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Fourier Transform InfraRed spectroscopy contribution to disentangle nanomaterial (DWCNT, TiO₂) impacts on tomato plants

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Abstract. Carbon nanotubes (CNTs) and titanium dioxide nanoparticles (TiO₂-NPs) are among the most used nanomaterials (NMs). However, their impacts especially on the terrestrial ecosystems and on plants are still controversial. Apart from obvious physico-chemical differences, a possible explanation of these contrasting results could be the wide range of methods used to evaluate the toxicity at different levels of plant physiology. Fourier Transformed InfraRed (FTIR) spectroscopy is a sensitive and widely informative technique that probes the chemical composition of plants. In this study, we investigated the impacts of CNTs and TiO₂-NPs (100 and 500 mg.kg⁻¹) on tomato plants after 5, 10, 15 and 20 days of exposure in soil. Using morphological parameters, no toxicity was found except after 15 days of exposure (-57% in height and -62% in foliar area for plants exposed to 100 mg.kg⁻¹ TiO₂-NPs, but no impact after CNT exposure) while FTIR revealed effects of the two NMs starting after 5 days of exposure and being maximum after 15 days. After spectral data treatment optimization, FTIR results suggested modifications in leaf cell wall components of plants subjected to both NMs. Microarray polymer profiling confirmed changes in xyloglucan and homogalacturonan levels for plants exposed to TiO₂-NPs. In summary, FTIR was an effective screening method to evaluate the impacts of NMs on tomato plants and to identify their implications on the plant cell walls.

30 **1. Introduction**

31 Over the last two decades, nanotechnologies have become increasingly important. Indeed, nanomaterials
32 (NMs) present unique properties such as a large specific surface area which can be useful in many domains
33 such as electronics, materials or food industry¹. In 2020, the Dutch Nanodatabase revealed that a total of
34 5000 consumer products officially contained NMs². Investigations about their possible use in medicine³ or
35 in agriculture⁴ are also in progress.

36 Carbon nanotubes (CNTs) and titanium dioxide nanoparticles (TiO₂-NPs) are among the most widely used
37 NMs². CNTs are part of the carbon-based NM family. They have remarkable optical, electrical, thermal,
38 mechanical and chemical properties⁵ and are mainly used in batteries, plastic additives or sporting goods⁶.
39 TiO₂-NPs are well known for photocatalytic applications⁷ and are included for example in food additives⁸
40 or cosmetics⁹. Since NM applications are steadily increasing, their release in the environment, intentionally
41 or not, is of great concern.

42 Assessing NM concentrations in the environment is a major bottleneck in ecotoxicology. Modeling studies
43 were carried out on some NMs to evaluate this information in different environmental compartments.
44 TiO₂-NPs have been identified as one of the most concerning NMs due to the high concentrations forecast:
45 around 61 mg.kg⁻¹ in sludge treated soils against 12 µg.kg⁻¹ for CNTs¹⁰.

46 NM impacts on terrestrial ecosystems are still controversial, in particular on plants¹¹. Indeed, some authors
47 reported higher germination rate and better yield after exposure to CNTs while other studies highlighted
48 decreased root length or oxidative stress¹¹. The same conclusions were reached for TiO₂-NP impacts on
49 plants¹²: while some beneficial effects were reported such as a higher germination rate or increased root
50 and shoot length¹³⁻¹⁶, other works described decreased germination rate, plant growth or genotoxic
51 effects¹⁷⁻¹⁹. Until now, the specific mechanisms implied in NM uptake (active vs. passive, apoplast vs.

52 symplast, among other questions) and impact (*e.g.* nano specific or ion related, oxidative stress mediated)
53 are still to be identified and require further research²⁰.

54 Apart from obvious physico-chemical differences, a possible explanation of these contrasting phytotoxicity
55 results may also be the method used to evaluate NM impacts on plants. Many biomarkers can be assessed
56 from the morphological to the gene scale showing variable sensitivity. Their use to evaluate plant health
57 is conclusive when many of them are combined. But in the literature, most of the studies use a limited
58 number of biomarkers leading to a potentially partial image of the toxicity effects and thus a biased risk
59 assessment. The availability of routine, standardized and widely informative analytical methods to
60 evaluate NM toxicity is a key to fill this current gap of knowledge²¹.

61 Fourier Transform InfraRed (FTIR) spectroscopy is a technique based on the vibrational state of molecules.
62 It allows the acquisition of a spectrum combining information on a multitude of compounds, unlike
63 chemical dosages which give access only to one compound (*e.g.* specific enzyme or secondary metabolite)
64 after a series of reactions²². There are two types of acquisition modes: either bulk analysis of the whole
65 plant (few minutes per sample) or 2D-imaging mode of cross sections (few hours per map)²³. In bulk mode,
66 sample preparation is very simple, consisting of grinding dry materials thus reducing artifacts. Therefore,
67 FTIR is a widely informative, easy to set-up and fast technique that could be used to screen NM effects on
68 different organisms. In plant biology, FTIR has been mainly used to characterize plant cell wall components
69 in a highly sensitive and more time-efficient manner than traditional methods which require isolation,
70 extraction and fractionation of the different cell wall components^{24–28}. Recently, FTIR has been used in
71 ecotoxicological studies to analyze changes occurring in biological materials after exposure to biotic or
72 abiotic stresses^{23,29–36}. For instance, Morales *et al.*, Servin *et al.* and Zhao *et al.* highlighted changes in the
73 chemical environment of carbohydrates of both cilantro and cucumber exposed to CeO₂, ZnO or TiO₂
74 NPs^{33,35,36}. Radish sprouts exposed to Ag-NPs also exhibited modifications of their IR spectral signature in
75 the region related to lipids, proteins and particularly structural component peaks such as lignin, pectin and

76 cellulose³⁴. Likewise, very recently, we applied FTIR to evaluate the influence of plant species on their
77 response to a CNT contamination highlighting the role of cell wall composition³². Indeed, it has been
78 demonstrated several times that cell walls play a crucial role in plant response to abiotic stresses³⁷. Plant
79 cell walls are composed of complex polysaccharides and a small amount of proteins and their composition
80 can be modified in response to stress³⁷⁻⁴⁰.

81 However, FTIR data processing is tedious due to spectrum complexity. Indeed, it contains overlapping
82 signals coming from many molecular bonds. A purely visual inspection of spectra is often insufficient to
83 draw a conclusion. Several factors could weaken this analysis and its subsequent conclusions: i)
84 sometimes, minor spectral differences not detected with the bare eye may contain critical information, ii)
85 the baseline may vary from one sample to another, iii) instrumental noise could induce bias. For these
86 reasons, it is important to find a way to process and analyze the data in a more systematic way using
87 statistical approaches (*i.e.* supervised classification, clustering method) in order to obtain meaningful
88 information⁴¹.

