (Fig. 3B), the nitrate concentrations at T0 were identical between planted soils,
253 but not in the unplanted soil without amendment, in which the concentration was lower (p-
254 value: 0.041). In addition, there was no difference in the nitrate concentration in unplanted
255 soils regardless of the concentration at T0. At TF, the nitrate concentrations in planted soils
256 were identical, regardless of the concentration, and significantly lower than those in unplanted
257 soils. In unplanted soils, the nitrate concentration of the soil amended with 87 kg ha⁻¹
258 procyanidins was higher than the concentration of the unplanted soil amended with 210 kg ha⁻¹
259 procyanidins (p-value: 0.047) and 142 % higher than that in the unplanted and unamended
260 soil (p-value: 0.046). Similarly, the nitrate concentration of the soil amended with
261 procyanidins at 210 kg ha⁻¹ was higher (by approximately 57 %) than that of untreated soil (p-
262 value: 0.049). It should be noted that between T0 and TF, all nitrate concentrations decreased
263 except for that in the unplanted and unamended soil and the unplanted soil with amendment at
264 87 kg ha⁻¹.

265

266

3.4. Effect of procyanidin addition on lettuce traits

267 In Manziat (Fig. 4A), the addition of 87 kg ha⁻¹ and 210 kg ha⁻¹ procyanidins increased the
268 fresh mass of the aerial parts of lettuce by 46% (p-value: 0.00012) and 33% (p-value: 0.025),
269 respectively, compared to unamended lettuce. Similarly, at SERAIL (Fig. 4A), compared to
270 the fresh mass of unamended lettuce, that of lettuce with the addition of procyanidins
271 increased by 16% (p-value: 0.092) with 87 kg ha⁻¹ and 25% (p-value: 0.00053) with 210 kg
272 ha⁻¹. In Manziat (Fig. 4B), amendment with procyanidins at 87 kg ha⁻¹ allowed a increase (p-
273 value: 0.0000026) in the fresh mass (approximately 73%) of roots compared to no
274 amendment. At SERAIL (Fig. 4B), amendment with 87 kg ha⁻¹ procyanidins allowed an
275 increase (p-value: 0.15) in the root fresh mass (approximately 22%), and amendment with 210
276 kg ha⁻¹ procyanidins allowed a increase (p-value: 0.017) in the fresh mass (approximately
277 28%) of roots compared to no amendment. Moreover, the specific toughness (J.m⁻³) of the
278 three leaves of the 4th crown did not differ between treated and untreated lettuces in either
279 field (Fig. 5). However, overall, the specific toughness of the leaves of the 4th crown in
280 Manziat was higher than that at SERAIL.

281

282

3.5. Nitrogen concentration in plants

283 In Manziat and at SERAIL (Fig. 6A), the N content in the leaves of amended lettuce was the
284 same, regardless of whether they were amended. However, the N content decrease from 2.1 to
285 1.8 % of N with the amendment concentration. Based on the gains in fresh mass of the aerial
286 parts of the lettuces, we can deduce that there was, on average, 1.46 g more N per lettuce for
287 the treatment with 87 kg ha⁻¹ procyanidins and 0.63 g more N for the treatment with 210 kg

288 ha⁻¹ procyanidins than in unamended lettuces. Similarly, in the SERAIL field, there was, on
289 average, 0.94 g more N per lettuce with 87 kg ha⁻¹ procyanidins and 0.56 g with 210 kg ha⁻¹
290 procyanidins than in unamended lettuces. In Manziat (Fig. 6B), the N content in lettuce roots
291 between treatments was the same. At SERAIL, although not significantly different, the N
292 concentrations seemed lower when lettuce was amended with procyanidins.

293

294 **3.6. Effect of procyanidins addition on soil porosities**

295 In Manziat, procyanidin amendment of planted and unplanted soils did not significantly
296 change the total porosity (Fig. 7A) or the capillary porosity (Fig. 7C). However, the
297 macroporosity (Fig. 7E) of the planted soil amended with procyanidins at 87 kg ha⁻¹ was
298 lower than the macroporosity of the unamended planted soil (p-value: 0.0083572), and
299 although not significant, it was also lower in the planted soil amended with 210 kg ha⁻¹
300 procyanidins. At SERAIL, procyanidin amendment of planted and unplanted soils did not
301 change the total porosity (Fig. 7B), capillary porosity (Fig. 7D) or macroporosity (Fig. 7F).

