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Fourier transform infrared spectroscopy contribution to disentangle nanomaterial (DWCNT, TiO_2) impacts on tomato plants†

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Carbon nanotubes (CNTs) and titanium dioxide nanoparticles (TiO_2-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 TiO_2-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} TiO_2-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 TiO_2-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.

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Environmental significance

Nanomaterial use is increasing continuously, implying their release in the environment. Today, the risk assessment of these new materials is lagging behind because of the lack of fast and reproducible techniques to assess their biological impacts. In this work, we report the use of Fourier transformed infrared (FTIR) spectroscopy as a fast, widely informative and easy to set up technique. Thanks to FTIR, early impacts of carbon nanotubes and TiO_2 nanoparticles on tomato plants exposed in soil were highlighted suggesting an alteration of plant cell walls. This information was further investigated by microarray polymer profiling confirming the relevance of FTIR data. FTIR appears thus as a very efficient technique to screen nanomaterial effects on organisms and speed up risk assessment.

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1. Introduction

Over the last two decades, nanotechnologies have become increasingly important. Indeed, nanomaterials (NMs) present unique properties such as a large specific surface area which can be useful in many domains such as electronics, materials or food industry.[1] In 2020, the Dutch Nanodatabase revealed that a total of 5000 consumer products officially contained NMs.[2] Investigations about their possible use in medicine[3] or in agriculture[4] are also in progress.

Carbon nanotubes (CNTs) and titanium dioxide nanoparticles (TiO_2-NPs) are among the most widely used NMs.[2] CNTs are part of the carbon-based NM family. They have remarkable optical, electrical, thermal, mechanical and

chemical properties[5] and are mainly used in batteries, plastic additives or sporting goods.[6] TiO_2-NPs are well known for photocatalytic applications[7] and are included for example in food additives[8] or cosmetics.[9] Since NM applications are steadily increasing, their release in the environment, intentionally or not, is of great concern.

Assessing NM concentrations in the environment is a major bottleneck in ecotoxicology. Modeling studies were carried out on some NMs to evaluate this information in different environmental compartments. TiO_2-NPs have been identified as one of the most concerning NMs due to the high concentrations forecast: around 61 mg kg^{-1} in sludge treated soils against 12 μg kg^{-1} for CNTs.[10]

NM impacts on terrestrial ecosystems are still controversial, in particular on plants.[11] Indeed, some authors reported higher germination rate and better yield after exposure to CNTs while other studies highlighted decreased root length or oxidative stress.[11] The same conclusions were reached for TiO_2-NP impacts on plants:[12] while some beneficial effects were reported such as a higher germination rate or increased root and shoot length,[13–16] other works described decreased germination rate, plant growth or genotoxic effects.[17–19] Until now, the specific mechanisms implied in NM uptake (active *vs.* passive, apoplast *vs.* symplast, among other questions) and impact (*e.g.* nano specific or ion related, oxidative stress mediated) are still to be identified and require further research.[20]

Apart from obvious physico-chemical differences, a possible explanation of these contrasting phytotoxicity results may also be the method used to evaluate NM impacts on plants. Many biomarkers can be assessed from the morphological to the gene scale showing variable sensitivity. Their use to evaluate plant health is conclusive when many of them are combined. But in the literature, most of the studies use a limited number of biomarkers leading to a potentially partial image of the toxicity effects and thus a biased risk assessment. The availability of routine, standardized and widely informative analytical methods to evaluate NM toxicity is a key to fill this current gap of knowledge.[21]

Fourier transform infrared (FTIR) spectroscopy is a technique based on the vibrational state of molecules. It allows the acquisition of a spectrum combining information on a multitude of compounds, unlike chemical dosages which give access only to one compound (*e.g.* specific enzyme or secondary metabolite) after a series of reactions.[22] There are two types of acquisition modes: either bulk analysis of the whole plant (few minutes per sample) or 2D-imaging mode of cross sections (few hours per map).[23] In bulk mode, sample preparation is very simple, consisting of grinding dry materials thus reducing artifacts. Therefore, FTIR is a widely informative, easy to set-up and fast technique that could be used to screen NM effects on different organisms. In plant biology, FTIR has been mainly used to characterize plant cell wall components in a highly sensitive and more time-efficient manner than traditional methods which require isolation, extraction and fractionation of the different cell wall components.[24–28] Recently, FTIR has been used in ecotoxicological studies to analyze changes occurring in biological materials after exposure to biotic or abiotic stresses.[23,29–36] For instance, Morales *et al.*, Servin *et al.* and Zhao *et al.* highlighted changes in the chemical environment of carbohydrates of both cilantro and cucumber exposed to CeO_2, ZnO or TiO_2 NPs.[33,35,36] Radish sprouts exposed to Ag-NPs also exhibited modifications of their IR spectral signature in the region related to lipids, proteins and particularly structural component peaks such as lignin, pectin and cellulose.[34] Likewise, very recently, we applied FTIR to evaluate the influence of plant species on their response to a CNT contamination highlighting the role of cell wall composition.[32] Indeed, it has been demonstrated several times that cell walls play a crucial role in plant response to abiotic stresses.[37] Plant cell walls are composed of complex polysaccharides and a small amount of proteins and their composition can be modified in response to stress.[37–40]