89 The main goal of this study was to develop the FTIR approach to evaluate the comparative impacts of two
90 types of NMs (CNTs and TiO₂-NPs) taking into account: (i) different NM concentrations and (ii) different
91 exposure durations. Seedlings of tomato (*Solanum lycopersicum* L.) were grown in soil contaminated with
92 CNTs or TiO₂-NPs at two different concentrations (100 and 500 mg.kg⁻¹ of soil) during different durations
93 (5, 10, 15 and 20 days). FTIR was used as the main technique to evaluate the impact of the two NMs on
94 tomato plants. Complementary morphological biomarkers were also assessed (height, biomass, number
95 of leaves, leaf surface area). Finally, to better understand the FTIR data, the cell wall composition was
96 further analyzed by microarray polymer profiling. Developing a reliable technique to assess in a screening
97 mode the biological effects of many different types of NMs is mandatory to accelerate the risk assessment
98 of these new materials being disseminated or intentionally introduced in our environment on a daily basis.

99 **2. Materials and methods**

100 **2.1. Nanomaterials**

101 TiO₂-NPs (ref 718467, Aeroxide P25, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were characterized
102 in a previous experiment (same batch) and were composed of 80% anatase and 20% rutile with a nominal
103 diameter of 25.0 ± 5.7 nm¹⁹. They had a specific surface area of 46 ± 1 m².g⁻¹ (Figure S1A)¹⁹.

104 Double walled CNTs were synthesized by catalytic chemical vapor deposition (CCVD) at 1000°C of a mixture
105 of CH₄ (18 mol.%) and H₂ using a Co:Mo/MgO-based catalyst (chemical composition:
106 Mg_{0.99}Co_{0.0075}MgO_{0.0025})⁴². The outer diameter ranged from 1 to 3 nm and the length varied from 1 to 100
107 microns (Figure S1B)⁴². The specific surface area was 980 m².g⁻¹ (Brunauer, Emmett and Teller (BET)
108 method; Micromeritics Flow Sorb II 2300, Micromeritics, Norcross, GA, USA).

109 Fresh NM suspension at 1 g.L⁻¹ were prepared with ultrapure water directly before use and dispersed using
110 a sonication bath for 10 min (Elmasonic S30H, 280 W, Elma, Singen, Germany).

111

112 **2.2. Soil characteristics and contamination**

113 A silty sand soil (according to the United State Department of Agriculture⁴³) was used for this experiment
114 (Lufa-Speyer, 2.1, Speyer, Germany) with a composition of 88.0% sand, 9.1% silt and 2.9% clay. It contained
115 0.71 ± 0.08 % organic carbon, 0.06 ± 0.01 % nitrogen, had a pH of 4.9 ± 0.3 and a cation exchange capacity
116 of 4.3 ± 0.6 meq/100 g of soil. The soil water capacity was 60 mL/100 g of soil.

117 CNT or TiO₂-NP suspensions were added to the dry soil to reach a concentration of 100 or 500 mg NMs/kg
118 dry soil (ratio liquid/soil = 1/1 in mass). After 2 h on a shaker table, the soil mixture was filtered to remove
119 the water in excess. This soil preparation protocol ensured a soil contamination as homogeneous as
120 possible. These concentrations were chosen to be relevant for TiO₂-NP contamination in sludge amended
121 soils¹⁰ and comparable between NMs.

122 **2.3. Plant material and cultivation**

123 Organic seeds of tomato *Solanum lycopersicum* L. (var. Red Robin) were obtained from the French seed
124 company Germinance (Soucelles, France) and surface-sterilized using $\text{Ca}(\text{ClO})_2$ (1%). Seedlings were first
125 grown in hydroponic conditions for 3 weeks until they reached the 5 leaf-stage. Plants were then placed
126 into control or contaminated soil until harvest after 5, 10, 15 or 20 days of exposure. Exposure durations
127 were chosen based on a literature study showing that more than 65% of articles studying CNT impacts on
128 plants used exposure duration of less than 15 days¹¹. Each exposure duration corresponded to an
129 independent experiment. The experiments were performed in a growth chamber with controlled
130 parameters: 10 h light/14 h dark photoperiod; 24°C during the day and 22°C during the night; and a
131 hygrometry of 85%.

132 Five different exposure conditions were set-up: control (only soil without NM contamination), 100 mg
133 CNTs/kg dry soil (CNT 100), 500 mg CNT/kg dry soil (CNT 500), 100 mg TiO_2 -NPs/kg dry soil (TiO_2 100) and
134 500 mg TiO_2 -NPs/kg dry soil (TiO_2 500). Five biological replicates were performed in each case.

135 Morphological parameters were monitored every day (plant height and number of leaves). Upon harvest,
136 other morphological parameters were measured (total fresh leaf biomass and foliar surface area using a
137 camera and ImageJ software⁴⁴). Leaves were dried at 50°C during 24 h prior to FTIR analysis.

138

139 **2.4. FTIR analysis**

140 About 20 mg dry leaves were ground 2 x 15 s at maximum speed using a FastPrep grinding machine (MP
141 Biomedicals, Illkirch-Graffenstaden, France). Each powdered sample was analyzed in attenuated total
142 reflectance (ATR) mode using a diamond crystal (Thermo Nicolet Nexus, Smart Orbit, Thermo Fisher
143 Scientific, Waltham, USA). Infrared spectra were collected in the range 4000 - 400 cm^{-1} . All the samples (5

144 biological replicates) were analyzed in (technical) triplicates and each spectrum was the sum of 64 scans.
145 OMNIC software (Thermo Fischer Scientific©) was used to export spectra.

146

147 **2.5. Chemometric analysis for FTIR data**

148 A chemometric analysis of FTIR spectra was developed using Orange software (BioLab, Ljubljana,
149 Slovenia)⁴⁵ including the add-on Spectroscopy⁴⁶. During the first step, data were pre-processed to
150 eliminate possible analytical biases (such as detector noise and atmospheric background)²³. For this, a
151 Savitzky-Golay filter was applied (point window: 21, polynomial order: 2, derivative order: 2). This filter is
152 based on simplified least square procedures and permits removal of various instrumental and scattering
153 effects. A vector normalization was then applied to minimize the effects of the source power fluctuations
154 as well as to overcome variations due to the amount of leaf powder analyzed. The last step of the pre-
155 processing was to select the region of interest in order to avoid background interferences^{23,47}. Here, we
156 focused on two regions of the spectra: between 2900 and 2700 cm^{-1} corresponding to the lipid region and
157 between 1800 and 800 cm^{-1} corresponding to the so-called fingerprint region (including proteins and
158 polysaccharides). The region between 1800 and 2700 cm^{-1} was removed because it mainly corresponded
159 to background interferences. With this pre-process, the robustness and accuracy of subsequent analyses
160 were improved and the interpretability of the data was increased by correcting issues associated with
161 spectral data acquisition.