302

303 **4. DISCUSSION**

304 **4.1. The addition of procyanidins induces BDI in soils of both textures**

305 As demonstrated in Galland et al. 2019, the addition of procyanidins to lettuce fields reduced
306 denitrification activity. This reduction was observed for both soil textures. In the SERAIL
307 field with a sandy clay loam-textured soil (the same soil as used in Galland et al. 2019), the
308 addition of procyanidins induced BDI from one year to the next, on the same orders of
309 magnitude (between 16 and 32%, depending on the procyanidin concentrations in the soil). In
310 addition, the results presented here showed that denitrification activity was also inhibited by

311 the addition of procyanidins to the loamy sand soil of Manziat, and the inhibition percentages
312 in the planted soils were the same between the two fields used in the experiment. Each plant
313 selects its antagonistic microorganisms, as described by Berg and Smalla (2009), which
314 would explain in our case the decrease in the abundance of the total bacterial community in
315 the RAS of lettuce with or without procyanidins in the SERAIL field, although as shown by
316 Buyer et al. (2002), plants such as soybean and corn increase the total community. This
317 phenomenon was not observed in Galland et al. (2019) because, as Dandie et al. (2008)
318 shows, bacterial communities in arable soil are likely to change from one year to the next.
319 However, the abundance of the total bacterial community did not differ between the RAS of
320 lettuce amended or unamended with procyanidins for either soil texture, which indicates that
321 the addition of procyanidins did not have an antimicrobial effect on the soil microfauna, as
322 shown by Lacombe et al. (2012), in which a procyanidin concentration much higher than that
323 used here had antimicrobial effects on some bacterial species. However, procyanidins act on
324 the abundance of denitrifying bacterial communities. As described by Wallenstein et al.
325 (2006), denitrifying bacterial communities have either copper-coupled nitrate reductase
326 (encoded by *nirK*) and the other with cytochrome cd1 (encoded by *nirS*), which is why we
327 targeted these two genes to measure denitrifier abundance. Thus, at a procyanidin
328 concentration of 210 kg ha⁻¹, whether in sandy clay loam or loamy sand, denitrifying
329 communities with the *nirK* gene and the *nirS* gene were less abundant in procyanidin-
330 amended soil than in unamended soil, identical to the result in Galland et al. (2019), despite
331 the fact that denitrifying communities can change from one year to the next, as Yoshida et al.
332 (2009) showed. This leads to the hypothesis that the loss of the denitrifying function of
333 bacteria *via* the BDI phenomenon would cause a lack of competitiveness and would thus be
334 counter-selected from the rhizosphere of amended lettuce. Denitrifying bacteria could no
335 longer use nitrate as an electron acceptor, which would reduce their multiplication and

336 persistence in the ecological niche of the rhizosphere, as observed in Dassonville et al. (2011).
337 We also observed that the abundances were not the same between the two soil textures; there
338 were more bacteria in the loamy sand soil of Manziat with the *nirS* gene and fewer with the
339 *nirK* gene than at the SERAIL, as Rosa et al. (2014) showed, indicating that the distribution
340 of denitrifier diversity is not the same among soils.

341 **4.2. The addition of procyanidins induces nitrate conservation and an increase in** 342 **lettuce morphological traits for both soil textures**

343 Bardon et al. (2017) showed in mesocosms that in a soil with a sandy clay loam texture,
344 procyanidin-induced BDI allows nitrate conservation in the soil of up to 6 times compared to
345 that in the same soil that has not been amended with procyanidins. In our experiment, there
346 were similar nitrate conservations in both soils at both concentrations, which were
347 homogeneous between the two soils. This conservation after BDI allowed the lettuces to grow
348 better in the amended soils, as observed in the previous year with another variety of lettuce. In
349 our case, after adding procyanidins, we observed that in the planted soils in the two fields,
350 lettuce consumed the nitrate present in the soil between the beginning and the end of the
351 experiment, as Jackson et al. (1994) observed in lettuces that use nitrate as the preferred
352 source of N during growth. In addition, in the unplanted soils of the two fields, there was a
353 loss of nitrate between the first measurement at the beginning of the experiment and the
354 second measurement taken at harvesting. Such loss over time is due to N losses in the system
355 such as leaching (Di and Cameron, 2002), denitrification (Kuypers et al., 2018), but this loss
356 was less significant when soils were amended with procyanidins, as expected. The protocol
357 tried to limit these losses by applying procyanidins to the increased N requirements of lettuce
358 (Despujols, 1997), knowing that the nitrate made available as a result of BDI is rapidly
359 assimilated by the plant (Bardon et al., 2018; Galland et al., 2019). In these two soils,
360 although lettuce yield varies depending on soil texture (Costigan, 1986), the supply of