However, FTIR data processing is tedious due to spectrum complexity. Indeed, it contains overlapping signals coming from many molecular bonds. A purely visual inspection of spectra is often insufficient to draw a conclusion. Several factors could weaken this analysis and its subsequent conclusions: i) sometimes, minor spectral differences not detected with the bare eye may contain critical information, ii) the baseline may vary from one sample to another, iii) instrumental noise could induce bias. For these reasons, it is important to find a way to process and analyze the data in a more systematic way using statistical approaches (*i.e.* supervised classification, clustering method) in order to obtain meaningful information.[41]

The main goal of this study was to develop the FTIR approach to evaluate the comparative impacts of two types of NMs (CNTs and TiO_2-NPs) taking into account: (i) different NM concentrations and (ii) different exposure durations. Seedlings of tomato (*S. lycopersicum* L.) were grown in soil contaminated with CNTs or TiO_2-NPs at two different concentrations (100 and 500 mg kg^{-1} of soil) during different durations (5, 10, 15 and 20 days). FTIR was used as the main technique to evaluate the impact of the two NMs on tomato plants. Complementary morphological biomarkers were also assessed (height, biomass, number of leaves, leaf surface area). Finally, to better understand the FTIR data, the cell wall composition was further analyzed by microarray polymer profiling. Developing a reliable technique to assess in a screening mode the biological effects of many different types of NMs is mandatory to accelerate the risk assessment of these new materials being disseminated or intentionally introduced in our environment on a daily basis.

2. Materials and methods

2.1. Nanomaterials

TiO_2-NPs (ref 718467, Aeroxide P25, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were characterized in a previous

experiment (same batch) and were composed of 80% anatase and 20% rutile with a nominal diameter of 25.0 ± 5.7 nm.[19] They had a specific surface area of 46 ± 1 m^2 g^{-1} (Fig. S1A†).[19]

Double walled CNTs were synthesized by catalytic chemical vapor deposition (CCVD) at 1000 °C of a mixture of CH_4 (18 mol.%) and H_2 using a Co:Mo/MgO-based catalyst (chemical composition: $Mg_{0.99}Co_{0.0075}MgO_{0.0025}$).[42] The outer diameter ranged from 1 to 3 nm and the length varied from 1 to 100 microns (Fig. S1B†).[42] The specific surface area was 980 m^2 g^{-1} (Brunauer, Emmett and Teller (BET) method; Micromerics Flow Sorb II 2300, Micromeritics, Norcross, GA, USA).

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

2.2. Soil characteristics and contamination

A silty sand soil (according to the United State Department of Agriculture[43]) was used for this experiment (Lufa-Speyer, 2.1, Speyer, Germany) with a composition of 88.0% sand, 9.1% silt and 2.9% clay. It contained 0.71 ± 0.08% organic carbon, 0.06 ± 0.01% nitrogen, had a pH of 4.9 ± 0.3 and a cation exchange capacity of 4.3 ± 0.6 meq/100 g of soil. The soil water capacity was 60 mL/100 g of soil.

CNT or TiO_2-NP suspensions were added to the dry soil to reach a concentration of 100 or 500 mg NMs per kg dry soil (ratio liquid/soil = 1/1 in mass). After 2 h on a shaker table, the soil mixture was filtered to remove the water in excess. This soil preparation protocol ensured a soil contamination as homogeneous as possible. These concentrations were chosen to be relevant for TiO_2-NP contamination in sludge amended soils[10] and comparable between NMs.

2.3. Plant material and cultivation

Organic seeds of tomato *S. lycopersicum* L. (var. Red Robin) were obtained from the French seed company Germinance (Soucelles, France) and surface-sterilized using $Ca(ClO)_2$ (1%). Seedlings were first grown in hydroponic conditions for 3 weeks until they reached the 5 leaf-stage. Plants were then placed into control or contaminated soil until harvest after 5, 10, 15 or 20 days of exposure. Exposure durations were chosen based on a literature study showing that more than 65% of articles studying CNT impacts on plants used exposure duration of less than 15 days.[11] Each exposure duration corresponded to an independent experiment. The experiments were performed in a growth chamber with controlled parameters: 10 h light/14 h dark photoperiod; 24 °C during the day and 22 °C during the night; and a hygrometry of 85%.

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

Morphological parameters were monitored every day (plant height and number of leaves). Upon harvest, other morphological parameters were measured (total fresh leaf biomass and foliar surface area using a camera and ImageJ software[44]). Leaves were dried at 50 °C during 24 h prior to FTIR analysis.

2.4. FTIR analysis

About 20 mg dry leaves were ground 2 × 15 s at maximum speed using a FastPrep grinding machine (MP Biomedicals, Illkirch-Graffenstaden, France). Each powdered sample was analyzed in attenuated total reflectance (ATR) mode using a diamond crystal (Thermo Nicolet Nexus, Smart Orbit, Thermo Fisher Scientific, Waltham, USA). Infrared spectra were collected in the range 4000–400 cm^{-1}. All the samples (5 biological replicates) were analyzed in (technical) triplicates and each spectrum was the sum of 64 scans. OMNIC software (Thermo Fischer Scientific©) was used to export spectra.