162 A multivariate analysis was then performed on the pre-processed spectra with first a principal component
163 analysis (PCA), followed by a linear discriminant analysis (LDA) when necessary⁴¹. PCA is an unsupervised
164 method which searches for directions where data have the largest variance, whose results can show data
165 structural information. While, LDA is a supervised method that looks for projections that maximize the
166 ratio between-class to within-class. The combination of both methods is particularly useful when the

167 number of variables is large, especially if the number of observations (samples) is lower than the number
168 of variables (wavenumbers) as in this work. PCA allows reducing the number of variables, in this analysis
169 from 1246 variables to 10 components, the reduced dataset being then analyzed by LDA to enhance
170 differences between the classes, if any.

171 In order to identify the wavenumbers contributing the most to differences among groups, a logistic
172 regression was run on the pre-processed spectra. The logistic regression is a predictive model that yields
173 the probability of occurrence of an event by fitting data to a logistic curve. The least absolute shrinkage
174 and selection operator (LASSO) method was used to perform the regularization and feature selection.
175 Most relevant wavenumbers were identified by obtaining logistic regression coefficients; this feature
176 extraction method has been already used in ATR-FTIR data analysis⁴⁸. For testing the robustness of the
177 statistical model used, the area under a receiver operating characteristics (ROC) curve (AUC) and K-fold
178 cross validation were used⁴¹. Finally, to compare the different spectra among them, the area under
179 differing absorption peaks was calculated by integrating the area starting from 0 on the pre-processed
180 spectra.

181

182 **2.6. Cell wall composition by polysaccharide microarray analysis**

183 The cell wall composition was assessed according to Moller et al.⁴⁹. This technique integrates the
184 sequential extraction of polysaccharides from cell walls, followed by generation of microarrays, which are
185 probed with monoclonal antibodies (mAbs) with specificities for cell wall epitopes.

186 Cell wall polysaccharides were sequentially extracted from homogenates using three solvents: (i) 50 mM
187 diamino-cyclo-hexane-tetra-acetic acid (CDTA), pH 7.5, (ii) 4 M NaOH with 1% v/v NaBH₄, and (iii) cadoxen
188 (31% v/v 1,2-diaminoethane with 0.78 M CdO). The three extraction solvents used are known to solubilize
189 pectins, non-cellulosic polysaccharides, and cellulose, respectively. For each extraction, a ratio of 6 μ L

190 solvent for 1 mg fresh biomass was added to each tube before incubation with shaking for 1 h. After
191 centrifugation at 2500 *g* for 10 min, the supernatants were removed prior to addition of the next solvent
192 to pellets. All the supernatants were finally stored at 4°C. Forty µL of diluted extracts (2/50, vol/vol) in TBS
193 (Tris-HCl 20 mM pH 7.5, NaCl 150 mM, pH 7.0) were then loaded in each well of a Bio-Dot apparatus (BIO-
194 RAD, Marnes-la-Coquette, France) onto nitrocellulose membranes (Sigma-Aldrich). After blocking TBS-
195 T/BSA 0.05% (0.05% Tween), the arrays were probed overnight at 4°C with primary mAbs directed against
196 different cell wall epitopes (<https://plantcellwalls.leeds.ac.uk/plantprobes/>) at a 1/250 dilution (vol/vol)
197 in TBS-T/BSA 0.05%: LM19 (for non-methylated homogalacturonans, HG), LM20 (for methylated HG,
198 mHG), LM25 for the XLLG, XXLG and XXXG motifs of xyloglucans, XG), LM15 (for the XXXG motif of XG and
199 to some extent single galactosyl substitution of the XXXG oligosaccharide, and LM24 (for the XLLG motif
200 of XG). After washing in TBS-T, the arrays were probed with anti-rat IgG secondary antibodies conjugated
201 to alkaline phosphatase (Sigma-Aldrich) at a 1/10,000 dilution (vol/vol) for 2 h before washing and
202 developing in a BCIP/NBT (5-bromo-4-chloro-3-*indolyphosphate*/nitro-blue tetrazolium chloride)
203 substrate. To check the activity of the mAbs, commercially purified polysaccharides were used as positive
204 controls: polygalacturonic acid (HG, Sigma-Aldrich), polygalacturonic acid methyl ester (mHG, Sigma-
205 Aldrich), and XG (Megazyme, Libios, Pontcharra-sur-Turdine, France). The arrays were scanned using an
206 Epson Perfection V370 Photo (Nagano, Japon). Color intensity of each spot was quantified thanks to ImageJ
207 software.

208

209 **2.7. Statistical analysis**

210 Data (morphological parameters) were checked for homoscedasticity and normality. When assumptions
211 were met for parametric analyses, a two-way ANOVA was used. Otherwise, a Kruskal-Wallis test was
212 applied. A PCA was also performed on the full dataset. All statistical analyses were carried out using the

213 RStudio statistical software⁵⁰ (version 1.1.453) with multcompView⁵¹, lsmeans⁵², pgirmess⁵³, ggplot2⁵⁴
214 packages.

215

216 **3. Results**

217 **3.1. Morphological responses**

218 Plant height and number of leaves were recorded during the time course of the four experiments (5, 10,
219 15 and 20 days of exposure) as well as plant biomass and leaf area at the end of the experiments. These
220 data are available in the Supplementary files (Figure S2, S3). No significant impact of NM exposure after 5,
221 10 and 20 days was evidenced for these parameters.

222 Differences were only detected after 15 days of exposure. Indeed, plants exposed to 100 mg.kg⁻¹ TiO₂-NPs
223 were significantly smaller than the control (-57%, p-value < 0.05, Figure 1A). Plants exposed to 500 mg.kg⁻¹
224 TiO₂-NPs were 28% smaller than the control plant but this decrease was not significantly different (2.3 ±
225 0.3 cm for the control and 1.6 ± 0.4 cm for 500 mg.kg⁻¹ TiO₂-NPs). Although plants exposed to both CNT
226 concentrations for 15 days were not significantly different in height from the control plants, there was an
227 increase of 26% and 28% in soils contaminated with 100 and 500 mg.kg⁻¹ CNT, respectively (2.3 ± 0.3 cm
228 for control, 2.9 ± 0.8 cm for 100 mg.kg⁻¹ CNT and 2.9 ± 0.5 cm for 500 mg.kg⁻¹ CNT).