361 procyanidins induced BDI, which allowed an increase in nitrate in the soil and induced a gain
362 in lettuce mass in the amended plots. As in Galland et al. (2019), there was a greater root
363 mass gain than aerial mass gain for lettuce roots in Manziat. Indeed, these traits exhibited a
364 78% increase in response to procyanidin amendment at 87 kg ha⁻¹ when the aerial parts
365 increased by 46%, whereas the previous study (Galland et al. 2019) discriminated against the
366 direct action of procyanidins on the roots, and this effect was assumed to be due solely to the
367 BDI phenomenon. It can therefore be deduced that procyanidin amendment at 87 kg ha⁻¹ is
368 sufficiently effective in loamy sand soil, while a higher concentration (such as 210 kg ha⁻¹) is
369 more effective in sandy clay loam soil. It was also observed that the percentages of N in
370 tissues did not differ between amended and unamended lettuce for either soil texture, meaning
371 that there was no plant physiological change due to the procyanidin amendment allowing
372 better N absorption by the lettuce. However, as lettuce grew better in plots amended with
373 procyanidins in both fields, the higher the mass, the higher the N concentration. Indeed, the
374 procyanidin treatment made it possible to conserve N in the form of nitrate due to BDI and
375 thus to make more nitrate available for lettuce, allowing better growth without modifying the
376 plants' ability to absorb N. This observation is consistent with the finding of a greenhouse
377 study conducted on several lettuce varieties where N use efficiency was not affected by soil
378 nitrate concentrations (Urlić et al., 2017). Similarly, although the specific toughness of lettuce
379 leaves was higher in Manziat, which was probably due to different growing conditions and
380 the fact that they were smaller in this soil, amending lettuce with procyanidins did not change
381 the quality of the leaves. Thus, treatment with procyanidins does not change the vulnerability
382 of leaves to potential herbivores and pests (Clissold et al., 2009; Onoda et al., 2011) and does
383 not lead to a gustatory quality difference detected by consumers for lettuce potentially placed
384 on the market (Lopez-Galvez et al., 1997; Rico et al., 2007).

385 **4.3. The addition of procyanidins did not induce a change in soil structure**

386 The measurement of soil porosity was carried out because soil structure plays a role in
387 denitrification (Laudone et al. 2011). In fact, the porosity of a soil is linked to soil aeration
388 and therefore to anaerobic conditions that are favourable for denitrification (Smith 1990).
389 Moreover, denitrifiers are more abundant in aggregates than in pores (Lecomte et al. 2018).
390 Therefore, a change in porosity between our two soils may have played a role in the
391 denitrification activity and consequently the inhibition of it by BDI. However, since two soils
392 with different textures necessarily do not have the same structure (Bouma, 1991), it is normal
393 for the total porosity to be slightly lower in sandy clay loam soil than in loamy sand soil.
394 Despite this, there was no difference in the efficiency of BDI, nitrate availability or the
395 efficiency of lettuce cultivation, suggesting that the BDI process is independent of soil
396 structure. Other abiotic factors such as pH, organic matter level may play a role in the
397 denitrification capacity of denitrifying bacteria (Zumft, 1997) but these factors have not
398 changed after the application of procyanidins. In addition, the initial N level may have an
399 effect on denitrification (Kuypers et al., 2018), in our case both soils have been set at an
400 equivalent starting N level, which excludes at most an effect by this factor. However, we
401 observed a decrease in the macroporosity of planted soils amended with 87 kg of procyanidins
402 per hectare, potentially due to the significant root growth under this condition. In addition, as
403 Hamblin (1986) explains, roots can, by growing, compact the soil and thus reduce
404 macroporosity.

405

406 **5. CONCLUSION**

407 As in our previous study, the use of procyanidin amendment allowed biological inhibition of
408 denitrification as well as a decrease in the competitiveness of denitrifiers in sandy clay loam-
409 textured soil but also in loamy sand-textured soil. This BDI induced the conservation of

410 nitrate in unplanted soils but allowed amended lettuce to grow better in planted soils.
411 Although the amendment at the lowest dose tested was most effective in loamy sand soil, the
412 addition of procyanidins to the field did not change leaf quality nor N levels in tissues in
413 either soil. Finally, soil porosities differed only slightly between treatments for both soils,
414 which means that regardless of soil texture or structure, the induction of BDI in fields is
415 applicable in two soil types and repeatable from one year to the next with another variety of
416 lettuce.

417

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419

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425 Ecology platform (UMR 5023). We thank Laurent Philippot and Delphine Moreau for
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427

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558

559 **Figure legends:**

560

561 Figure 1: Effects of different concentrations of procyanidins (0, 87 and 210 kg of
562 procyanidins per hectare) applied to unplanted soil and soil planted with lettuces in the
563 Manziat field on the left and the SERAIL field on the right on biological denitrification
564 inhibition (BDI, % in relation to the corresponding untreated soil) by communities colonizing
565 the root-adhering soil of treated lettuces. n=4 for each treatment. Vertical bars indicate
566 standard deviation. Different letters indicate a difference between treatments (Nemenyi test;
567 $\alpha < 0.05$).

