Comparative study of response of four crop species exposed to carbon nanotube contamination in soil

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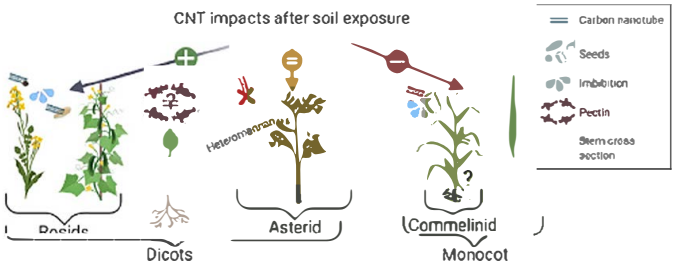
HIGHLIGHTS

- Different plants exposed to CNT contaminated soil exhibited different responses.
- CNT presence lead to a decreased development for monocot while enhanced for dicots.
- FTIR analysis evidenced differences in cell walls correlated with plant sensitivity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Carbon nanotube
Cell wall
FTIR
Plant
Toxicity
Soil

ABSTRACT

Crop plants are exposed to a variety of contaminants through sewage sludge spreading but very little is known about the impact of emerging contaminants such as nanomaterials. To date their impact on plants is still very controversial with many works claiming negative impacts while some authors suggest their use as plant growth regulator in agriculture. In this study, aiming to better understand where these discrepancies may come from, we investigated the influence of plant species (tomato, rapeseed, cucumber and maize) on plant response to a carbon nanotube contamination in soil condition. Our results demonstrate that the same CNT contamination can lead to different effects depending on plant species with positive impacts on cucumber and rapeseed (more than 50% increase in leaf biomass and surface area and 29% increase in chlorophyll for cucumber) but negative impact on maize (14% for plant height), while tomato was insensitive. FTIR analysis of biomacromolecule composition suggested that these differences could be related with plant cell wall composition (in particular: pectins, xyloglucans and lignins). As a summary, no overall conclusion can be drawn about the toxicity of a specific nanomaterial for all plant species.

1. Introduction

Research about nanomaterials, and especially carbon nanomaterials, has intensively increased over the last few decades. Among the carbon nanomaterials family, carbon nanotubes (CNTs) are one of the most promising (Titirici et al., 2015). CNT market has become a billion value industry and is expected to develop and reach 9 billion dollars by 2023 (MarketsandMarkets™ 2019).

CNTs can be described as seamless rolled layers of graphene forming nanotubes with a nanometric diameter and a typical length of few microns (Dresselhaus, Dresselhaus, and Avouris

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https://doi.org/10.1016/j.chemosphere.2021.129854

2003). Thanks to their outstanding thermal, electrical and mechanical properties, CNTs are used in many applications such as batteries, plastic additives or sporting goods (Ajayan and Tour 2007; De Volder et al., 2013). Agriculture is also one of the potential sectors for the use of CNTs. Indeed, they might be used as fertilizers to enhance plant growth, pesticides for pest and disease management or as sensors to monitor plant health and soil quality (Mukherjee et al., 2016; Abd Elsalam 2020; Giraldo et al., 2014).

As a consequence of their increased use, CNTs are now seen as an emerging contaminant in the environment. Data on actual CNT concentrations in the environment is not yet available due to the analytical issue of detecting CNTs in complex carbonaceous matrices (Sun et al., 2016). Sun et al. used modelling to predict CNT concentrations in different environmental compartments and established that in urban and natural soils, the concentration could be around 35 ng/kg while in sludge treated soil (*i.e.* in agriculture), it could reach 11.7 μg/kg (Sun et al., 2016). Gogos, Knauer, and Bucheli (2012) calculated that the application dose of CNTs as plant protection products or fertilizers could be 3–12 g/ha which would correspond to an additional flux of 1.1–4.3 μg/kg per year. Therefore, it is crucial to understand the toxicity of this emerging contaminant in agrosystems and evaluate related health risk for humans.

Despite this increasing concern, CNTs effects on plant morphological, physiological, and molecular processes and their mechanisms of action are far from being fully understood (Liné et al. 2017; Verma et al., 2019). It has been reported several times that exposure to CNTs can lead to an enhancement of plant productivity in both hydroponic medium (McGehee et al., 2017; Pandey et al., 2018; Lahiani et al., 2018) and soil conditions (Khodakovskaya et al., 2013; Pandey et al., 2019). However, other studies have shown that CNTs can lead to phytotoxic effects: decreased plant growth, increased generation of reactive oxygen species or decreased cell dry weight (Hao et al., 2018; Wang et al., 2017; Lin et al., 2009). Finally, some authors highlighted that CNTs exhibited no effect on different plant species (Lin and Xing 2007; Larue et al., 2012; Hamdi et al., 2015). Such controversial results could be explained by differences in terms of the type of CNTs used, experimental set up as well as by the type of plants. Indeed, Canas et al. (2008) screened six crop species (cabbage, carrot, cucumber, lettuce, onion and tomato) and concluded that CNTs inhibited root elongation in tomato but enhanced it in onion and cucumber. Likewise, Begum et al. (2014) demonstrated that red spinach and lettuce were more sensitive to CNTs than rice and cucumber, with a decrease in root and shoot lengths. They also observed no toxic effect on chili, lady's finger and soybean. Plants belonging to different families could have specific traits (foliar area, stomata distribution, nutrient acquisition strategy or structure, among others) which could lead to different interactions with CNTs. It is also striking to note that almost all these studies were performed in hydroponic conditions and focused for most of them only on the impacts on seeds (germination, seedling root and shoot length) (Canas et al., 2008; Begum et al. 2014; Lahiani et al. 2015, 2018). Indeed, the presence of a soil matrix will influence plant and nanomaterial interactions at different levels: root structure will be different in soil *vs.* in hydroponics, CNT behaviour will be different in the presence of ions from the soil solution (surface charge, agglomeration); and they will also interact with soil particles, ions and organic matter possibly making them less bioavailable for plants (Baysal et al. 2020). Furthermore, bacterial activity, especially in the rhizosphere with the secretion of root exudates, may lead to material alteration. In a review, Vithanage et al., 2018 concluded that CNTs tended to stimulate plant growth in most cases, but that their exact physiological functions depended on the genetic traits of a particular plant species, what is largely unknown.

The aim of this study was thus to try to identify relevant biological parameters influencing plant response to a CNT contamination using exposure conditions as realistic as possible: exposure in soil and during a period covering more than the seedling stage (5 weeks). We selected crop plants divided in three dicot species: tomato (*Solanum lycopersicum L.),* rapeseed (*Brassica napus L.*), cucumber (*Cucumis sativus L.*) and one monocot: maize (*Zea mays L.*). These plant species were selected to address different issues: (i) the natural ratio between monocot and dicot species (*i.e.* around 60,000 *vs.* 200,000 species (The angiosperm phylogeny group 2016), respectively and 1 *vs.* 3 in our study), (ii) represent both fruit and seed plants (tomato and cucumber *vs.* rapeseed and maize), and (iii) among the dicots, have plants from the same or differing clades (rapeseed and cucumber are part of the Rosids while tomato is an Asterid) to make possible the identification of common or diverging features. CNT phytotoxicity was evaluated at different biological levels: (i) plant morphology: germination rate, plant height, number of leaves, fresh and dry biomass as well as leaf area; (ii) plant metabolism: chlorophyll, flavonoid, total phenolic compound and tannin concentrations and finally (iii) plant biomacromolecule composition using Fourier transformed infrared spectroscopy (FTIR).