2.5. Chemometric analysis for FTIR data

A chemometric analysis of FTIR spectra was developed using Orange software (BioLab, Ljubljana, Slovenia)[45] including the add-on Spectroscopy.[46] During the first step, data were pre-processed to eliminate possible analytical biases (such as detector noise and atmospheric background).[23] For this, a Savitzky–Golay filter was applied (point window: 21, polynomial order: 2, derivative order: 2). This filter is based on simplified least square procedures and permits removal of various instrumental and scattering effects. A vector normalization was then applied to minimize the effects of the source power fluctuations as well as to overcome variations due to the amount of leaf powder analyzed. The last step of the pre-processing was to select the region of interest in order to avoid background interferences.[23,47] Here, we focused on two regions of the spectra: between 2900 and 2700 cm^{-1} corresponding to the lipid region and between 1800 and 800 cm^{-1} corresponding to the so-called fingerprint region (including proteins and polysaccharides). The region between 1800 and 2700 cm^{-1} was removed because it mainly corresponded to background interferences. With this pre-process, the robustness and accuracy of subsequent analyses were improved and the interpretability of the data was increased by correcting issues associated with spectral data acquisition.

A multivariate analysis was then performed on the pre-processed spectra with first a principal component analysis (PCA), followed by a linear discriminant analysis (LDA) when necessary.[41] PCA is an unsupervised method which searches for directions where data have the largest variance, whose results can show data structural information. While, LDA is a supervised method that looks for projections that maximize the ratio between-class to within-class. The combination of both methods is particularly useful when the number of

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

In order to identify the wavenumbers contributing the most to differences among groups, a logistic regression was run on the pre-processed spectra. The logistic regression is a predictive model that yields the probability of occurrence of an event by fitting data to a logistic curve. The least absolute shrinkage and selection operator (LASSO) method was used to perform the regularization and feature selection. Most relevant wavenumbers were identified by obtaining logistic regression coefficients; this feature extraction method has been already used in ATR-FTIR data analysis.[48] For testing the robustness of the statistical model used, the area under a receiver operating characteristics (ROC) curve (AUC) and K-fold cross validation were used.[41] Finally, to compare the different spectra among them, the area under differing absorption peaks was calculated by integrating the area starting from 0 on the pre-processed spectra.

2.6. Cell wall composition by polysaccharide microarray analysis

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

Cell wall polysaccharides were sequentially extracted from homogenates using three solvents: (i) 50 mM diamino-*cyclo*-hexane-tetra-acetic acid (CDTA), pH 7.5, (ii) 4 M NaOH with 1% v/v NaBH4, and (iii) cadoxen (31% v/v 1,2-diaminoethane with 0.78 M CdO). The three extraction solvents used are known to solubilize pectins, non-cellulosic polysaccharides, and cellulose, respectively. For each extraction, a ratio of 6 μL solvent for 1 mg fresh biomass was added to each tube before incubation with shaking for 1 h. After centrifugation at 2500*g* for 10 min, the supernatants were removed prior to addition of the next solvent to pellets. All the supernatants were finally stored at 4 °C. Forty μL of diluted extracts (2/50, vol/vol) in TBS (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-RAD, Marnes-la-Coquette, France) onto nitrocellulose membranes (Sigma-Aldrich). After blocking TBS–T/BSA 0.05% (0.05% Tween), the arrays were probed overnight at 4 °C with primary mAbs directed against different cell wall epitopes (https://plantcellwalls.leeds.ac.uk/plantprobes/) at a 1/250 dilution (vol/vol) in TBS–T/BSA 0.05%: LM19 (for non-methylated homogalacturonans, HG), LM20 (for methylated HG, mHG), LM25 for the XLLG, XXLG and XXXG motifs of xyloglucans, XG), LM15 (for the XXXG motif of XG and to some extent single galactosyl substitution of the XXXG oligosaccharide, and LM24 (for the XLLG motif of XG). After washing in TBS-T, the arrays were probed with anti-rat IgG secondary antibodies conjugated to alkaline phosphatase (Sigma-Aldrich) at a 1/10000 dilution (vol/vol) for 2 h before washing and developing in a BCIP/NBT (5-bromo-4-chloro-3¢-indolyphosphate/nitro-blue tetrazolium chloride) substrate. To check the activity of the mAbs, commercially purified polysaccharides were used as positive controls: polygalacturonic acid (HG, Sigma-Aldrich), polygalacturonic acid methyl ester (mHG, Sigma-Aldrich), and XG (Megazyme, Libios, Pontcharra-sur-Turdine, France). The arrays were scanned using an Epson Perfection V370 Photo (Nagano, Japon). Color intensity of each spot was quantified thanks to ImageJ software.

2.7. Statistical analysis

Data (morphological parameters) were checked for homoscedasticity and normality. When assumptions were met for parametric analyses, a two-way ANOVA was used. Otherwise, a Kruskal-Wallis test was applied. A PCA was also performed on the full dataset. All statistical analyses were carried out using the RStudio statistical software[50] (version 1.1.453) with multcompView,[51] lsmeans,[52] pgirmess,[53] ggplot2 (ref. 54) packages.

3. Results

3.1. Morphological responses

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

Differences were only detected after 15 days of exposure. Indeed, plants exposed to 100 mg kg^{-1} TiO_2-NPs were significantly smaller than the control (57%, p-value < 0.05, Fig. 1A). Plants exposed to 500 mg kg^{-1} TiO_2-NPs were 28% smaller than the control plant but this decrease was not significantly different (2.3 ± 0.3 cm for the control and 1.6 ± 0.4 cm for 500 mg kg^{-1} TiO_2-NPs). Although plants exposed to both CNT concentrations for 15 days were not significantly different in height from the control plants, there was an increase of 26% and 28% in soils contaminated with 100 and 500 mg kg^{-1} CNT, respectively (2.3 ± 0.3 cm 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).