568 Figure 2: Effects of procyanidins applied to the Manziat and SERAIL fields (210 kg of
569 procyanidins per hectare) to unplanted soil and soil planted with lettuces on the (A) whole
570 bacterial community (copy numbers of 16S rRNA g⁻¹ of dry soil), (B) denitrifying bacteria
571 (copy numbers of *nirS* g⁻¹ of dry soil), and (C) denitrifying bacteria (copy numbers of *nirK* g⁻¹
572 of dry soil), with n = 3 for each treatment. Vertical bars indicate standard deviation. Different
573 letters indicate different means (Nemenyi test, $\alpha < 0.05$).

574 Figure 3: Effects of different concentrations of procyanidins applied to the field (0, 87 and
575 210 kg of procyanidins per hectare) of A) Manziat and B) SERAIL on the nitrate
576 concentration of unplanted soil and soil planted with lettuce (rhizospheric soil), measured at
577 time 0 (T0) and the final time (TF). n = 4 for each treatment. Vertical bars indicate standard
578 deviation. Different letters indicate a difference between treatments (Nemenyi test; $\alpha < 0.05$).

579 Figure 4: Effects of different concentrations of procyanidins applied to soil planted with
580 lettuces in the Manziat field (left) and the SERAIL field (right) (0, 87 and 210 kg of
581 procyanidins per hectare) on the fresh mass of (A) shoots (g) and (B) roots (g). n= 16 for the
582 root system and n= 48 for the shoots in each treatment. Vertical bars indicate standard
583 deviation. Different letters indicate a difference between treatments (Tukey's test; $\alpha < 0.05$).

584 Figure 5: Effects of different concentrations of procyanidins applied to soil planted with
585 lettuces in the Manziat field (left) and the SERAIL field (right) (0, 87 and 210 kg of
586 procyanidins per hectare) on specific toughness ($J.m^{-3}$). n = 16 for each treatment. Vertical
587 bars indicate standard deviation. Different letters indicate a difference between treatments
588 (Tukey's test; $\alpha < 0.05$).

589 Figure 6: Effects of different concentrations of procyanidins applied to soil planted with
590 lettuces in the Manziat field (left) and the SERAIL field (right) (0, 87 and 210 kg of
591 procyanidins per hectare) on the (A) leaf and (B) root N content (% of dry mass). n = 4 for
592 each treatment. Vertical bars indicate standard deviation. No significant effect (Nemenyi test;
593 $\alpha < 0.05$).

594

595 Figure 7: Effects of different concentrations of procyanidins applied to unplanted soil and soil
596 planted with lettuces (0, 87 and 210 kg of procyanidins per hectare) on: total porosity

597 ($\text{cm}^3.\text{cm}^{-3}$) in the Manziat field (A) and SERAIL field (B); capillary porosity ($\text{cm}^3.\text{cm}^{-3}$) in the
598 Manziat field (C) and SERAIL field (D) and macroporosity ($\text{cm}^3.\text{cm}^{-3}$) in the Manziat field
599 (E) and SERAIL field (F). $n = 4$ for each treatment. Vertical bars indicate standard deviation.
600 Different letters indicate a difference between treatments (Nemenyi test; $\alpha < 0.05$).