2. Material and methods

2.1. CNT preparation and characterization

CNTs were synthetized by catalytic chemical vapor deposition according to (Flahaut et al., 2003). They were thoroughly characterized: according to transmission electron microscope observations, the mean outer diameter of the CNTs was 2.05 ± 0.70 nm (Figure S1), with a length ranging from 1 to 100 μm (Flahaut et al., 2003). The sample was mainly composed of double walled CNTs (80%) (Flahaut et al., 2003). The specific surface area was measured at 985 m^2/g. CNTs zeta potential measured in deionized water was 27.5 mV at pH 6.7 while in soil solution it was 32.1 mV. More details about CNTs synthesis and characteristics in SI.

2.2. Soil characteristics and contamination

Experiments were carried out on a silty sand soil (LUFA Speyer 2.1) with a composition of 88.0% sand, 9.1% silt and 2.9% clay. It contained 0.71 ± 0.08% of organic carbon, 0.06 ± 0.01% of nitrogen, had a pH of 4.9 ± 0.3 and a cation exchange capacity of 4.3 ± 0.6 meq/100 g. The soil water capacity was 60 mL for 100 g of soil.

The amount of CNT suspension used to contaminate the medium was calculated to add half of the water holding capacity to the soil (here 30 mL for 100 g of soil) to avoid deconstructing the soil and to reach a final concentration of 100 mg CNT/kg of dry soil. This concentration was chosen to be comparable to the published literature on that topic (Liné et al. 2017). To obtain a CNT distribution in the soil as homogeneous as possible, the suspension was spread on a tray of soil with a maximum soil thickness of 2 cm. The soil was then mixed thoroughly.

2.3. Plant material and cultivation

Organic seeds of tomato *Solanum lycopersicum* (var. Red Robin), cucumber *Cucumis sativus* (var. Le Genereux), rapeseed *Brassica napus* (var. KALIF) and maize *Zea mays* (var. PROSIL) were surface sterilized using $Ca(ClO)_2$ (1%). The experiment was performed in an environmental chamber with controlled parameters (10 h/14 h day/night cycle, 24°/22 °C and a hygrometry rate of 85%). The exposure

duration was set to 5 weeks. Two conditions were used: control plants and plants exposed to 100 mg CNTs/kg with 5 biological replicates per condition. Four seeds were introduced per pot. After the appearance of the cotyledons, only one plant was kept per pot.

Morphological parameters including germination, plant height (from day 14 for accurate measurements) and number of leaves were monitored along the experiment. Upon harvest, roots and shoots were weighted in order to obtain the fresh biomass. The foliar area was measured using a camera and ImageJ software. Part of the leaves was frozen in liquid nitrogen and stored at 80 °C for further biochemical analyses. Remaining leaves and roots were dried at 50 °C for 24 h and weighted to obtain the dry biomass weight as well as for FTIR analyses.

2.4. Biochemical analyses

Biochemical analyses were performed on liquid nitrogen frozen leaves using a high throughput biomarker set. A high throughput grinding step was used with a bead mill and 4 mm diameter glass beads. Five biomarkers were assessed to get information on two main metabolic processes: photosynthesis (chlorophyll *a* and *b*) and secondary metabolites, which are good stress markers (total phenolic compounds, flavonoids and tannins). Briefly, around 20 mg of ground fresh leaves were introduced into a 96 well microplate (2 mL wells, 3 technical replicates per plant). 1.5 mL of methanol (95%) were added in each well. Plates were shaken for 2 min and covered with aluminum foil in order to avoid light induced degradation. Incubation time was set to 24 h in the dark for photosynthetic pigments and 48 h for secondary metabolites. After incubation, plates were centrifuged at 4500 rpm for 5 min. For pigments analysis, 100 µL of supernatant were transferred into microplates and absorbance was measured at 652 and 666 nm (Lichtenthaler 1987). The concentration was expressed as milligram per gram of fresh weight (mg/g f. wt.) using calibration curves.

For total phenolic compounds, concentrations were calculated based on Folin Ciocalteu assay (Ainsworth and Gillespie 2007). Briefly, 20 µL of supernatant were mixed with 40 µL of Folin reagent (10% v/v) and 0.10 mmol of sodium hydrogen carbonate ($NaHCO_3$). The mixture (final volume: 200 µL) was incubated for 2 h at room temperature until color development. Absorbance was then measured at 760 nm. Concentrations were calculated using a cali bration curve of gallic acid and expressed as milligram of gallic acid equivalent (GAE) per gram of fresh weight (mg GAE/g f. wt.).

Flavonoid concentrations were determined based on aluminum chloride method (Settharaksa et al., 2014). The reaction mixture (final volume: 200 µL) contained 25 µL of supernatant, 7.25 µmol of sodium nitrite ($NaNO_2$), 0.11 µmol of aluminum chloride ($AlCl_3$) and 0.02 mmol of sodium hydroxide (NaOH). The mixture was ho mogenized for 1 min and absorbance was read at 595 nm. Con centrations were calculated using a calibration curve of catechine and expressed as milligram of catechine equivalent (CE) per gram of fresh weight (mg CE/g f. wt.).

Finally for tannins analysis, reaction mixture (final volume: 100 µL) contained 50 µL of methanolic extract and 6.57 µmol of vanillin (El Euch, Bouajila, and Bouzouita 2015). The mixture was left in the dark for 15 min and absorbance was measured at 500 nm. Tannins concentrations were calculated using a calibration curve of catechine. Results were expressed as milligram catechin equivalent per gram of fresh weight (mg CE/g f.wt.).

2.5. FTIR measurements and chemometric analysis

Around 20 mg of dry leaves were ground using a FastPrep equipment (2 × 15 s at maximum speed). FTIR analyses were per formed in attenuated total reflectance (ATR) mode with a diamond crystal (Thermo Nicolet, Nexus, Smart Orbit) using a conventional IR source. The infrared spectra were collected from 4000 cm^{-1} to 400 cm^{-1}. All samples were analyzed in (technical) triplicates and each spectrum was the sum of 64 scans. OMNIC software was used to export experimental data.

FTIR spectra were analyzed with Orange software (BioLab) (Demšar et al., 2013). First, they were pre processed by restricting the area of interest between 1800 and 800 cm^{-1}, corresponding to the protein region and corresponding to most of the differences observed among samples. Data were then normalized using vector normalization and a Savitzky Golay filter was applied (window: 21, polynomial order: 2, derivative order: 2). After pre processing, a principal component analysis (PCA) was applied. This analysis permitted to check if different groups could be identified among experimental conditions.

2.6. Statistical analysis

Data were checked for homoscedasticity and normality. When assumptions were met for parametric analyses, a student T test was used. Otherwise, a Wilcoxon test was applied to compare between control and treated plants. For comparison among species, a one way ANOVA or a Kruskal Wallis test was used. Additionally, a PCA was performed with all the data (morphological, biochemical and from biomacromolecule composition). All statistical analyses were performed using the RStudio statistical software (version 1.1.453) with car (Fox 2002), multcompView (Graves et al. 2015), pgirmess (Giraudoux et al., 2018), agricolae (de Mendiburu 2020) and ggplot2 (Wickham 2009) packages.

3. Results

3.1. CNT impact on plant morphological response

Among the 9 morphological parameters investigated here, CNT exposure had no significant impact on germination rate (Figure S2A), number of leaves (Figure S2B, Figure S3), root fresh biomass (Figure S2C), total fresh biomass (Figure S2D) and water content in roots (Figure S2E) whatever the plant species (see SI for more details).

However, plant height was a more sensitive parameter with exposed maize plants being significantly smaller than control plants all along the experiment (Figure S4D), resulting in a decrease of 14% in plant size after 5 weeks (p 0.016, Fig. 1A). An opposite trend was observed for all the other species, with exposed plants being overall taller than the control plants, although this difference was not significant (Fig. 1A and Figure S4A, B, C).