229 The number of additional leaves at the end of the treatments was not significantly different between
230 conditions (Figure S2B); plants displayed an average of 1.8 additional leaf after 15 days.

231 On the one hand, the total leaf area of plants exposed to the two TiO₂-NP concentrations was decreased
232 after 15 days of exposure: 7.2 ± 2.4 cm² for 100 mg.kg⁻¹ TiO₂-NPs and 8.9 ± 2.6 cm² for 500 mg.kg⁻¹ TiO₂-
233 NPs while that of the control was at 19.0 ± 2.7 cm² (p-value < 0.001, Figure 1B). On the other hand, no
234 significant difference was found for plants exposed to CNTs.

235 Plant biomass was not significantly different for treated plants compared to the control after 15 days of
236 exposure but different in between exposed plants (p -value < 0.001 , Figure 1C). However, plants exposed
237 to 500 mg.kg^{-1} CNTs exhibited a trend for higher biomass compared to the control (+30%) while plants
238 exposed to 100 mg.kg^{-1} TiO_2 -NPs tended to be lighter than the control (-64%).

239 The PCA analysis of the different morphological parameters highlighted a significant impact of TiO_2 -NPs
240 on tomato morphology after 15 days of exposure with a decrease in most of the assessed parameters
241 while CNTs had a more mitigated impact at this developmental level (Figure 1D).

242

243 **3.2. Leaf chemical composition after FTIR analysis**

244 Again the highest differences were visible after 15 days of treatment, even though NM impact was visible
245 already after 5 days, opposite to what was observed from morphological parameters. PC-LDA analyses on
246 FTIR data for 5, 10 and 20 days of NM exposure are available in supplementary data (Figure S4).

247 After the different contaminant exposure, the composition of leaves was significantly different between
248 the three treatments according to the PC-LDA (Figure 2A). Looking at the distance of the barycenter of the
249 ellipses, the plants exposed to 500 mg.kg^{-1} CNT exhibited the highest differences in comparison to the
250 control while those exposed to 100 mg.kg^{-1} CNT showed the lowest differences. Both groups of the TiO_2 -
251 NP conditions were almost at the same distance from the control, but in the opposite direction to CNT
252 groups along the component 1 axis. This result suggests that the leaf composition is different between
253 plants exposed to CNTs and TiO_2 -NPs, confirming the different impacts seen at the morphological level
254 (decreased growth after TiO_2 -NP exposure vs. trend for an increase after CNT exposure).

255 Once a significant cluster structure was identified for the 15 day treatment case, feature extraction was
256 performed with a logistic regression model. This model was tested by a stratified 3-fold cross-validation

257 method, obtaining average over classes scores of 0.932 of AUC, 0.889 of precision and a recall of 0.852,
258 notice that a strong and robust model have scores close to one⁴¹. A difference was highlighted in the so-
259 called “lipid region” (Figure 2B, peak A, Table 1) with higher relative amounts for plants exposed to the
260 four different treatments in comparison to the control with the highest amount in leaves of plants grown
261 on soil contaminated by CNTs at 500 mg.kg⁻¹ (+ 29 ± 2.3% of the area under the peak for CNT 500 in
262 comparison to the control). In the amide II peak^{55,56}, leaves of plants grown on contaminated soils
263 exhibited an increase in peak area in comparison to the control except for CNT 500 (12 ± 1% increase for
264 CNT 100, 6 ± 7 % for TiO₂ 100 and 6 ± 1% TiO₂ 500) (Figure 2B, peak B; Table 1). Polysaccharides^{38,47,56,57}
265 also seemed to be impacted with differences in the areas of peaks C (1320 - 1312 cm⁻¹) and D (1160 - 1155
266 cm⁻¹). Exposed plants displayed an increase in peak C area while a slight decrease in the peak D area was
267 observed. Between 1080 and 1070 cm⁻¹ corresponding to hemicellulose^{38,47,56} (Figure 2B, peak E; Table 1),
268 a slight decrease in the peak area was detected for the leaves of plants grown on all contaminated soils.
269 Finally, a decrease in areas of peak F corresponding to pectin and various polysaccharides^{47,56,57} (1052 –
270 990 cm⁻¹, Figure 2B, peak F; Table 1) was noticed for the plants grown in soil contaminated with CNTs
271 whereas an increase in peak F area was detected for the plants grown with TiO₂-NPs. Altogether, most of
272 the differences observed between the FTIR spectra were related to cell wall components (pectin, cellulose
273 or hemicellulose).

274 The signals obtained here were averaged on the whole leaf biomass. For further analysis, chemical
275 composition of the leaves was observed considering their age on the two 500 mg.kg⁻¹ NM treatments
276 (Figure 3). Overall, the oldest and the youngest (at early development stage during contaminant exposure)
277 leaves were the least impacted by NM contamination; leaves of plants exposed to TiO₂-NPs, in particular,
278 had a chemical composition very similar to those of control plants (Figure 3A,D). However, intermediate
279 leaves (Figure 3B, C) exposed to NM displayed different FTIR signatures than control plants, but similar in-
280 between them according to the PC-LDA with overlapping ellipses for both CNT and TiO₂-NP treated leaves.

281

282 **3.3. Cell wall composition by polysaccharide microarray analysis**

283 As FTIR analyses suggested a strong impact of NM treatments on cell wall components, a more precise
284 characterization was carried out on three cell wall fractions enriched in pectin, hemicellulose or cellulose.
285 Several mAbs recognizing the main polysaccharides found in dicot cell walls were used, namely HG, mHG
286 and different XG epitopes. No significant signal was obtained with LM20 (recognition of mHG), and LM24
287 (recognition of XLLG motifs of XG) mAbs (results not shown). Signals were observed with the three other
288 mAbs: LM19 (recognition of HG), LM25 and LM15 (recognition of XLLG, XXLG and XXXG motifs and of XXXG
289 motifs of XG, respectively) (Figure 4).

290 Significant differences were found for HG (LM19) in the pectin-enriched fraction of leaves of plants
291 exposed for 15 days to 500 mg.kg⁻¹ TiO₂-NPs with a 58% increase (p= 0.028). The LM19 signal also increased
292 by nearly two-fold in the hemicellulose-enriched fraction although this increase was not significant.
293 Significant differences were also detected with LM25 for the same condition (500 mg.kg⁻¹ TiO₂-NPs) in the
294 hemicellulose-enriched fraction (+37% in comparison to the control, p= 0.046). However, no significant
295 difference was found with LM15 specific for the XXXG motif of XG. It can be concluded that XXLG is the
296 only XG motif whose amount was modified in leaves when plants were exposed to 500 mg.kg⁻¹ TiO₂-NPs
297 for 15 days. For CNT, no significant difference was found with all the mAbs tested here.