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

On the one hand, the total leaf area of plants exposed to the two TiO_2-NP concentrations was decreased after 15 days of exposure: 7.2 ± 2.4 cm^2 for 100 mg kg^{-1} TiO_2-NPs and 8.9 ± 2.6 cm^2 for 500 mg kg^{-1} TiO_2-NPs while that of the control was at 19.0 ± 2.7 cm^2 (p-value < 0.001, Fig. 1B). On the other

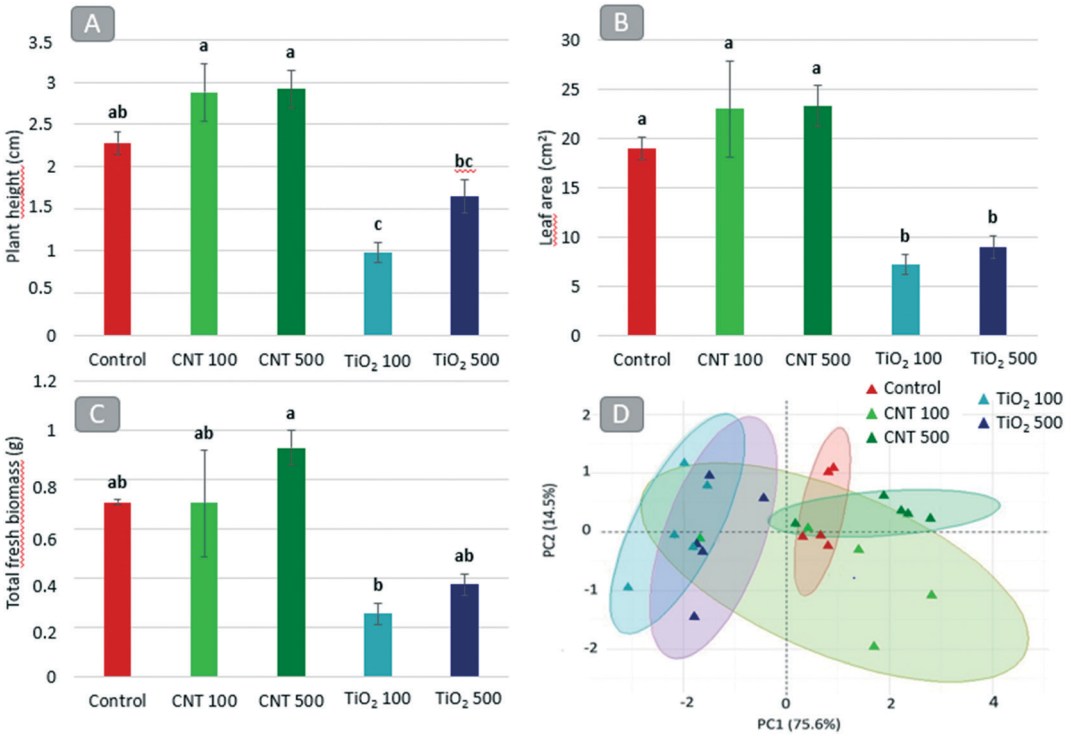

Fig. 1 Morphological responses of tomato plants: plant height (A), total leaf area (B) and total fresh biomass (C) after exposure during 15 days in soil contaminated with CNTs or TiO_2 NPs at 100 or 500 mg kg^{-1} (CNT 100, CNT 500, TiO_2 100, TiO_2 500) with standard errors (n = 5). Different letters imply statistical differences ($p < 0.05$). D is the PCA using all the morphological parameters (leaf number, plant height, leaf area and biomass).

hand, no significant difference was found for plants exposed to CNTs.

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

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

3.2. Leaf chemical composition after FTIR analysis

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

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

Once a significant cluster structure was identified for the 15 day treatment case, feature extraction was performed with a logistic regression model. This model was tested by a stratified 3-fold cross-validation method, obtaining average over classes scores of 0.932 of AUC, 0.889 of precision and a recall of 0.852, notice that a strong and robust model have scores close to one.[41] A difference was highlighted in the so-called "lipid region" (Fig. 2B, peak A, Table 1) with higher relative amounts for plants exposed to the four different treatments in comparison to the control with the highest amount in leaves of plants grown on soil contaminated by CNTs at 500 mg kg^{1} (+ 29 ± 2.3% of the area under the peak for CNT 500 in comparison to the control). In the amide II peak,[55,56] leaves of plants grown on contaminated soils exhibited an increase in peak area in comparison to the control except for CNT 500 (12 ± 1% increase for CNT 100, 6 ± 7% for TiO_2 100 and 6 ± 1% TiO_2 500) (Fig. 2B, peak B; Table 1). Polysaccharides[38,47,56,57] also seemed to be impacted