After exposure, leaf fresh biomass (Fig. 1B) was also significantly impacted with an increase of 55% for rapeseed and 71% for cu cumber (p value 0.021 and 0.041, respectively) but remained unchanged for the other two species with a trend to decrease for maize. Maize had the highest leaf fresh biomass (1225 mg on average, 5.6 times more than rapeseed, 6 times more than cu cumber and 7.8 times more than tomato, $p < 0.001$).

The total leaf area at the end of the experiment was increased by 58% for rapeseed and 64% for cucumber in comparison to their respective control plants (p value 0.033 and 0.040, respectively; Fig. 1C). Likewise, the mean leaf area per leaf was significantly more developed by 63% in exposed rapeseed in comparison with the control plant (p value 0.040, Figure S2F). Again, a non significant decrease was noted for maize leaves exposed to CNTs.

Overall, maize was the plant with the most important devel opment (height, biomass, leaf area, $p < 0.001$).

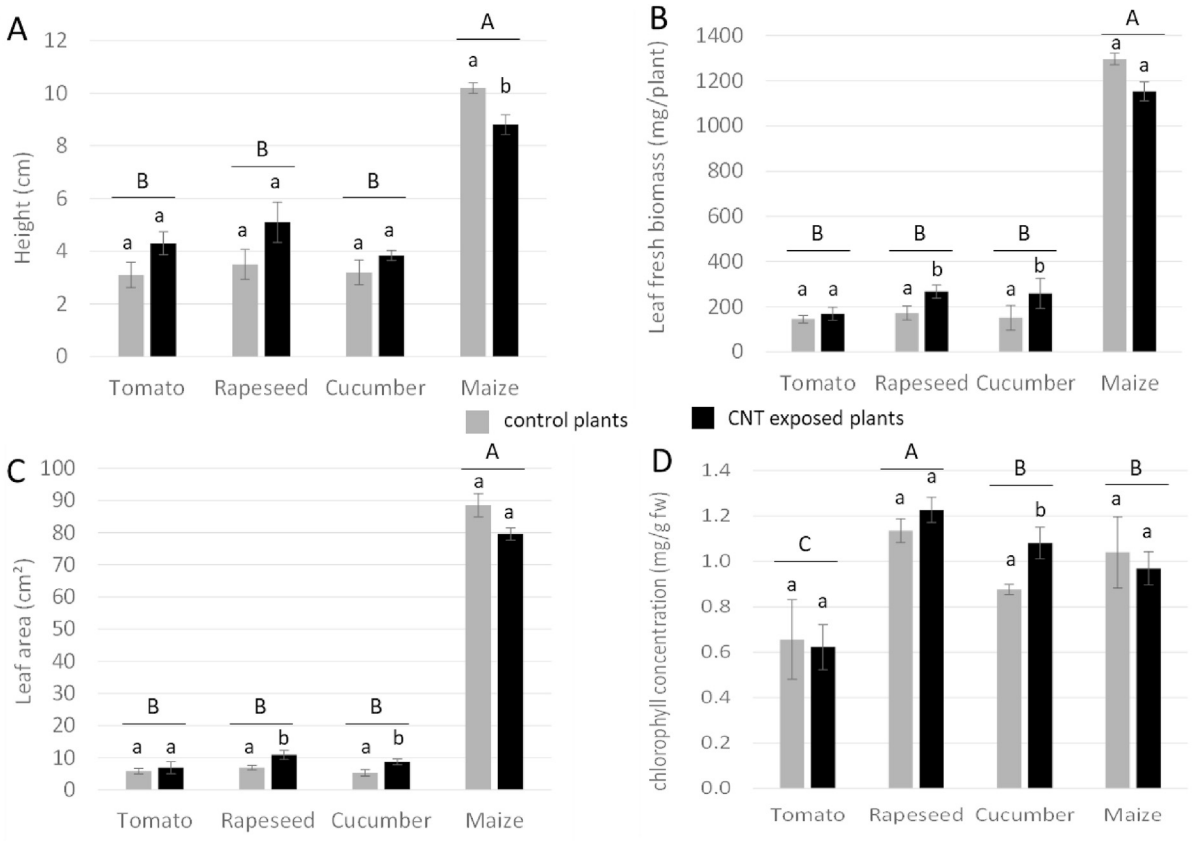

Fig. 1. Morphological (A. plant height, B. leaf fresh biomass, C. leaf area) and biochemical (D. total chlorophyll concentration) biomarkers in control plants or in plants exposed in a soil contaminated by 100 mg carbon nanotubes/kg after a 5 week exposure. Lowercase letters indicate significant differences ($p < 0.05$) within a species and between treatments (Student test or wilcoxon test). Uppercase letters indicate significant differences ($p < 0.05$) among species (ANOVA 1 way or Kruskal Wallis test). (mean ± standard error, n 5).

3.2. CNT impacts on plant biochemical response

No impact of CNT exposure was visible on both flavonoids and tannins concentrations whatever the species (Figure S5A, C). The flavonoids concentration was the highest in maize in comparison to the other plant species (32.5 mg CE/g f. wt. for maize and 14.2, 19.9 and 23.2 mg CE/g f. wt. for cucumber, tomato and rapeseed, respectively; $p < 0.001$). For tannins, tomato was the species containing the highest concentration and rapeseed the lowest ($p < 0.001$).

Chlorophylls of cucumber were significantly impacted with an increase in total chlorophyll concentration of 29% (p value 0.033) (Fig. 1D). Rapeseed was the plant with the highest chlorophyll concentration in comparison to the other plants ($p < 0.001$): 1.18 mg/g f. wt. *vs*. 0.99 for cucumber and maize on average and 0.64 mg/g f. wt. for tomato.

Finally, the concentrations of phenolic compounds were lower in rapeseed exposed for 5 weeks to CNTs in comparison to control rapeseed (Figure S5B). However, rapeseed remained one of the species containing the highest concentrations of total phenol together with maize (51.0 and 62.5 mg GAE/g f. wt., respectively) against tomato and cucumber (12.1 and 16.0 mg GAE/g f. wt., respectively; $p < 0.001$).

A PCA was performed with both morphological and biochemical biomarkers, highlighting a clear separation between maize and the 3 other species (Fig. 2). The factor mainly driving the PCA along axis 1 was plant development with maize (the only monocot species) which performed best on most assessed parameters segregating on the right hand side of the PC1 while other species (all dicots) were

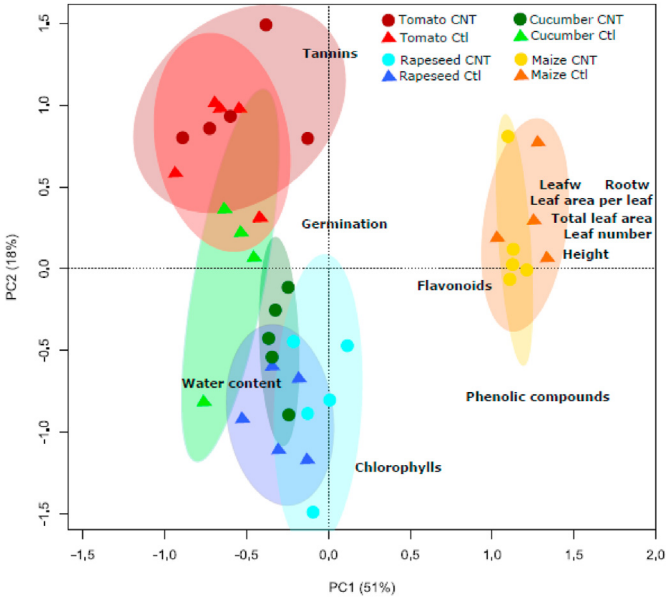

Fig. 2. Principal component analysis on morphological (leaf number, height, leafw: leaf fresh weight, rootw: root fresh weight, root water content, total leaf area, leaf area per leaf, germination rate) and biochemical (chlorophyll, flavonoid, phenolic compound and tannin concentrations) markers in plants after a 5 week exposure in control condition (Ctl) or exposed to 100 mg carbon nanotubes/kg soil (CNT).

overlapping in the left hand side. Along PC2, plants segregated according to the chlorophyll concentration to the bottom with

mainly rapeseed and according to tannins concentration towards the top with tomato plant, with maize and cucumber being intermediate. Taking plant species into account, differences arising from CNT contamination played a minor role in the PCA, and was only noticeable on cucumber.