Figure 1

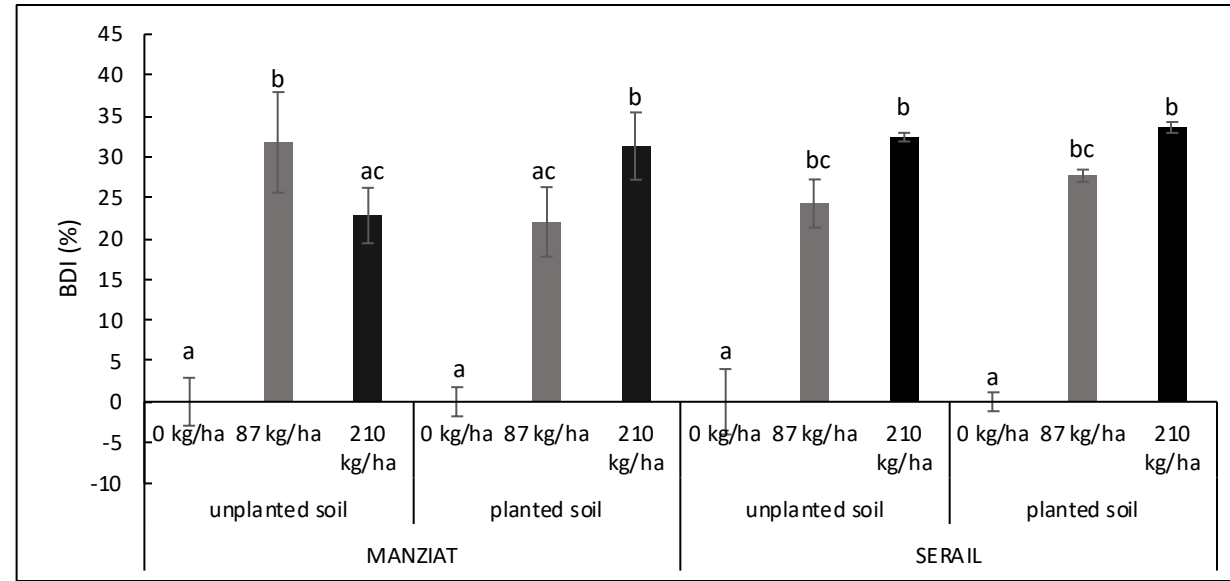


Figure 1

Figure 2

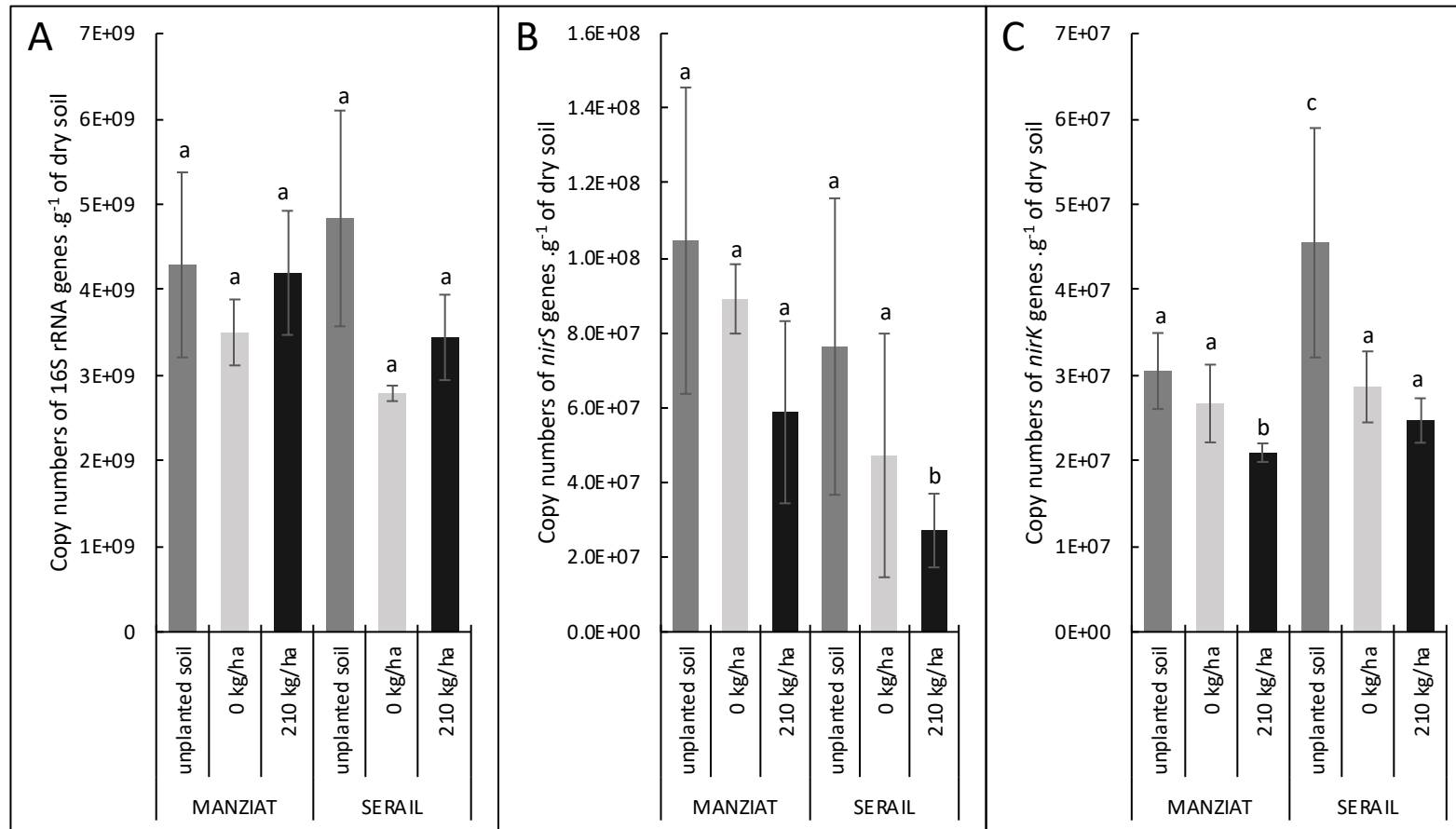


