

Evaluation of parameters influencing plant response to carbon nanotube contamination

Clarisse Liné

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Abstract

Carbon nanotubes (CNTs) are among the most used nanomaterials (NMs) thanks to their excellent properties (*i.e.* optical, mechanical, electrical and thermal). All along their lifecycle, they may be spread unintentionally in the environment. They could also be introduced intentionally due to novel applications (e.g. growth regulators in agriculture, remediation of polluted soil, etc.). For this reason, it is essential to assess their behavior and potential impacts on ecosystems and particularly on agroecosystem. Plants are found at the interface between soil, air and water and at the basis of the food chains. The possible accumulation of such emerging contaminants in edible plants places this topic in the food safety field, and thus makes it a potential public health problem. Overall, behaviour and effects of CNTs in plants are not well understood and still very controversial. This can be explained by the influence of several parameters on plant response to CNT exposure. For instance, plant response can be different if CNTs are internalized or not. However, their detection in plants is still a challenge. Several CNT detection techniques were reviewed in this work and their pros and cons tested. To review the different detection techniques, we used hydroponic exposure while all the other experiments were conducted in soil. Plant response can also vary according to the type of NMs used. To investigate this hypothesis, tomato plant response to two TiO₂-NPs and double walled CNTs (DWCNTs) was evaluated using classical morphological and biochemical markers and Fourier-transformed infrared spectroscopy (FTIR). Our results highlighted that even if the two NMs were different for several parameters (*i.e.* shape, size, surface chemistry), tomato plants tended to exhibit a common response with a strong impact on cell wall components. CNTs can also have different impacts according to the **plant species** considered. Responses of different plant species (tomato, canola, maize and cucumber) to a DWCNT contamination were assessed highlighting differences among species: maize displayed a decreased development while for the other species, enhanced development was mostly identified. However, plant cell wall components were impacted in the four species. Many different CNTs are currently available on the market, varying in their **physicochemical parameters**. Five types of CNTs with different diameter/number of walls, functionalization and length were used to investigate their impacts on canola. Canola was more sensitive to CNTs with the smallest diameter and the highest specific surface area, but it was also observed that the functionalization greatly modulated plant response. Finally, the influence of a concomitant environmental stress on CNT toxicity was investigated. Canola plants were grown under optimal conditions or under heat stress. Under optimal conditions, the different CNTs did not canola impact canola growth. However, under heat stress, CNT exposure led to significant differences in plant development according to CNT type. Functionalized double walled CNTs were able to alleviate the effects of the abiotic stress on the plants while the non-functionalized CNTs increased the inhibition on plant development. Plants were thus more sensitive to CNTs when they were submitted to a concomitant heat stress.

Keywords: carbon nanotubes, detection, plant response, FTIR, plant species, physicochemical parameters, combined stress

Resume

Les nanotubes de carbone (NTC) sont parmi les nanomatériaux (NM) les plus utilisés grâce à leurs excellentes propriétés optiques, mécaniques, électriques et thermiques. Tout au long de leur cycle de vie, ils peuvent se propager de façon involontaire ou intentionnelle dans l'environnement. Il est donc essentiel d'évaluer leur comportement et leurs impacts potentiels sur les écosystèmes et en particulier sur l'agroécosystème. Les plantes se trouvent à l'interface entre le sol, l'air et l'eau et à la base des chaînes alimentaires. L'accumulation possible d'un tel contaminant émergent dans les plantes place ce sujet de recherche dans le domaine de la santé publique. Le comportement et les effets des NTC chez les plantes ne sont pas bien compris et demeurent très controversés. Ceci peut s'expliquer par l'influence de plusieurs paramètres sur la réponse des plantes à une contamination. Ainsi, la réponse peut être différente si les NTC sont internalisés ou non. Cependant, leur détection dans les plantes reste un défi. Plusieurs techniques de détection de ces NTC ont été examinées dans ce travail afin d'en déterminer les avantages et les inconvénients. Pour passer en revue les différentes techniques, nous avons utilisé des conditions d'exposition hydroponiques alors que toutes les autres expériences ont été réalisées en sol. La réponse des plantes peut également varier selon le type de NM utilisé. Les impacts de deux types de NM (TiO₂-NPs et NTC) ont été évalués sur des plants de tomate en utilisant des marqueurs morphologiques, biochimiques et la spectroscopie infrarouge par transformée de Fourier (FTIR). Nos résultats ont mis en évidence que même si les deux NM étaient différents pour plusieurs paramètres (forme, taille, chimie de surface), les tomates avaient tendance à présenter une réponse commune avec un fort impact sur les composants des parois cellulaires. Les NTC peuvent également avoir un impact différent selon les espèces végétales considérées. Différentes espèces végétales (tomate, colza, maïs et concombre) ont été exposées à une contamination en NTC mettant en évidence des différences de sensibilité entre les espèces. Le développement du maïs a diminué tandis que pour les autres espèces, le développement a été augmenté pour la plupart. Cependant, les composants des pariétaux ont été modifiés chez les quatre espèces. De nombreux NTC avec des paramètres physico-chimiques différents sont actuellement disponibles sur le marché. Cinq types de NTC variant en diamètre, fonctionnalisation et longueur ont été utilisés pour étudier leur impact sur le colza. Le colza s'est avéré plus sensible aux NTC ayant le plus petit diamètre, mais la fonctionnalisation modulait grandement la réponse de la plante. Enfin, l'influence d'un stress environnemental (stress thermique) sur la toxicité des NTC a été étudiée. Dans les conditions de croissance optimales, le colza n'a montré aucune réponse aux différents NTC en termes de développement des plantes. Cependant, sous l'effet du stress thermique, l'exposition aux NTC a entraîné des différences de croissance significatives selon le type de NTC : les NTC fonctionnalisés ont permis d'atténuer les effets du stress abiotique sur les plantes, tandis que les NTC non fonctionnalisés ont augmenté l'inhibition du développement des plantes. Les plantes étaient donc plus sensibles aux NTC lorsqu'elles étaient soumises à un stress thermique concomitant.