3.3. CNT impacts on plant biomacromolecules

FTIR spectra obtained on the 4 crops were analyzed using a PCA approach highlighting differences among plant macromolecules with a clear distinction between monocot (maize) and dicots (tomato, cucumber and rapeseed) along PC1, explaining 94% of the variance (Fig. 3A). The main differences were evidenced by a significant shift of about +11 cm^{-1} for maize in comparison to the other dicot species at 1014 cm^{-1} as well as at 1151 cm^{-1}, peaks attributed respectively to pectins (Alonso Simón et al., 2011) and other various cell wall polysaccharides (symmetric bonding of aliphatic CH_2, OH, or C—O stretch of various groups) (Türker Kaya and Huck 2017). Along PC1, the wavenumber 1078 cm^{-1} also greatly contributed to the segregation of the groups suggesting different xyloglucan composition (Alonso Simón et al., 2011). Finally, at 1515 cm^{-1} a peak was more pronounced for maize related to aromatic system (semi circle ring stretch) from lignin (Regvar et al., 2013; Türker Kaya and Huck 2017). Furthermore, dicots were also distributed along PC2 (explaining only 3% of the variance) with rapeseed and cucumber rather towards positive values of PC2 while tomato was more individualized towards negative values of PC2.

Exposure to CNTs did not lead to major differences when plant species was considered. However, this parameter set apart, biomacromolecule composition for plants grown in control conditions *vs*. exposed to CNTs varied especially for rapeseed. The differences in between exposed plants and control plants could be mainly observed in the region 1020–800 cm^{-1} which is related to pectins and various polysaccharides (Regvar et al., 2013). The area under the peak at these wavenumbers was lower for plants grown in contaminated soil, which indicates that the relative amount of cell wall related compounds decreased with exposure to CNTs.

4. Discussion

Our results highlighted, for a same contamination, a significant decreased in maize height while a significant increased biomass and leaf area (and to some extent chlorophyll concentration) were detected for rapeseed and cucumber. Tomato plant appeared to be the less sensitive plant species with no significant impact of CNT exposure upon harvest.

In the literature, most of the studies evaluating the impact of CNTs on plants focused on one single plant species. It can be tricky to compare effects of CNTs on different plant species from the literature since many parameters usually vary from one study to another (*e.g.* exposure time, growth media, type of CNTs). In the rare studies comparing the phytotoxicity of CNTs using different plant species, similar effects were most of the time described. Lahiani et al. (2013) established that seed germination was activated for soybean, barley and maize after CNT deposition on seed surface. Using the same 3 species, these authors also demonstrated an enhanced development as well as an increase in photosynthesis efficiency after exposure to up to 100 $mg.L^{-1}$ of CNTs in hydroponic conditions (Lahiani et al., 2018). In 2015, they also identified "positive" impacts of a different type of carbon nanomaterial (single walled carbon nanohorns, 25, 50 and 100 $mg.L^{-1}$) on soybean, tomato, maize and rice but no impact was found for barley and switchgrass (Lahiani et al., 2015). Srivastava and Rao (2014) also reported an enhancement of plant growth and biomass for wheat, maize, garlic and peanut exposed to 50 $mg.L^{-1}$ of CNTs.

Contrasting with earlier published data, our current work focused on plants exposed in soil condition. Nanomaterials behavior in soil is not yet fully understood (Shrivastava et al., 2019); nevertheless, we can expect that it is different in soil compared to suspension or in agar growth medium, thus affecting their interactions and finally their impact on plants. Likewise, Garcia Gomez et al., studied the influence of plant species upon ZnO nanoparticles exposure in soil and also highlighted different impacts according to plant species (García Gómez et al., 2018).

Several hypotheses can be stated to explain the different sensitivity observed here:

(i) **seed surface**: interactions between seeds and CNTs will be increased with larger seeds and could lead to a higher CNTs sensitivity. Indeed, maize seeds had a surface area of about 120 mm^2 while the other species had a significantly smaller surface area (50 mm^2 for cucumber, 10 mm^2 for rapeseed and 8 mm^2 for tomato, according to our measurements). However, in the literature, this hypothesis was not confirmed with no difference in the toxicity reported according to seed surface area (Liné et al. 2017; Chen et al., 2018). In particular, Jain et al. (2017) established no correlation between seed size and ZnO nanoparticles toxicity. Cell wall composition of seeds also differ among species and could explain some differences. For instance, tomato seed endosperm cell walls contain abundant heteromannan unlike Brassicaceae (Lee et al., 2012), family to which rapeseed belongs. This compound affects cell wall porosity so it could contribute to limit

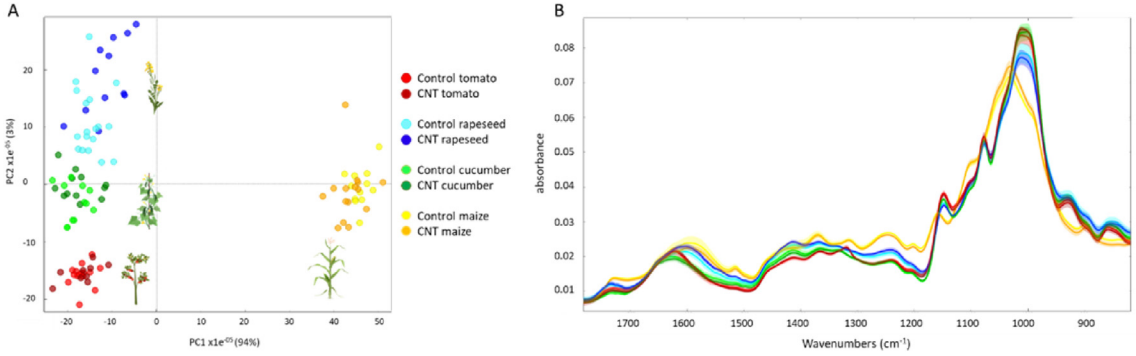

Fig. 3. FTIR analysis of leaf biomacromolecule composition after a 5-week exposure in control condition or exposed to 100 mg carbon nanotubes/kg soil (CNT). A. Principal component analysis, B. FTIR spectra in the 1800-800 cm^{-1} range.

CNTs absorption into tomato seeds and it may explain the absence of observed effects of CNTs contamination on this species. Jain et al., also reported that seed surface anatomy played a crucial role in determining nanomaterial phyto toxicity. In their study, a lower toxicity was observed in pearl millet seeds which had a thick and smooth testa (seed coat) while a higher toxicity was observed in wheat seeds, explained by the presence of crease on one side of the seed which may facilitate the interactions between ZnO nano particles and seeds (Jain et al., 2017). Likewise, rapeseed, cucumber and maize have a smooth seed surface while to mato has "hairy" seeds.