Figure 2

Figure 3

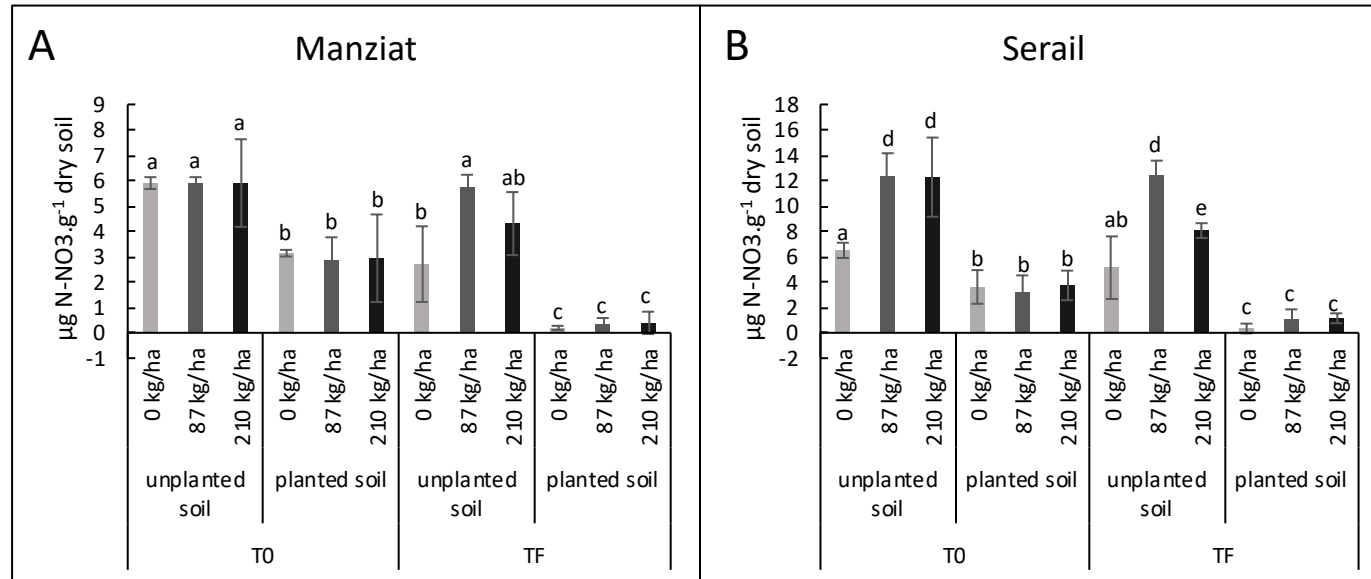


Figure 3

Figure 4

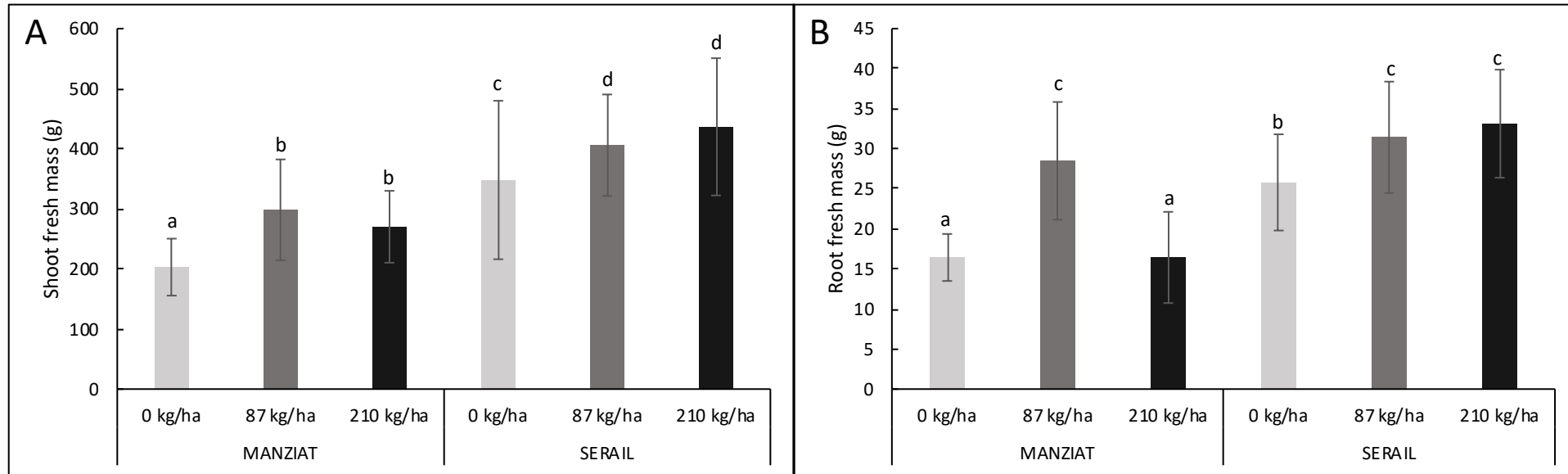