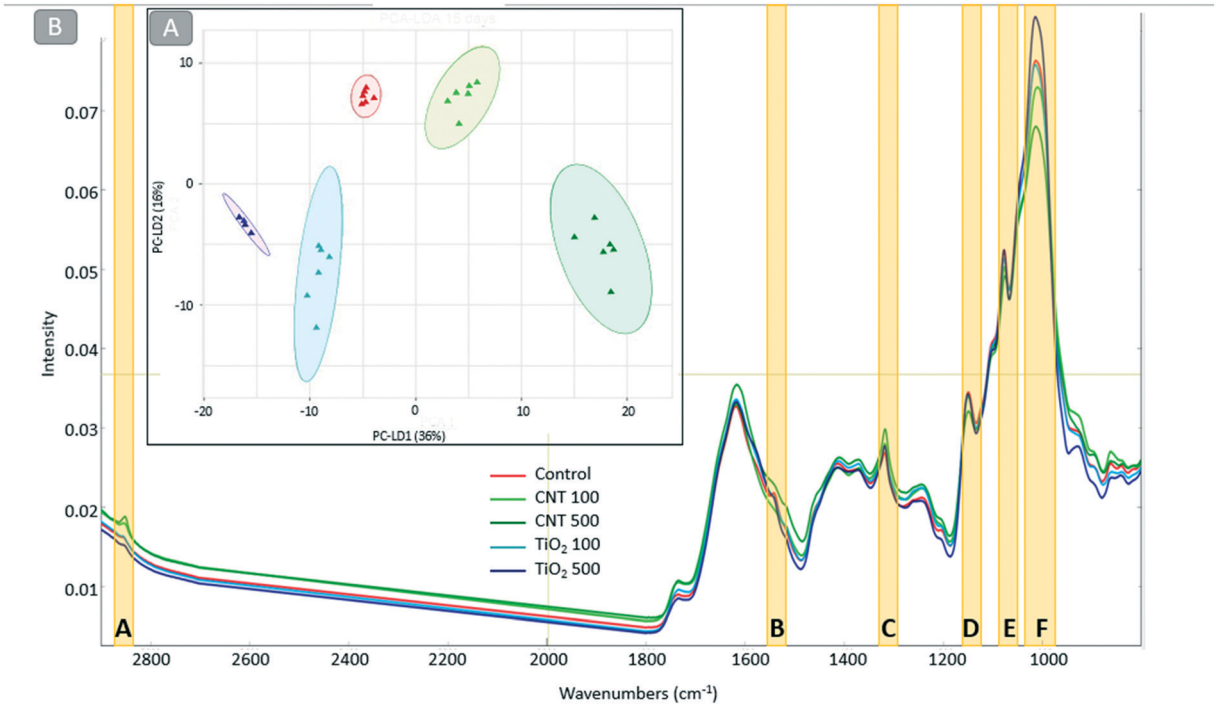

Fig. 2 (A) PC LDA analysis of the normalized FTIR spectra for tomato leaves after 15 days of exposure in soil contaminated with CNTs or TiO_2 NPs at 100 or 500 mg kg^{-1} (CNT 100, CNT 500, TiO_2 100, TiO_2 500) including also the barycenter of the ellipse for each treatment. (B) Normalized FTIR spectra for tomato leaves after 15 days of exposure in soil contaminated with CNTs or TiO_2 NPs. Peaks contributing the most to differences among groups are highlighted in yellow. Peak A = 2852 2848 cm^{-1}, lipid region. Peak B = 1550 1537 cm^{-1}, amide II region. Peak C = 1320 1312 cm^{-1}, carboxyl region. Peak D = 1160 1155 cm^{-1}, polysaccharide region (cellulose). Peak E = 1082 1070 cm^{-1}, polysaccharide region (hemicelluloses). Peak F = 1052 990 cm^{-1}, pectin and various polysaccharides region.

Table 1 Peaks contributing the most to differences among treatments extracted from the logistic regression for 15 days of exposure with the band letter corresponding to the Fig. 2 together with the area under the absorption peak extracted from normalized FTIR spectra the for the five different conditions (control, CNT 100, CNT 500, TiO_2 100 and TiO_2 500). Areas are expressed in % in comparison to the control with standard errors

Wavenumbers (cm^{-1})	Band	Assignment	Main compounds	Ref.	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

with differences in the areas of peaks C (1320–1312 cm^{-1}) and D (1160–1155 cm^{-1}). Exposed plants displayed an increase in peak C area while a slight decrease in the peak D area was observed. Between 1080 and 1070 cm^{-1} corresponding to hemicellulose[38,47,56] (Fig. 2B, peak E; Table 1), a slight decrease in the peak area was detected for the leaves of plants grown on all contaminated soils. Finally, a decrease in areas of peak F corresponding to pectin and various polysaccharides[47,56,57] (1052–990 cm^{-1}, Fig. 2B, peak F; Table 1) was noticed for the plants grown in soil contaminated with CNTs whereas an increase in peak F area was detected for the plants grown with TiO_2-NPs. Altogether, most of the differences observed between the FTIR spectra were related to cell wall components (pectin, cellulose or hemicellulose).

The signals obtained here were averaged on the whole leaf biomass. For further analysis, chemical composition of the leaves was observed considering their age on the two 500 mg kg^{-1} NM treatments (Fig. 3). Overall, the oldest and the youngest (at early development stage during contaminant

exposure) leaves were the least impacted by NM contamination; leaves of plants exposed to TiO_2-NPs, in particular, had a chemical composition very similar to those of control plants (Fig. 3A and D). However, intermediate leaves (Fig. 3B and C) exposed to NM displayed different FTIR signatures than control plants, but similar in-between them according to the PC-LDA with overlapping ellipses for both CNT and TiO_2-NP treated leaves.