(ii) **plant clade**: plant response may change according to its clade: monocots *vs.* dicots. Indeed, several mechanisms as well as plant architecture are different between monocots and dicots. Here, there was a significant difference in CNT phytotoxicity with a decreased development for the monocot species (maize, member of the Commelinids) while the di cots experienced either an enhanced development (for cu cumber and rapeseed, both being part of the Rosids) or no impact (tomato, member of the Asterids). Monocots usually exhibit a larger root system, with thin and numerous long roots whereas dicots have one large primary root and several smaller lateral roots (Bouguerra et al., 2016). In our study, maize root biomass was significantly higher than the root biomass of the other species ($p < 0.001$, except for cucumber, Figure S2C). Since plant exposure was made through the roots, a more developed root system could potentially imply enhanced interaction between plants and CNTs in the soil. Therefore, more CNTs could potentially penetrate or accu mulate in the roots of monocots. Furthermore, the xylem system in monocots is made of several circles of conducting vessels while dicots have a single one (Scarpella and Meijer 2004). Thus, the transport of water and CNTs is potentially faster in monocots than in dicots, leading to enhanced accumulation in the aerial parts. We tested this hypothesis by analyzing xylem sap by transmission electron microscopy, but this technique unfortunately did not allow us to detect CNTs in any case.

(iii) **plant genus/species**, each species is different from one another (*e.g.* height, number of leaves, foliar area, *etc.*). For example, a higher leaf surface area can enhance the water exchange between soil and atmosphere, thus leading to a higher CNT accumulation in the leaves and possibly more toxic effects. As a matter of fact, maize which was the most sensitive species, also had the highest foliar area (≈ 17 cm^2, $p < 0.001$) in comparison with the others (<4 cm^2, Figure S2F). In the literature, several studies have also shown that nanomaterials uptake varied according to plant species (Pérez de Luque 2017).

(iv) **leaf cell wall composition**, interestingly, the segregation among plant species evidenced on the PCA based on morphological and biochemical biomarkers (demonstrating different plant sensitivity) followed the trend on the PCA performed using biomacromolecule composition. Indeed, the cell wall composition of monocot Poaceae as maize dif fers from other monocots and dicots. The cell walls of Poa ceae contain cellulose, hemicellulose with a majority of xylan and very low levels of pectin and structural proteins while the cell walls of dicots and non commelinid monocots consist of cellulose fibers encased in a network of hemicel lulose, in particular xyloglucan, pectin and structural pro teins (Tolbert 1980; Vogel 2008). This difference of cell wall composition between monocots and dicots was highlighted by the FTIR analysis (along PC1 of Fig. 3). Furthermore, the structure of xyloglucans differs depending on the species: xyloglucans in the primary walls of most dicots have a XXXG core structure (where X represents a α D Xylp (1 → 6) β D Glc*p* and G a β D Glc*p*), and are substituted with fucose while they have a XXGG structure without fucose in monocots (Fry et al., 1993). However, there is an exception in dicots: the Asterids, clade to which tomato belongs, have xyloglucans with a XXGG structure without fucose (Rose 2003), which could explain why cucumber and rapeseed formed one large group along PC2 while tomato segregated to the bottom of PC2. Also considering the wavenumber 1317 cm^{-1} charac teristic of the xyloglucan (Alonso Simón et al., 2011), it can be seen that tomato and maize exhibited the same type of feature, which was different from the other two Rosids.

Cell walls play a crucial role in plant response to contam ination, which has been well documented for heavy metals. According to Colzi et al., the essential capacity of cell walls for binding divalent and trivalent metal cations depends mainly on the amount of pectins and polysaccharides rich in carboxylate groups (thus bearing negatively charged sites); this accumulation of cations in cell walls allowing plants to better resist to heavy metal contamination (Colzi et al., 2012). Likewise, root binding capacity for trace metal ions is usually higher in dicots than in monocots and this difference is also attributed to a higher pectin content in the dicot cell walls (20−35% of the dry mass) than in monocot cell walls (5% of the dry mass) (Vogel 2008; Rabęda et al., 2015). Furthermore, hemicellulose acts as a heavy metal binding site, in particular xyloglucans and, according to Wan et al., the absence of fucose decreases the capacity of xyloglucans to sequester heavy metals and thus increases plant sensitivity (Wan et al., 2018). Concerning CNTs, they were negatively charged in soil suspension (Figure S1) but once in plants, diverse molecules may adsorb onto their surface leading to modifications of their overall surface charge. Hence, CNTs might interact with pectins and fucosylated xyloglucans and accumulate in plant cell walls similarly to heavy metals. Being a monocot Poa ceae, maize has cell walls with a low content of pectins and non fucosylated xyloglucans; the capacity of CNTs seques tration in cell walls would thus be less important in this species compared to dicots, which may contribute to make this plant species more sensitive to a contamination by CNTs.

5. Conclusion

Overall, in this study performed in soil condition, exposure to CNTs did not lead to drastic effects with only 4 biomarkers out of 13 being significantly modified. However, plant species were impacted in different ways: rapeseed and cucumber exhibited an enhanced development while maize experienced symptoms of CNT phyto toxicity, and tomato was not sensitive. FTIR analysis was used to explain the observed differences of sensitivity. Indeed, cell wall composition seemed to play an important role in plant response to contamination and in particular the quantity of pectins. Further more, other hypotheses were explored. Altogether, we propose that tomato seed envelope containing relatively high quantities of het eromannan could prevent early CNT penetration in the plant and its high pectin content would permit to store the contaminants in cell walls further defending the plant during its development leading to the absence of visible effect of exposure to CNTs for this species. On the other side, the increased development observed for rapeseed and cucumber may be explained by the capability of CNTs to penetrate seed coat and to promote water uptake (Ma et al., 2010) and also by the possible CNT sequestration in cell walls thanks to a

high content in pectins and fucosylated xyloglucans. Finally, in maize, a monocot species, many morphological parameters (developed root system, xylem system and leaf area) and cell wall composition (low content of pectins, xyloglucans without fucose) could lead to CNT penetration in roots, clogging of the root trans port pathways (possibly decreasing water and nutrient uptakes) (Asli and Neumann 2009), and translocation to shoot where they could induce cell damages and oxidative stress (Liné et al. 2017).

To further confirm these hypotheses, the development of effi cient techniques is needed to image carbon based nanomaterial distribution in plants (both at organ and cell levels) and to go deeper into cell wall composition and behavior when confronted to soil contamination by nanomaterials. The main implication of our results is that risk assessment studies should include multiple plant species to reach a robust conclusion about a specific contaminant toxicity. Furthermore, a larger screening could help in determining which plant parameters are the most important to govern nano material toxicity.

Author's contribution

Clarisse Liné; , Conceptualization, Data acquisition, Data cura tion, Formal analysis, Writing – original draft. Fanny Manent, Data acquisition, Data curation, Formal analysis. Adèle Wolinski, Formal analysis, Writing – original draft. Emmanuel Flahaut, Conceptual ization, Supervision, Reviewing, Editing. Camille Larue, Conceptu alization, Data curation, Formal analysis, Writing – original draft, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Clarisse Liné received a grant from the Région Occitanie and the Université Fédérale de Toulouse. Authors have no competing in terests to declare. Authors thank Dr Hiram Castillo Michel for his help in FTIR data acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.129854.

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Comparative study of response of four crop species exposed to carbon nanotube contamination in soil

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Supporting information

2. Material and Methods

2.1. CNT preparation and characterization

Double walled CNTs were synthetized at 1000°C by catalytic chemical vapor deposition (CCVD) of a mixture of CH_4 (18 mol.%) and H_2 using a Co:Mo MgO-based catalyst composed of $Mg_{0.99}Co_{0.0075}MgO_{0.0025}$ (Emmanuel Flahaut et al. 2003). After CCVD, the composite powder was treated with an aqueous HCl solution (Sigma-Aldrich, ACS reagent, 37%) for 12h to dissolve oxides and non-protected residual catalyst nanoparticles without degrading CNTs. The sample was then filtered through a cellulose nitrate membrane (Merck Milipore, 0.45 µm) and washed few times with deionized water until neutrality. Suspensions were prepared by dispersing the wet sample in the required amount of deionized water using a BRANDSON digital sonifier S-250D equipped with a 1/8-inch tapered microtip (200 W; amplitude: 35%; 1s/1s on/off). Before use, suspensions were re-dispersed in a sonication bath for 15 min (Elmasonic S30H, 280 W).