Figure 4

Figure 5

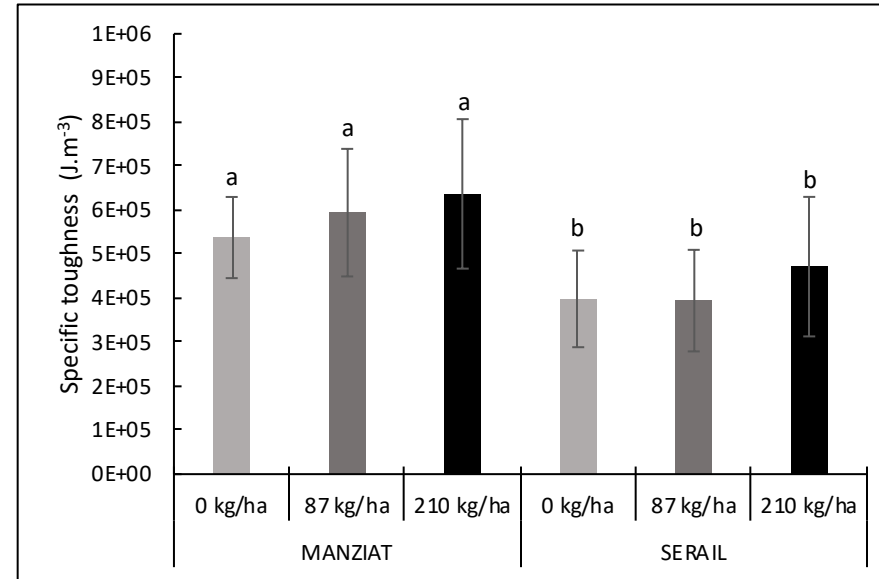


Figure 5

Figure 6