298 Altogether, it was not possible to detect mHG (LM19) or the XLLG motif of XG (LM24) in the tomato leaves
299 whatever the treatment. Significant changes were only observed after the treatment with 500 mg.kg⁻¹
300 TiO₂-NPs, corresponding to an increase in the amount of HG (LM19) and of the XXLG motif of XG (LM25,
301 vs LM15 and LM24).

302 **4. Discussion**

303 In this study, **FTIR** spectroscopy appeared to be a more sensitive technique to detect the impact of NM
304 treatment on plant compared to the traditionally used morphological biomarkers. Indeed, FTIR analysis
305 revealed a plant response to NM contamination even at the shortest time of exposure (5 days). Looking at
306 the morphological parameters, few differences were visible only after 15 days but not earlier which
307 seemed to be compensated later. FTIR also allowed assessing NM impacts on several biomacromolecules
308 (*i.e.* lipids, polysaccharides) in one single analysis, thus permitting to dedicate further research efforts to
309 look at the modifications of cell wall composition under the influence of NM exposure. The developed
310 chemometric analysis was quite powerful in highlighting differences between the experimental conditions
311 in an automated way, which would have not been possible by visual inspection of the FTIR spectra. FTIR
312 spectroscopy is thus a relevant method to identify early impacts of NMs on plants in a fast and reliable
313 way, thereby permitting a screening approach.

314 In this soil experiment, plant response to NMs was not **dose-dependent** since most of the time, impacts
315 were not higher at the highest concentration. One hypothesis possibly explaining this result is that NMs
316 can have different behaviors in the environment depending on the concentration used. Indeed, when the
317 concentration is increased, it also leads to more chances for hetero- and homo-agglomeration phenomena
318 which would result in decreasing NM mobility and bioavailability in soils^{58,59}.

319 The impacts of both NMs tended to increase with **time** until 15 days of exposure, and then decreased
320 (20 days: no detectable difference in morphological biomarkers and lower impact on biomacromolecules
321 such as lipids, polysaccharides or proteins as demonstrated by FTIR analysis). This decrease in impacts after
322 15 days of exposure could suggest a plant recovery. Likewise, when studying NM impact on individual
323 leaves of different ages, the oldest one (*i.e.* exposed for the longest period) was the least impacted in its
324 chemical composition while clear differences were visible on other fully-expanded leaves. This could also
325 correspond to a recovery or adaptation at the leaf level. Very little has been done so far to study plant
326 recovery after a NM exposure. One study reported that TiO₂-NPs had no major impact on tomato plants

327 upon harvest (after 5 months of exposure), but some markers indicated that plants might have gone
328 through oxidative stress earlier in their life cycle²⁹. It has also been shown after exposure to different heavy
329 metals (Zn, Co, Cd, Ni, Mn) that the detoxification response was triggered during the first days of exposure
330 and then decreased back nearly to its basal level after 9 days⁶⁰. It would thus be interesting to investigate
331 NM impacts on biomacromolecules under chronic exposure conditions to confirm this hypothesis and
332 further improve risk assessment strategies.

333 **CNTs and TiO₂-NPs** have been chosen here as they are two very different NMs; in particular, they vary in
334 shape (tubular for CNTs vs. spherical for TiO₂-NPs), in surface chemistry (carbon vs. metal oxide), in
335 diameter (1-3 nm diameter for CNTs vs. 25 nm for TiO₂-NPs) but they are both very insoluble. Their
336 behavior and impacts are thus expected to be quite different. Indeed, at the morphological level, TiO₂-NPs
337 inhibited tomato development while CNTs tended to stimulate it. These results are consistent with
338 previously published literature^{19,61}. However, regarding biomacromolecule composition NM triggered
339 quite similar impacts, especially on cell wall components, which might suggest a common response of
340 plants upon exposure to CNTs or TiO₂-NPs.

341 FTIR spectra showed that the relative amount of lipids in leaves was increased following exposure to both
342 NMs. This result is in agreement with studies performed on spinach, where TiO₂-NPs also increased the
343 level of lipids after a foliar contamination^{33,35,62}. Using FTIR analysis, several studies also reported that
344 metal-based NMs increased the relative amount of lipids in *Raphanus sativus* (Ag-NPs)³⁴ and in *Coriandrum*
345 *sativum* (CeO₂-NPs)³³. Lipid accumulation is one of the plant responses to various stresses such as high
346 temperature, drought or heavy metals^{63,64}. Changes in the lipid composition and/or interactions between
347 lipids and specific membrane proteins can occur in order to reinforce the phospholipid membrane to resist
348 the stress⁶⁵.

349 Differences in the FTIR spectra also occurred in the protein region. Several studies reported that NMs can
350 impact proteins (increased or decreased content; *i.e.* proteins involved in redox regulation), depending on
351 the exposure dose and the type of plant species⁶⁶. In particular, FTIR analysis also demonstrated a decrease
352 in amide (both primary and secondary) in cucumber fruits and tomato leaves after exposure to TiO₂-
353 NPs^{29,36}.

354 **Plant cell wall components** were the most impacted after exposure to both NMs. It has been reviewed
355 several times that abiotic and biotic stresses can modify content of primary and secondary cell wall
356 components like cellulose and hemicellulose³⁷ which can in turn influence plant growth and biomass.
357 Indeed, it has been shown that cell wall stress feeds back to regulate microtubule organization, auxin
358 transport, cellulose deposition, and future growth directionality⁶⁷. For instance, in the case of drought
359 stress, plants developed mechanisms leading to differential cell wall modifications allowing the reduction
360 of the aerial parts while underground parts were increased to further investigate for residual water in the
361 soil³⁹. Here, cell walls of plants exposed to TiO₂-NPs were the most impacted and subsequently their
362 growth was reduced up to 28% as well as their leaf area and biomass. These results are also in agreement
363 with nanoecotoxicology studies which reported that Ag-NPs also affected cellulose and hemicellulose
364 regions of FTIR spectra in radish sprouts (*Raphanus sativus*)³⁴. TiO₂-NP exposure also led to an increase
365 in the lignin band area of the FTIR spectra of cucumber fruit³⁶; however, they decreased lignin relative
366 content in tomato leaves but did not impact tomato fruit after exposure to TiO₂-contaminated sludge²⁹.
367 Cell wall components of rapeseed exposed to CNTs were also modified with a particular decrease in pectin
368 relative amount³². It has finally been reported that metal lignin complexes may be formed which could be
369 responsible for changes in plant chemical environment and could lead to modifications in their nutritional
370 properties^{33,36}.