Characterization was realized on CNT suspension immediately before use since the different steps of the preparation protocol may modify their physicochemical properties. Transmission Electron

Microscopy (TEM) was used to assess the shape, diameter and purity (JEOL TEM 1400; 120 kV, Centre de microcaractérisation Raimond Castaing, Toulouse). The specific surface area was determined using Brunauer-Emmett-Teller (BET) method (Micrometrics Flow Sorb II 2300; 2h degassing at 100°C in N_2 and adsorption of nitrogen gas at the temperature of liquid nitrogen; measurement accuracy ± 3%). The mass contents of carbon, oxygen and nitrogen were determined using organic micro-analyzers (total combustion at 1050°C under helium/oxygen flux for C and N dosage; total pyrolysis at 1080°C under nitrogen flux for O dosage; SCA CNRS Lyon). Metal concentrations (Co and Mo) were determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES; Crealins, Lyon). Raman signature was analyzed to get information on the structural quality of the nanotubes (Labram HR800 Horiba Yvon Jobin, λ = 633 nm). Thermal analysis of the CNTs was carried out by thermogravimetric analysis (SERATAM TAG 16; ramp from RT to 1000°C under air flux at 1°C/min). X-Ray photoelectron spectroscopy (XPS) was used to determine the quantitative atomic composition of the CNTs (XPS Kalpha ThermoScientific). Finally, zeta potential was determined in ultrapure water (Zetameter ZETACAD, CIRIMAT, Toulouse).

According to TEM observations, the mean outer diameter of the CNTs was 2.05 ± 0.7 nm (Figure S1A). The median inner diameter was 1.35 nm and the length between 1 and 100 µm (Emmanuel Flahaut et al. 2003). The sample was composed of 15% of CNT triple-walled, 80% double-walled and 15% single-walled (Emmanuel Flahaut et al. 2003).

Using Raman spectroscopy, the three main bands characteristics of CNTs were determined: D, G and 2D bands respectively at 1320, 1590 and 2610 cm^{-1} (Figure S1B). Typical RBM peaks were also measured between 50 and 250 cm^{-1}. The ratio intensities between the D and the G bands gives some information about the sample structural quality (Costa et al. 2008) : a ratio close to 1 indicates the presence of a lot of structural defects. Here, the ratio was 0.23, suggesting very little structural defects in the sample.

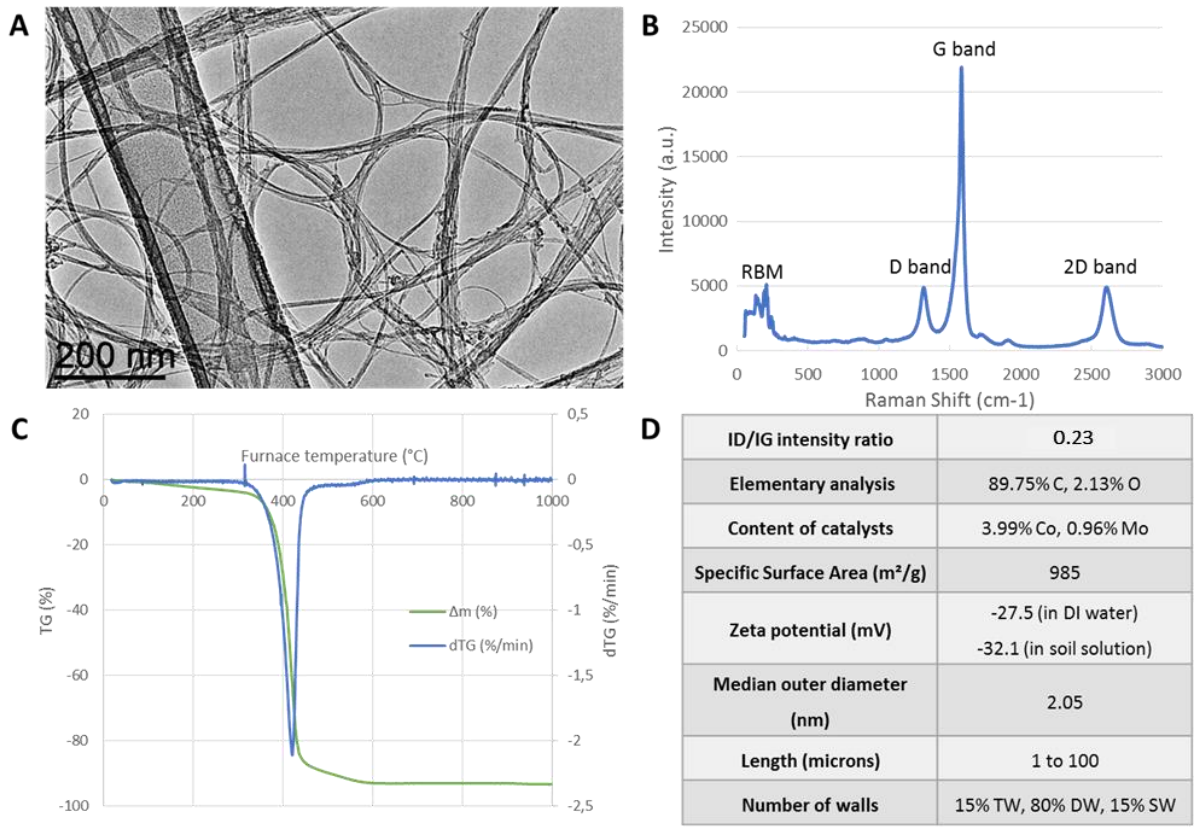
The first derivative of the TGA curve demonstrates that the CNTs were thermally stable up to *ca.* 310°C

and the maximum rate of decomposition of the nanotubes was at 421°C (Figure S1C).

The specific surface area was 985 m^2/g (Figure S1D).

The elemental analysis evidenced the composition of the CNTs: 89.75% carbon and 2.13% oxygen (Figure S1D). The catalyst amount remaining in the sample was 3.99% for Co and 0.96% for Mo. These metals were tightly encapsulated within graphitised layers of carbon and fully protected from their environment (no possible leak) (E. Flahaut et al. 2002).

The CNT zeta potential measured in deionized water was -27.5 mV at pH 6.7 while in the soil solution it was -32.1 mV (Figure S1D). The soil solution was obtained by mixing soil with ultrapure water (1:1 weight) during 3 hours and then filtrating through filter paper.



ID/IG intensity ratio	0.23
Elementary analysis	89.75% C, 2.13% O
Content of catalysts	3.99% Co, 0.96% Mo
Specific Surface Area (m^2/g)	985
Zeta potential (mV)	-27.5 (in DI water) -32.1 (in soil solution)
Median outer diameter (nm)	2.05
Length (microns)	1 to 100
Number of walls	15% TW, 80% DW, 15% SW

***Figure S1**. CNT characterization (A) TEM image of the purified CNTs. (B) CNT powder Raman scattering spectrum obtained using a 633 nm wavelength laser. (C) The weight loss profile obtained from TGA analysis. (D) Table summarizing the physicochemical characteristics (TW = triple walled, DW = double walled, SW = single walled).*

3. Results

3.1. CNT impact on plant morphological response

Germination started for all plants 3 days after the beginning of exposure. The germination rates were not significantly impacted by CNT exposure (Figure S2A). On average, the germination rate was 75% for tomato, 70% for rapeseed, 94% for cucumber and 78% for maize.