3.3. Cell wall composition by polysaccharide microarray analysis

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

Significant differences were found for HG (LM19) in the pectin-enriched fraction of leaves of plants exposed for 15 days to 500 mg kg^{1} TiO_2-NPs with a 58% increase (p = 0.028). The LM19 signal also increased by nearly two-fold in the hemicellulose-enriched fraction although this increase was not significant. Significant differences were also detected with LM25 for the same condition (500 mg kg^{1} TiO_2-NPs) in the hemicellulose-enriched fraction (+37% in comparison to the control, p = 0.046). However, no significant difference was found with LM15 specific for the XXXG motif of XG. It can be concluded that XXLG is the only XG motif whose amount was modified in leaves when plants were exposed to 500 mg kg^{1} TiO_2-NPs for 15 days. For CNT, no significant difference was found with all the mAbs tested here.

Altogether, it was not possible to detect mHG (LM19) or the XLLG motif of XG (LM24) in the tomato leaves whatever the treatment. Significant changes were only observed after the treatment with 500 mg kg^{1} TiO_2-NPs, corresponding to an increase in the amount of HG (LM19) and of the XXLG motif of XG (LM25, *vs.* LM15 and LM24).

4. Discussion

In this study, **FTIR** spectroscopy appeared to be a more sensitive technique to detect the impact of NM treatment on plant compared to the traditionally used morphological biomarkers. Indeed, FTIR analysis revealed a plant response to NM contamination even at the shortest time of exposure (5

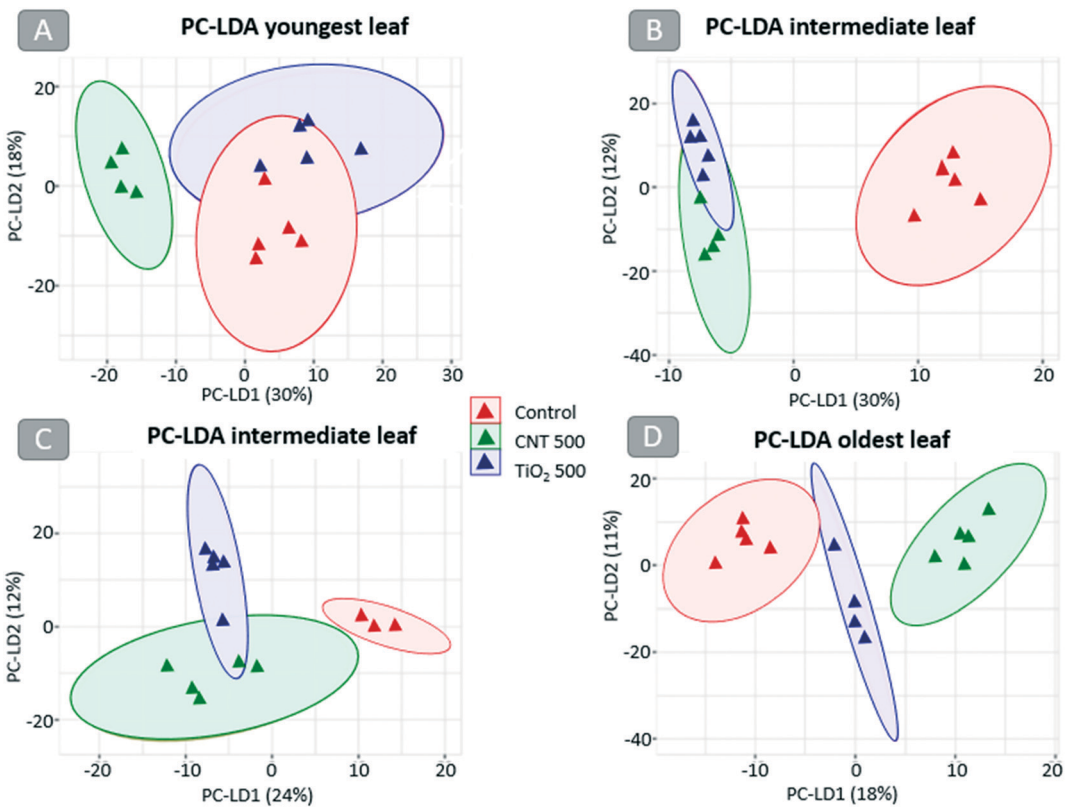

Fig. 3 PC LDA of the FTIR spectra (between 1800 800 and 2900 2700 cm^{-1}) acquired on individual tomato leaves after 15 days of exposure in soil containing 500 mg kg^{-1} CNTs or TiO_2 NPs (control, CNT 500 and TiO_2 500) (A youngest leaf, B intermediate leaf, C intermediate leaf and D oldest leaf). PC LDA were run with Orange software and drawn with RStudio (ggplot2).