371 Cellulose and hemicellulose are located inside primary cell walls and are responsible for the cell wall
372 rigidity³⁹. Cellulose provides mechanical strength for load-bearing due to the cross-linking by

373 hemicelluloses⁶⁸. Cell wall thickening represents a way for plant to resist both biotic and abiotic stress⁴⁰.
374 In fact, a thickening has been observed in plants as a response to mechanical intrusion of pathogens⁶⁹. It
375 has also been demonstrated that cellulose-deficient mutant plants are more sensitive to abiotic stress than
376 wild type plants³⁷. The increase in cellulose relative amount highlighted by FTIR could thus be a reaction
377 of plant exposed to NMs to limit their entry through cell walls. This hypothesis is consistent with the result
378 of the microarray profiling which demonstrated an increase in the LM19 labeling, *ie.* of lowly esterified
379 HG, also responsible for cell wall stiffening through the formation of the so-called egg boxes with calcium
380 ions⁷⁰.

381 An alternative hypothesis to the increased accumulation of this cell wall component is that it represents
382 the main negatively charged molecule of cell walls. Indeed, HG with a low degree of methylesterification
383 contains some amount of free carboxyl groups which can bind cations. As such, it plays a crucial role as a
384 buffer by sequestering positively charged molecules such as most heavy metals^{40,71,72}. Using quantum dots
385 (QD, NPs with diameter < 10 nm), some authors showed that NPs can directly interact with cell walls either
386 through hydrogen bonds with cellulose –OH groups or via the conjugated C-C or C=C chains in lignin^{73,74}.
387 Furthermore, a recent study assessed the influence of NP surface charge on their fate in plants and
388 demonstrated an accumulation of negatively charged QD in cell walls⁷⁵. Here, both NMs bear negative
389 charges when analyzed in suspension. However, so far, we have no data about their status in soil. Thanks
390 to their large surface area, NMs exhibit high adsorption properties and could thus adsorb many molecules
391 from the soil which in turn could influence their overall surface charge. In our experiment, the increase in
392 the amount of LM19 epitopes could indicate a higher sequestration capability in response to the presence
393 of NM in the medium.

394 Another phenomenon that can be responsible for cell wall modification is the oxidative stress caused by
395 NMs. Indeed, all types of NMs (*e.g.* carbon-based and metal based) have been reported to generate an
396 excess of reactive oxygen species (ROS)⁷⁶. For instance, CNTs increased ROS content in epidermis cells of

397 *Onobrychis arenaria* as well as the activity of antioxidant enzymes such as peroxidases (POX) after 15 days
398 of exposure in hydroponic conditions⁷⁷. TiO₂-NPs also increased the level of catalase (CAT) and ascorbate
399 peroxidase (APX) activities in leaves of cucumber exposed for 150 days in sandy loam soil³⁶. Besides, ROS
400 can be associated with cell wall modifications since a sudden burst of ROS can lead to catalytic oxidation
401 of various substrates of the cell wall which results in cross-linking of cell wall components and growth
402 arrest⁷⁸. Class III peroxidases, also involved in the regulation of oxidative stress, can promote cell wall
403 loosening via the hydroxylic cycle⁴⁹. Indeed, this has been demonstrated in *A. thaliana* exposed to nZVI
404 (nano zero valent iron) in agar medium⁷⁹. The authors concluded that root elongation was related to the
405 potential for nZVI to lead to H₂O₂ release causing OH radical-induced cell wall loosening in roots. This was
406 confirmed by the degradation of pectin-polysaccharides and a decrease in cell wall thickness. The
407 modification identified in the cell wall compounds in this work may thus also be explained by the oxidative
408 stress caused by the NMs tested which could be independent of NM internalization.

409

410 **5. Conclusion**

411 The use of FTIR spectroscopy in this study has allowed to identify similar impacts of CNTs and TiO₂-NPs on
412 tomato leaf cell walls despite their different physico-chemical properties. Microarray profiling confirmed
413 FTIR results and demonstrated significant modification in HG and XG for plants exposed to TiO₂-NPs
414 associated with a transiently reduced plant development (particularly visible after 15 days of exposure).
415 The same trend in cell wall modification was noticed for plants exposed to CNTs, though not significantly,
416 and with no impact on plant development. FTIR is a relatively easily accessible, fast and powerful technique
417 for a first screening approach. Although data processing is not straightforward, we have proposed a
418 strategy based on simple statistical analysis of the data which highlighted very slight modifications induced

419 by NM exposure and permitted us to focus the analysis further on the cell wall composition for a more
420 precise description of the physiological response.

421

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Figure captions

629 Figure 1. Morphological responses of tomato plants: plant height (A), total leaf area (B) and total fresh
630 biomass (C) after exposure during 15 days in soil contaminated with CNTs or TiO₂-NPs at 100 or 500 mg.kg⁻¹
631 (CNT 100, CNT 500, TiO₂ 100, TiO₂ 500) with standard errors (n = 5). Different letters imply statistical
632 differences (p<0.05). D is the PCA using all the morphological parameters (leaf number, plant height, leaf
633 area and biomass).

634

635 Figure 2. (A) PC-LDA analysis of the normalized FTIR spectra for tomato leaves after 15 days of exposure in
636 soil contaminated with CNTs or TiO₂-NPs at 100 or 500 mg.kg⁻¹ (CNT 100, CNT 500, TiO₂ 100, TiO₂ 500)
637 including also the barycenter of the ellipse for each treatment. (B) Normalized FTIR spectra for tomato
638 leaves after 15 days of exposure in soil contaminated with CNTs or TiO₂-NPs. Peaks contributing the most
639 to differences among groups are highlighted in yellow. Peak A = 2852-2848 cm⁻¹, lipid region. Peak B =
640 1550-1537 cm⁻¹, amide II region. Peak C = 1320-1312 cm⁻¹, carboxyl region. Peak D = 1160-1155 cm⁻¹,
641 polysaccharide region (cellulose). Peak E = 1082-1070 cm⁻¹, polysaccharide region (hemicelluloses). Peak F
642 = 1052-990 cm⁻¹, pectin and various polysaccharides region.