Over the course of the experiment, the leaf number was rapidly higher for cucumber plants exposed to CNTs in comparison to the control plants with a significant difference (p-value = 0.0161) at 16 days of exposure (Figure S3C). However, after 5 weeks of exposure, this difference disappeared with in average 2 leaves for both conditions (Figure S2B). The same trend was visible for tomato plants; a higher leaf number was detected at 19 and 20 days of exposure (Figure S3A) while after 35 days, plants growing in both conditions had on average 3 leaves per plant (Figure S2B). For rapeseed and maize, no difference in leaf number was evidenced along the experiment (Figure S3B and Figure S3D, respectively) with in average 4.2 and 5 leaves respectively at the end of exposure (Figure S2B).

The root fresh biomass as well as the total fresh biomass were not impacted by CNT exposure (Figure S2C and Figure S2D). Maize had the highest fresh root and total biomass in comparison with other plants species (6326 mg for maize total biomass *vs.* 830 mg for the other species, $p<0.001$). Likewise, the root water content was similar between control and exposed plants ranging from 87% for maize to 93% for cucumber (Figure S2E).

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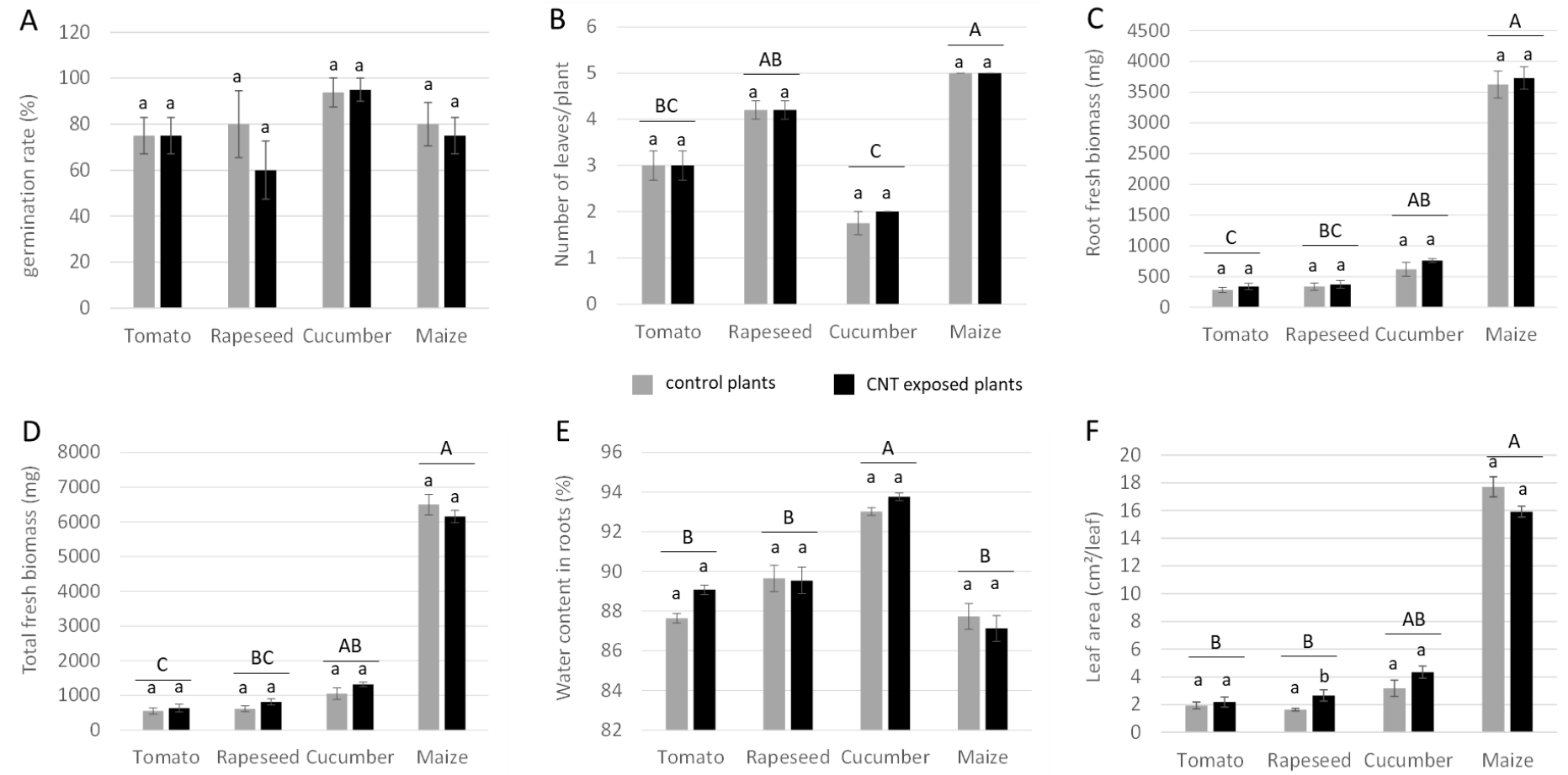

Figure S2. *Morphological parameters recorded after a 5 week exposure in control conditions or in a soil contaminated with 100 mg carbon nanotubes/kg (CNT). A Germination rate, B. Number of leaves, C. Root fresh biomass, D. Total fresh biomass, E. Water content in roots, F. Leaf area per leaf. Lowercase letters indicate significant differences ($p<0.05$) within a species and between treatments (student test or wilcoxon test). Uppercase letters indicate significant differences ($p<0.05$) among species (ANOVA 1 way or Kruskal Wallis test). (mean ± standard error, n=5)*

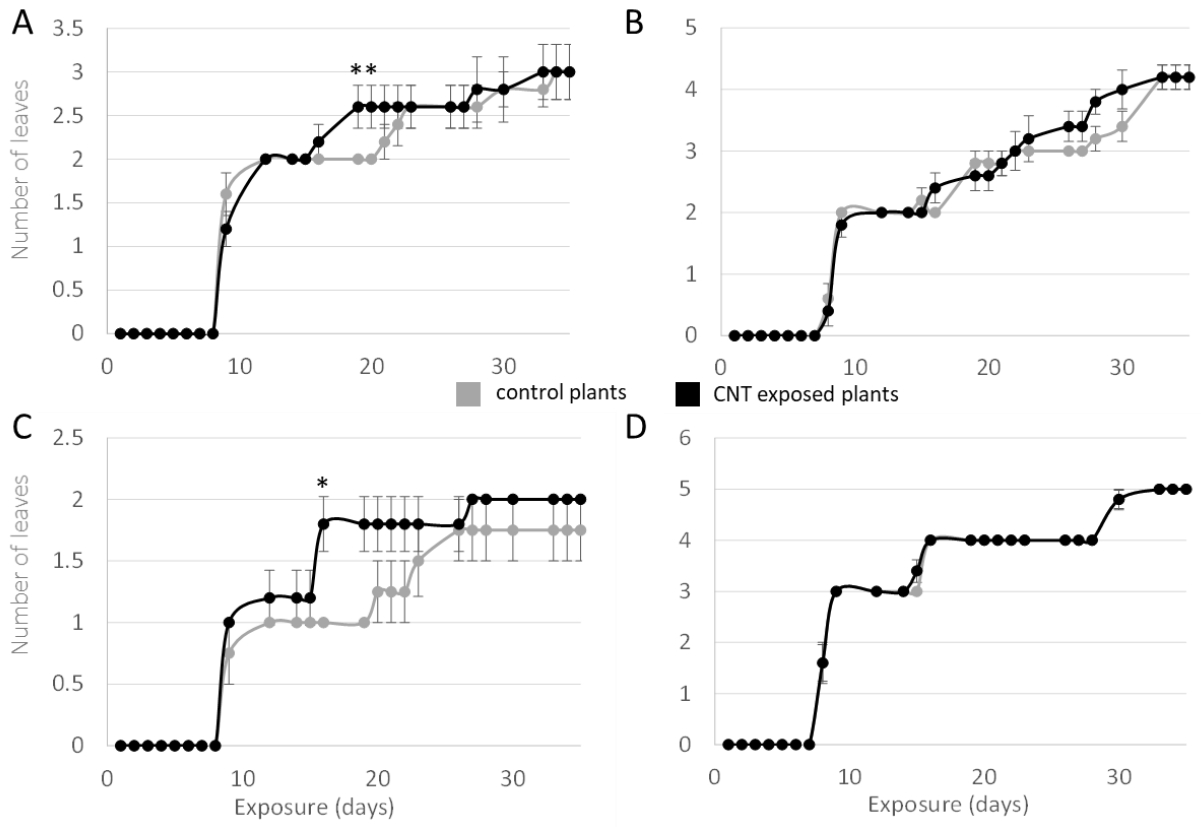

Figure S3. *Leaf number recorded along a 5 week exposure in control conditions or in a soil contaminated with 100 mg carbon nanotubes/kg (CNT). A. Tomato, B. Rapeseed, C. Cucumber, D. Maize. Stars indicate significant differences ($p<0.05$) between treatments for a given day (mean ± standard error, n=5, student test or wilcoxon test).*