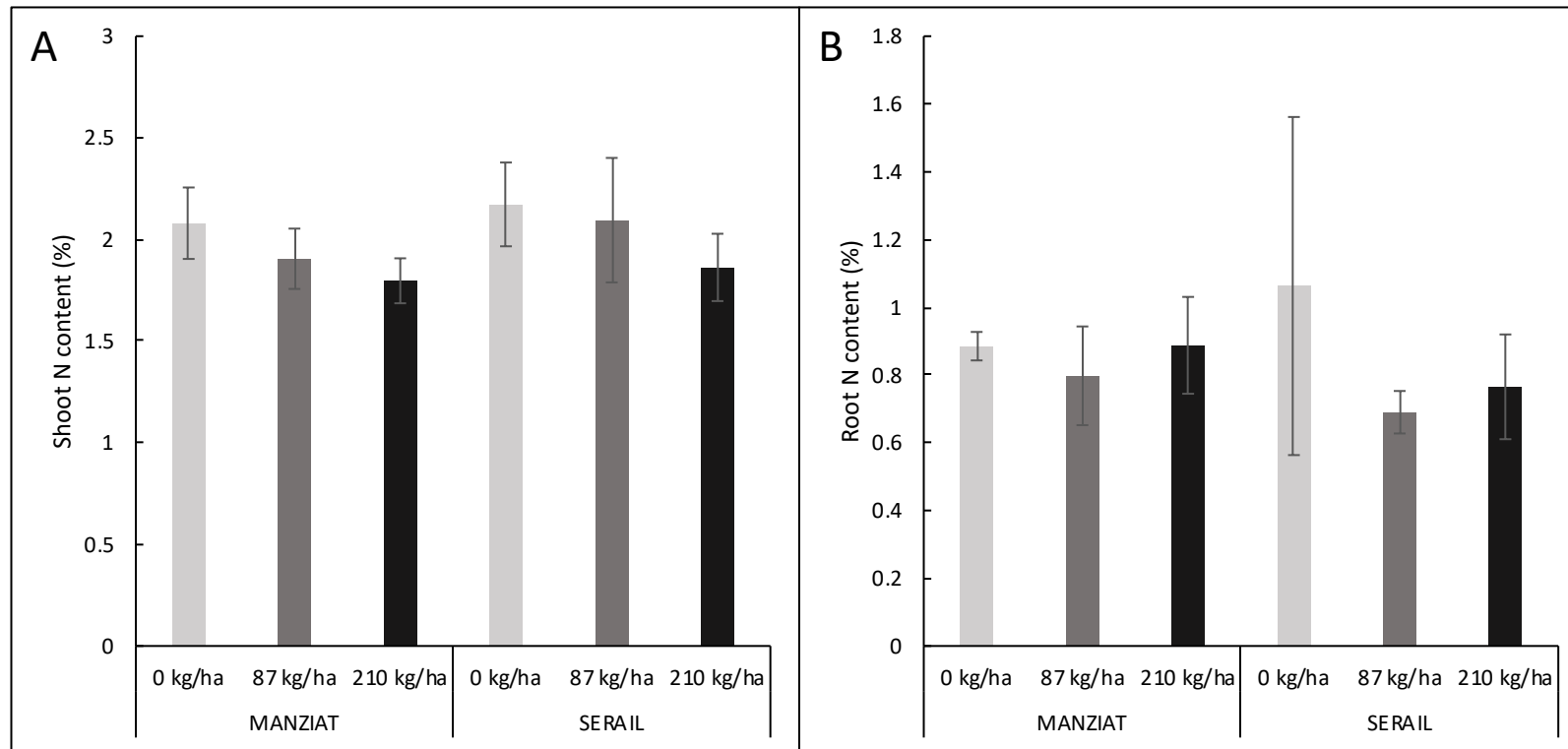


Figure 6

Figure 7

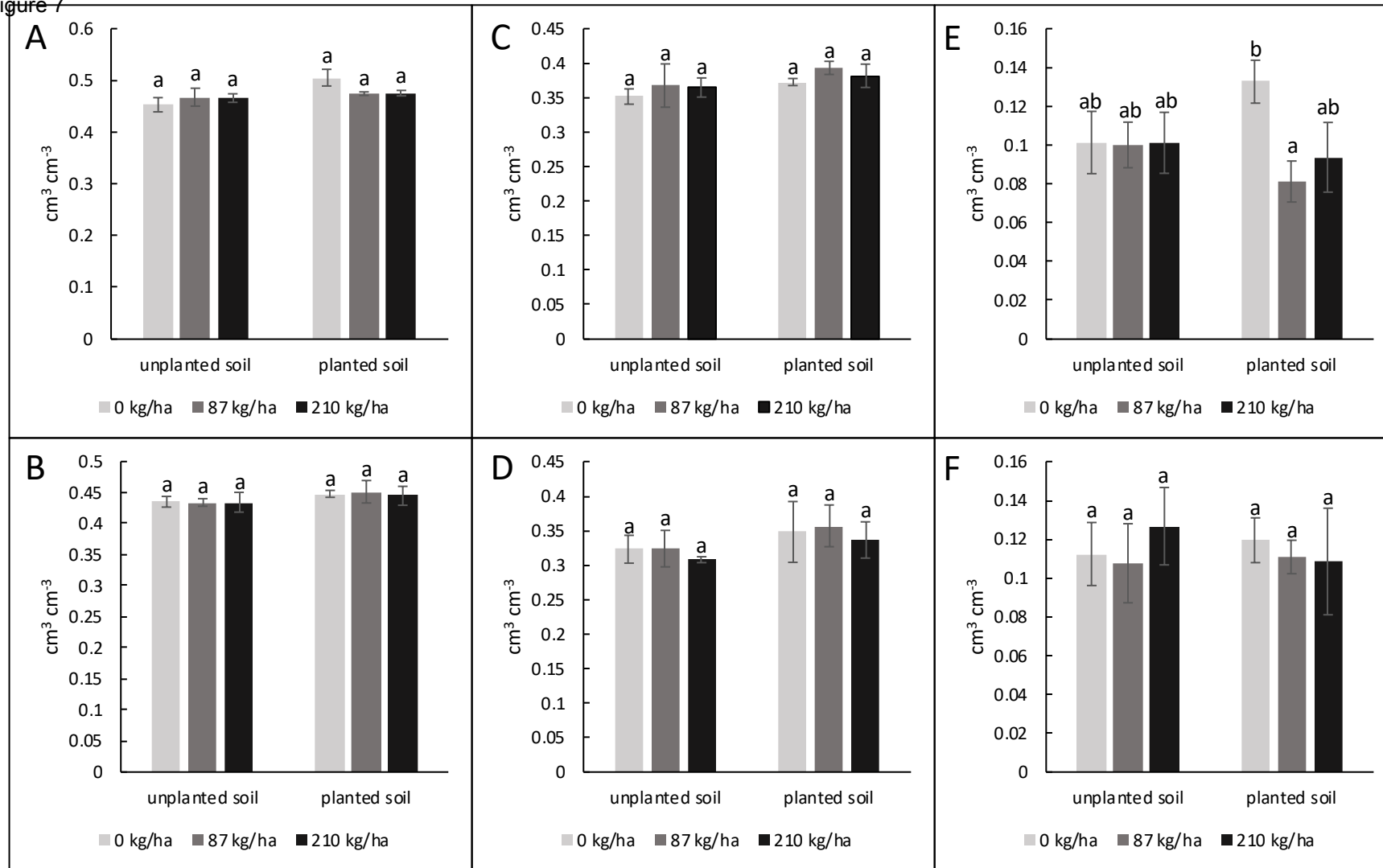


Figure 7