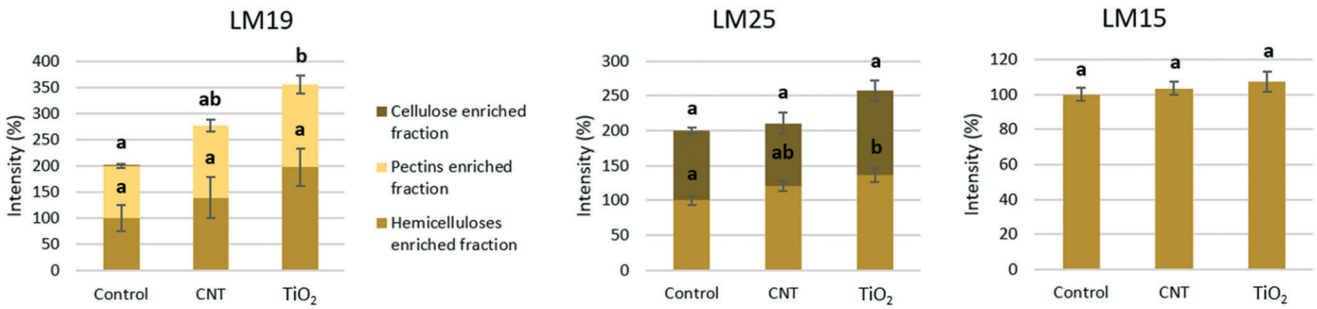

Fig. 4 Polysaccharide microarray analysis of cellulose , hemicelluloses and pectin enriched fractions of tomato cell wall leaves exposed for 15 days to 500 mg kg^{-1} CNT or TiO_2 NPs. The detection was performed on nitrocellulose membranes which were subsequently scanned. The signals were then quantified. Results are expressed in intensity in comparison to the control and standard errors are indicated (n = 5). LM25 is specific for XG (motifs XLLG, XXLG and XXXG), LM15 for XG (motif XXXG) and LM19 for HG.

days). Looking at the morphological parameters, few differences were visible only after 15 days but not earlier which seemed to be compensated later. FTIR also allowed assessing NM impacts on several biomacromolecules (*i.e.* lipids, polysaccharides) in one single analysis, thus permitting to dedicate further research efforts to look at the modifications of cell wall composition under the influence of NM exposure. The developed chemometric analysis was quite powerful in highlighting differences between the experimental conditions in an automated way, which would have not been possible by visual inspection of the FTIR spectra. FTIR spectroscopy is thus a relevant method to identify early impacts of NMs on plants in a fast and reliable way, thereby permitting a screening approach.

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

The impacts of both NMs tended to increase with **time** until 15 days of exposure, and then decreased (20 days: no detectable difference in morphological biomarkers and lower impact on biomacromolecules such as lipids, polysaccharides or proteins as demonstrated by FTIR analysis). This decrease in impacts after 15 days of exposure could suggest a plant recovery. Likewise, when studying NM impact on individual leaves of different ages, the oldest one (*i.e.* exposed for the longest period) was the least impacted in its chemical composition while clear differences were visible on other fully-expanded leaves. This could also correspond to a recovery or adaptation at the leaf level. Very little has been done so far to study plant recovery after a NM exposure. One study reported that TiO_2-NPs had no major impact on tomato plants upon harvest (after 5 months of exposure), but some markers indicated that plants might have gone through oxidative stress earlier in their life cycle.[29] It has also been shown after exposure to different heavy metals (Zn, Co, Cd, Ni, Mn) that the detoxification response was triggered during the first days of exposure and then decreased back nearly to its basal level after 9 days.[60] It would thus be interesting to investigate NM impacts on biomacromolecules under chronic exposure conditions to confirm this hypothesis and further improve risk assessment strategies.

CNTs and TiO_2-NPs have been chosen here as they are two very different NMs; in particular, they vary in shape (tubular for CNTs *vs.* spherical for TiO_2-NPs), in surface chemistry (carbon *vs.* metal oxide), in diameter (1–3 nm diameter for CNTs *vs.* 25 nm for TiO_2-NPs) but they are both very insoluble. Their behavior and impacts are thus expected to be quite different. Indeed, at the morphological level, TiO_2-NPs inhibited tomato development while CNTs tended to stimulate it. These results are consistent with previously published literature.[19,61] However, regarding biomacromolecule composition NM triggered quite similar impacts, especially on cell wall components, which might suggest a common response of plants upon exposure to CNTs or TiO_2-NPs.

FTIR spectra showed that the relative amount of lipids in leaves was increased following exposure to both NMs. This result is in agreement with studies performed on spinach, where TiO_2-NPs also increased the level of lipids after a foliar contamination.[33,35,62] Using FTIR analysis, several studies also reported that metal-based NMs increased the relative amount of lipids in *R. sativus* (Ag-NPs)[34] and in *C. sativum* (CeO_2-NPs).[33] Lipid accumulation is one of the plant responses to various stresses such as high temperature, drought or heavy metals.[63,64] Changes in the lipid composition and/or interactions between lipids and specific membrane proteins can occur in order to reinforce the phospholipid membrane to resist the stress.[65]

Differences in the FTIR spectra also occurred in the protein region. Several studies reported that NMs can impact proteins (increased or decreased content; *i.e.* proteins involved in redox regulation), depending on the exposure dose and the type of plant species.[66] In particular, FTIR analysis also demonstrated a decrease in amide (both primary and secondary) in cucumber fruits and tomato leaves after exposure to TiO_2-NPs.[29,36]