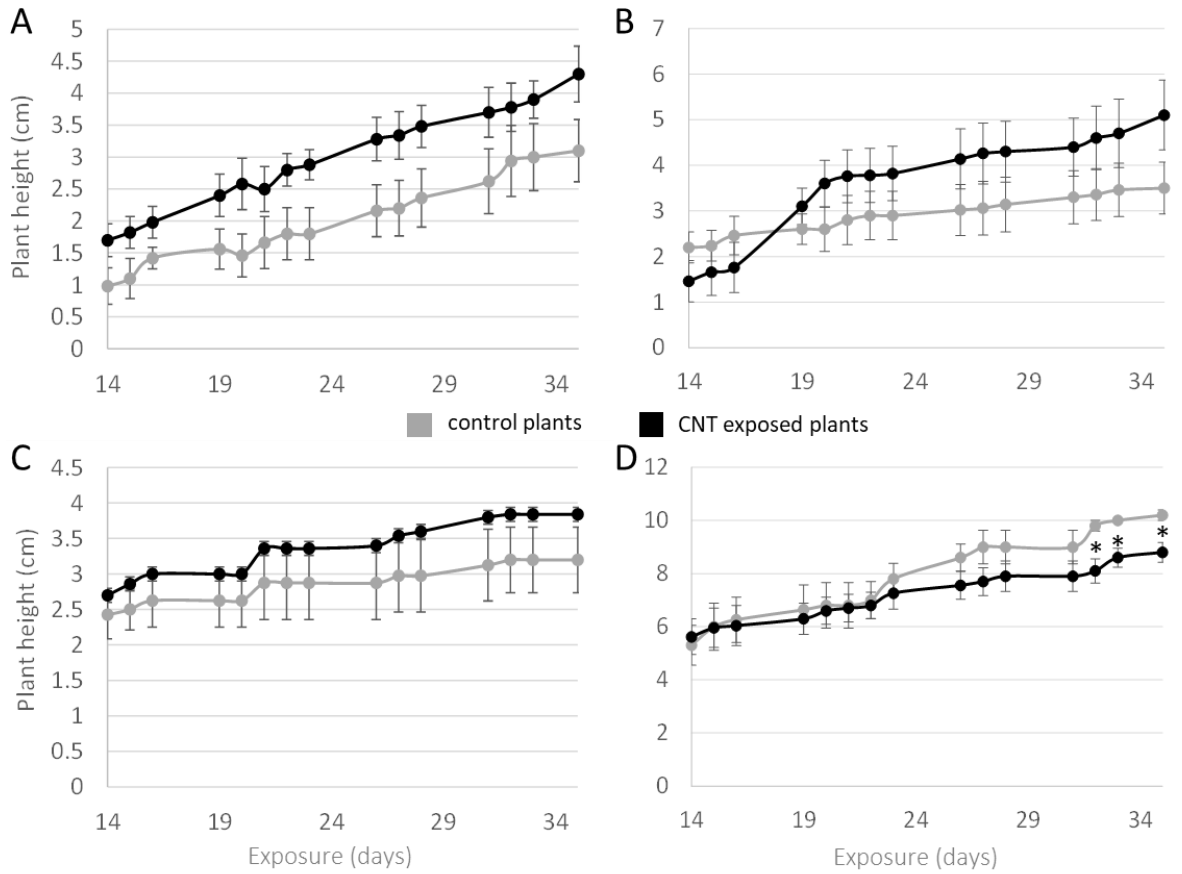

***Figure S4**. Plant height recorded along a 5 week exposure in control conditions or in a soil contaminated with 100 mg carbon nanotubes/kg (CNT). A. Tomato, B. Rapeseed, C. Cucumber, D. Maize. Stars indicate significant differences ($p<0.05$) between treatments for a given day (mean ± standard error, n=5, student test or wilcoxon test).*

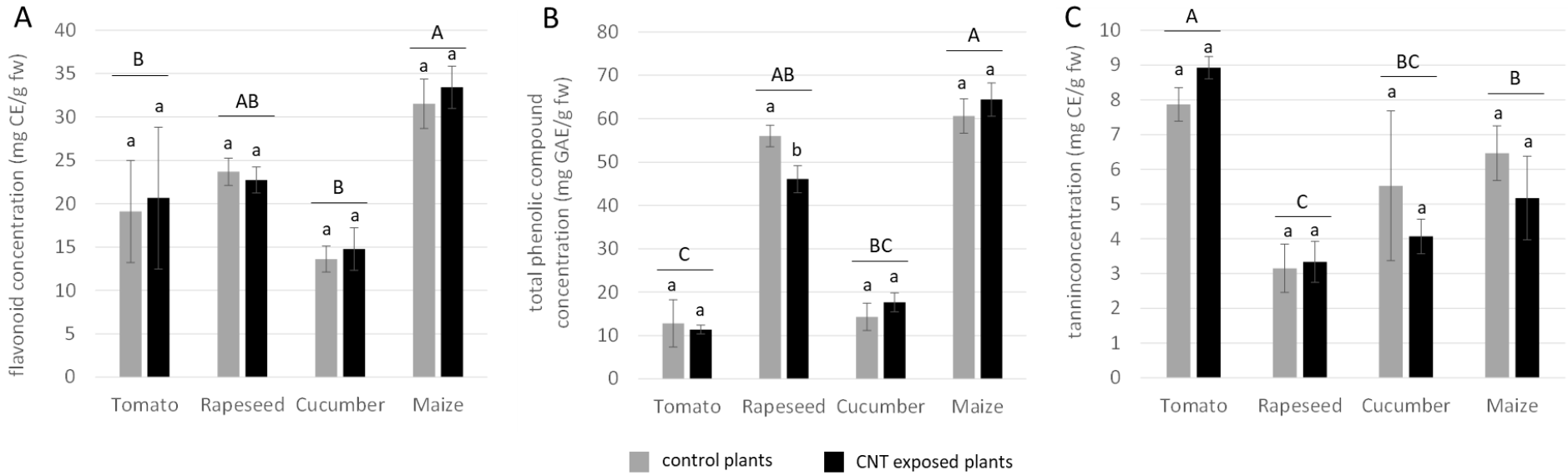

Figure S5. *Biochemical parameters recorded after a 5 week exposure in control conditions or in a soil contaminated with 100 mg carbon nanotubes/kg (CNT). Lowercase letters indicate significant differences (p<0.05) within a species and between treatments (student test or wilcoxon test). Uppercase letters indicate significant differences (p<0.05) among species (ANOVA 1 way or Kruskal Wallis test). CE: catechine equivalent, GAE: gallic acid equivalent (mean ± standard error, n=5)*