643

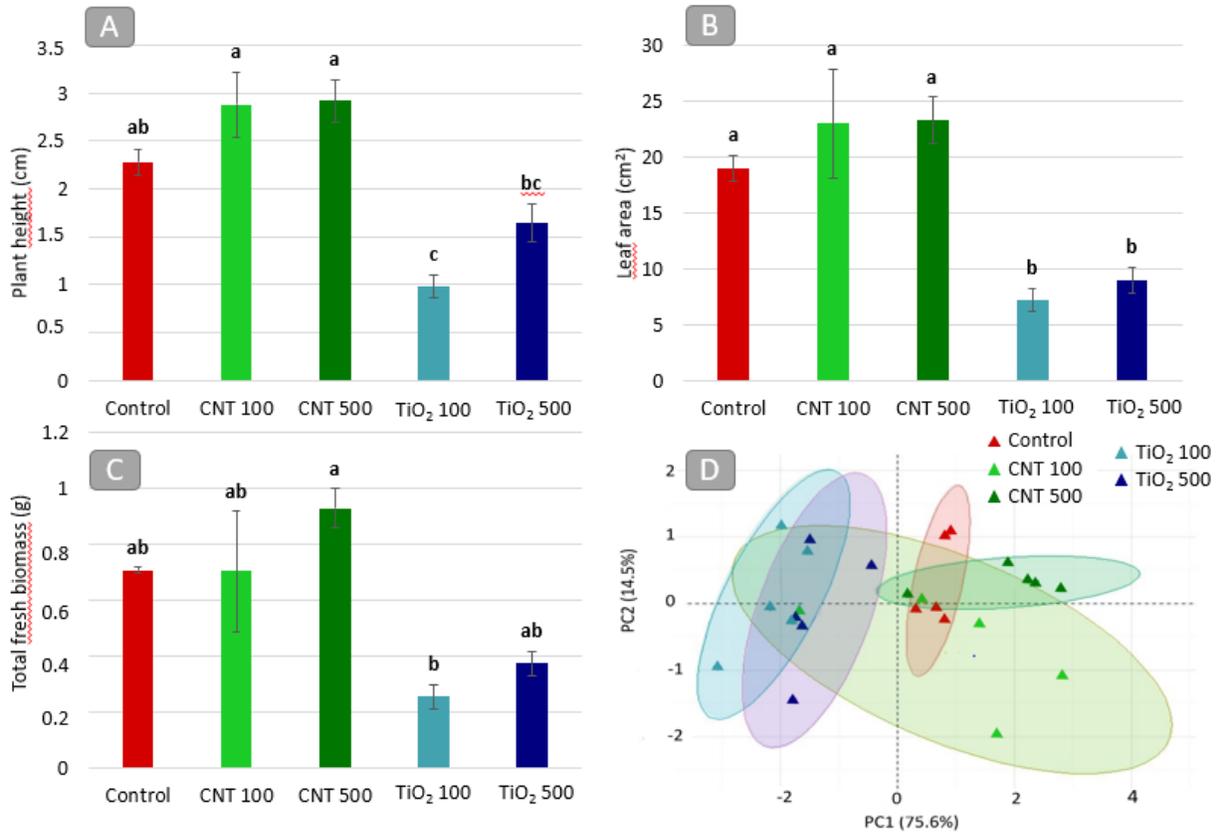
644 Figure 3. PC-LDA of the FTIR spectra (between 1800-800 and 2900-2700 cm⁻¹) acquired on individual
645 tomato leaves after 15 days of exposure in soil containing 500 mg.kg⁻¹ CNTs or TiO₂-NPs (Control, CNT 500
646 and TiO₂ 500) (A youngest leaf, B intermediate leaf, C intermediate leaf and D oldest leaf). PC-LDA were
647 run with Orange software and drawn with RStudio (ggplot2).

648

649 Figure 4. Polysaccharide microarray analysis of cellulose-, hemicelluloses- and pectin-enriched fractions of
650 tomato cell wall leaves exposed for 15 days to 500 mg.kg⁻¹ CNT or TiO₂-NPs. The detection was performed
651 on nitrocellulose membranes which were subsequently scanned. The signals were then quantified. Results
652 are expressed in intensity in comparison to the control and standard errors are indicated (n=5). LM25 is
653 specific for XG (motifs XLLG, XLG and XXXG), LM15 for XG (motif XXXG) and LM19 for HG.

654

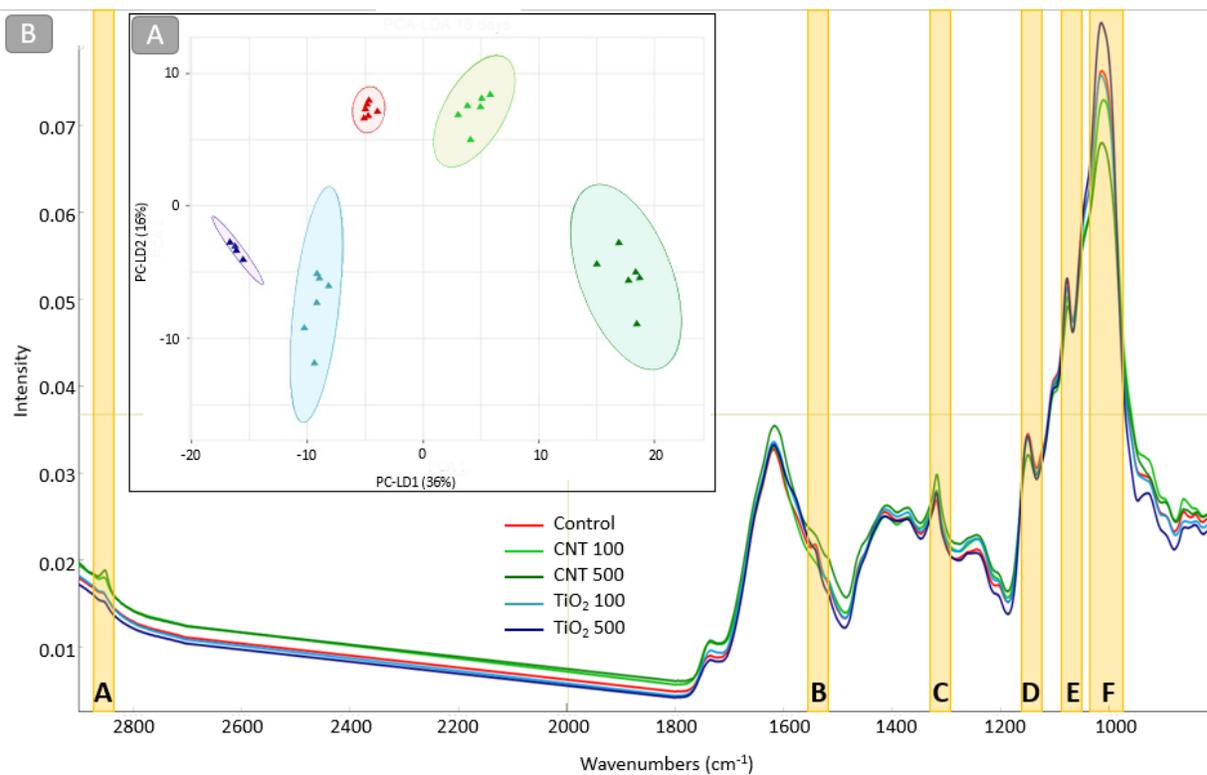
655 Figure 1.