Plant cell wall components were the most impacted after exposure to both NMs. It has been reviewed several times that abiotic and biotic stresses can modify content of primary and secondary cell wall components like cellulose and hemicellulose[37] which can in turn influence plant growth and biomass. Indeed, it has been shown thatcell wall stress feeds back to regulate microtubule organization, auxin transport, cellulose deposition, and future growth directionality.[67] For instance, in the case of drought stress, plants developed mechanisms leading to differential cell wall modifications allowing the reduction of the aerial parts while underground parts were increased to further investigate for residual water in the soil.[39] Here, cell walls of plants exposed to TiO_2-NPs were the most impacted and subsequently their growth was reduced up to 28% as well as their leaf area and biomass. Thess results are also in agreement with nanoecotoxicology studies which reported that Ag-NPs also affected cellulose and hemicellulose regions of FTIR spectra in radish sprouts (*R. sativus*).[34] TiO_2-NP exposure also lead to an increase in the lignin band area of the FTIR spectra of cucumber fruit;[36] however, they decreased lignin relative content in tomato leaves but did not impact tomato fruit after exposure to TiO_2-contaminated sludge.[29] Cell wall components of rapeseed exposed to CNTs were also modified with a particular decrease in pectin relative amount.[32] It has finally been reported that metal lignin complexes may be formed which could be responsible for changes in plant chemical environment and could lead to modifications in their nutritional properties.[33,36]

Cellulose and hemicellulose are located inside primary cell walls and are responsible for the cell wall rigidity.[39] Cellulose provides mechanical strength for load-bearing due to the cross-linking by hemicelluloses.[68] Cell wall thickening represents a way for plant to resist both biotic and abiotic stress.[40] In fact, a thickening has been observed in plants as a response to mechanical intrusion of pathogens.[69] It has also been demonstrated that cellulose-deficient mutant plants are more sensitive to abiotic stress than wild type plants.[37] The increase in cellulose relative amount highlighted by FTIR could thus be a reaction of plant exposed to NMs to limit their entry through cell walls. This hypothesis is consistent with the result of the microarray profiling which demonstrated an increase in the LM19 labeling, *i.e.* of lowly esterified HG, also responsible for cell wall stiffening through the formation of the so-called egg boxes with calcium ions.[70]

An alternative hypothesis to the increased accumulation of this cell wall component is that it represents the main negatively charged molecule of cell walls. Indeed, HG with a low degree of methylesterification contains some amount of free carboxyl groups which can bind cations. As such, it plays a crucial role as a buffer by sequestrating positively charged molecules such as most heavy metals.[40,71,72] Using quantum dots (QD, NPs with diameter <10 nm), some authors showed that NPs can directly interact with cell walls either through hydrogen bonds with cellulose –OH groups or *via* the conjugated C–C or C═C chains in lignin.[73,74] Furthermore, a recent study assessed the influence of NP surface charge on their fate in plants and demonstrated an accumulation of negatively charged QD in cell walls.[75] Here, both NMs bear negative charges when analyzed in suspension. However, so far, we have no data about their status in soil. Thanks to their large surface area, NMs exhibit high adsorption properties and could thus adsorb many molecules from the soil which in turn could influence their overall surface charge. In our experiment, the increase in the amount of LM19 epitopes could indicate a higher sequestration capability in response to the presence of NM in the medium.

Another phenomenon that can be responsible for cell wall modification is the oxidative stress caused by NMs. Indeed, all types of NMs (*e.g.* carbon-based and metal based) have been reported to generate an excess of reactive oxygen species (ROS).[76] For instance, CNTs increased ROS content in epidermis cells of *O. arenaria* as well as the activity of antioxidant enzymes such as peroxidases (POX) after 15 days of exposure in hydroponic conditions.[77] TiO_2-NPs also increased the level of catalase (CAT) and ascorbate peroxidase (APX) activities in leaves of cucumber exposed for 150 days in sandy loam soil.[36] Besides, ROS can be associated with cell wall modifications since a sudden burst of ROS can lead to catalytic oxidation of various substrates of the cell wall which results in cross-linking of cell wall components and growth arrest.[78] Class III peroxidases, also involved in the regulation of oxidative stress, can promote cell wall loosening *via* the hydroxylic cycle.[49] Indeed, this has been demonstrated in *A. thaliana* exposed to nZVI (nano zero valent iron) in agar medium.[79] The authors concluded that root elongation was related to the potential for nZVI to lead to H_2O_2 release causing OH radical-induced cell wall loosening in roots. This was confirmed by the degradation of pectin-polysaccharides and a decrease in cell wall thickness. The modification identified in the cell wall compounds in this work may thus also be explained by the oxidative stress caused by the NMs tested which could be independent of NM internalization.

5. Conclusion

The use of FTIR spectroscopy in this study has allowed to identify similar impacts of CNTs and TiO_2-NPs on tomato leaf cell walls despite their different physico-chemical properties. Microarray profiling confirmed FTIR results and demonstrated significant modification in HG and XG for plants exposed to TiO_2-NPs associated with a transiently reduced plant development (particularly visible after 15 days of exposure). The same trend in cell wall modification was noticed for plants exposed to CNTs, though not significantly, and with no impact on plant development. FTIR is a relatively easily accessible, fast and powerful technique for a first screening approach. Although data processing is not straightforward, we have proposed a strategy based on simple statistical analysis of the data which highlighted very slight modifications induced by NM exposure and permitted us to

focus the analysis further on the cell wall composition for a more precise description of the physiological response.

Conflicts of interest

Authors have no competing interests to declare.

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