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658 Figure 2.



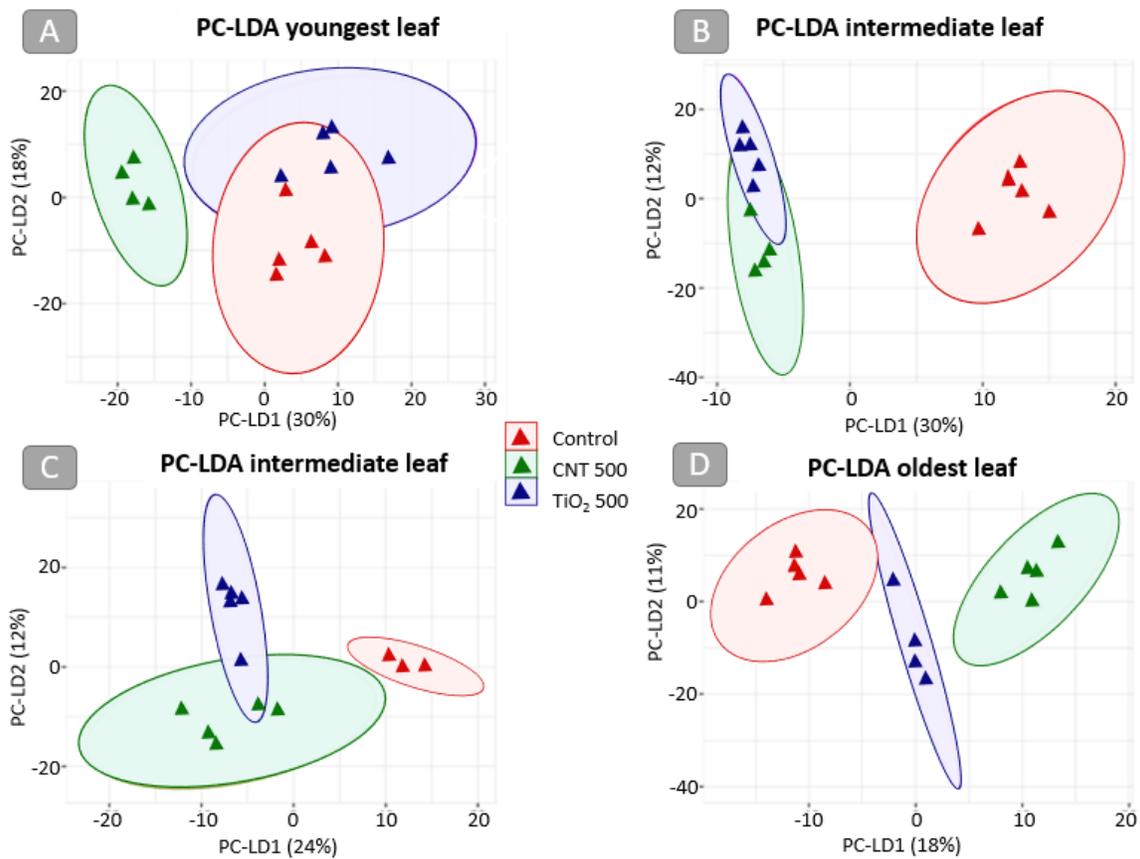
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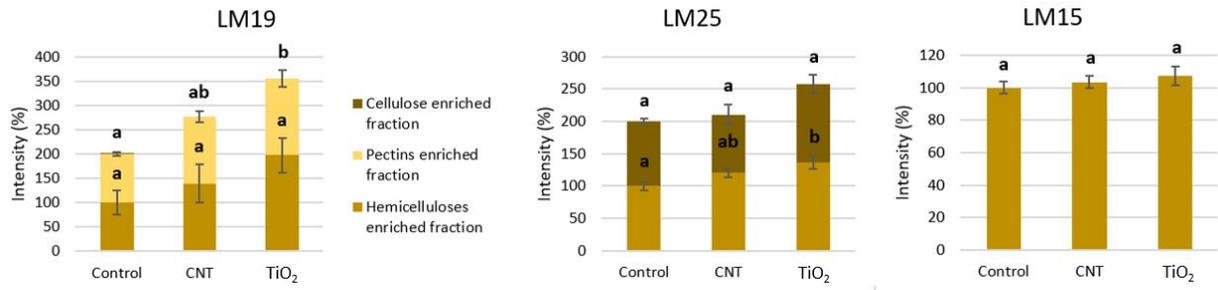
663 Figure 3.



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666 Figure 4.



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670 Table 1. Peaks contributing the most to differences among treatments extracted from the logistic
 671 regression for 15 days of exposure with the band letter corresponding to the figure 2 together with the
 672 area under the absorption peak extracted from normalized FTIR spectra the for the five different
 673 conditions (Control, CNT 100, CNT 500, TiO₂ 100 and TiO₂ 500). Areas are expressed in % in comparison to
 674 the control with standard errors.

Wavenumbers (cm ⁻¹)	Band	Assignment	Main compounds	References	CNT 100	CNT 500	TiO ₂ 100	TiO ₂ 500	P-value
2852 - 2848	A	CH ₂ symmetric stretch	Lipids	47,57	+25 ± 0%	+29 ± 2%	+12 ± 2%	+6 ± 0%	<0.001
1550 - 1537	B	N-H and C=N	Amide II	55,56	-12 ± 1%	+1 ± 1%	-6 ± 7%	-6 ± 1%	0.041
1320 - 1312	C	C-H bend	Carboxyl groups from ligands, proteins, various polysaccharides (cellulose)	38,47,56, 57	+5 ± 1%	+15 ± 3%	+3 ± 5%	+3 ± 1%	0.018
1160 - 1155	D	OH or C-O stretch	Various polysaccharides (mainly cellulose)	38,47,56, 57	-1 ± 0%	-5 ± 1%	-1 ± 1%	-1 ± 1%	0.004
1082 - 1070	E	C-O ring stretch	Various polysaccharides (hemicelluloses in particular)	38,47,56	-5 ± 1%	-6 ± 1%	-3 ± 1%	-1 ± 0%	0.033
1052 - 990	F	O-H and C-OH stretch	Pectin, various polysaccharides	47,56,57	-1 ± 2%	-6 ± 1%	+2 ± 3%	+9 ± 1%	<0.001

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