Mots-clés : nanotubes de carbone, détection, réponse des plantes, FTIR, espèces végétales, paramètres physico-chimiques, stress combiné

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UNDER OPTIMAL CONDITIONS AND HEAT STRESS

INTRODUCTION (VERSION FRANÇAISE RÉSUMÉE)

1. LES NANOTECHNOLOGIES

Afin de répondre au développement et à l'augmentation de la population, notre société moderne est en constante évolution avec pour objectif l'amélioration des connaissances et des méthodes dans de nombreux domaines. Dans ce but, les industries et la science se tournent de plus en plus vers les nanotechnologies (Das *et al*, 2015). Un nanomatériau (NM) est défini comme étant un matériau naturel, formé accidentellement ou manufacturé contenant des particules libres, sous forme d'agrégat ou sous forme d'agglomérat, dont au moins 50 % des particules, dans la distribution en nombre par taille, présentent une ou plusieurs dimensions externes se situant entre 1 et 100 nm (European Commision, 2011). Le secteur des NM est en pleine expansion depuis quelques années. Le nombre de produits de la vie quotidienne contenant des NM a augmenté passant de 54 en 2005 à plus de 3000 en 2019 (Danish Consumer Council, The Ecological council, 2019). Au-delà de leur taille nanométrique, la caractéristique la plus importante des NM est leur rapport surface/volume très important. En effet, plus la taille du matériau est faible, plus le pourcentage d'atomes en surface augmente (Figure 1).



Figure 1 Évolution du pourcentage d'atomes en surface des particules en fonction de leur diamètre en nanomètres. La barre rouge représente le seuil expérimental en dessous duquel les propriétés exceptionnelles des nanoparticules apparaissent (Auffan *et al.*, 2009).

Cette forte augmentation d'atomes en surface rend les matériaux beaucoup plus réactifs et leur confère des propriétés nouvelles, ou accentue celles déjà existantes. Les propriétés des NM peuvent être très variées. On peut ainsi trouver des NM très résistants mécaniquement, très conducteurs (électricité, chaleur) ou encore agissant comme des catalyseurs très performants. Ces propriétés particulières offrent de nouvelles possibilités dans de très nombreux domaines d'applications. On les retrouve par exemple en agroalimentaire, en électronique, dans des articles de sport ou encore dans le médical. L'agriculture porte également une attention grandissante aux NM. Ils pourraient être utilisés pour l'optimisation des engrais

et des produits phytosanitaires (lavicoli *et al.*, 2017). Les NM les plus utilisés sont les ceux à base d'oxyde de métaux ou de métaux (argent, dioxyde de titane) ainsi que les NM carbonés (Vance *et al.*, 2015). Parmi ces derniers, les nanotubes de carbone (NTC) constituent une des classes les plus importantes au niveau de leurs potentiels applicatifs. Ils sont aussi les seuls à permettre la combinaison de plusieurs propriétés physiques telles par exemple une résistance mécanique exceptionnelle et une bonne conductivité électrique.

2. LES NANOTUBES DE CARBONE

2.1 Description

Les NTC sont décrits comme des feuillets de graphène enroulés sur eux-mêmes pour former des tubes (Figure 2). Il existe une multitude de NTC différents. Ils peuvent varier selon plusieurs paramètres physicochimiques. Ils ont une longueur souvent comprise entre 1 et 100 µm. Ils peuvent présenter un nombre de parois différent, on trouve ainsi trois types de NTC : les NTC mono paroi (un seul feuillet de graphène), les NTC double parois (deux tubes de graphène concentriques) et les NTC multi parois (plus de 2 tubes de graphène concentriques). Ils présentent un diamètre pouvant varier de moins de 1 nm pour les mono parois jusqu'à plusieurs dizaines de nm pour les multi parois. Il est également possible de fonctionnaliser les NTC (greffage de groupements chimiques sur la face extérieure du tube externe), ce qui va modifier leurs propriétés de surface. Hydrophobes par nature, la fonctionnalisation peut ainsi leur conférer un caractère plus hydrophile (dans le cas du greffage de fonctions oxygénées par exemple), ce qui permet, entre autres, de les utiliser plus facilement en suspension et d'améliorer leur dispersion dans une matrice.



Figure 2 Représentation d'un NTC mono paroi.

2.2 Synthèse des NTC

Des techniques de préparation à très hautes températures (pouvant atteindre jusqu'à 6000°C) ont tout d'abord été utilisées comme l'ablation laser et l'arc électrique (Prasek *et al.*, 2011). Ces méthodes ont progressivement été remplacées par une technique plus "basses températures" de dépôt chimique catalytique en phase vapeur (Chemical Vapor Deposition, CVD) (Laurent *et al.*, 1998). Avec cette dernière, l'orientation, l'alignement, la longueur, la pureté et la densité des tubes peuvent être modulés. La technique CVD est expliqué dans le chapitre 2 matériels et méthodes (1.2).

2.3 Propriétés et applications des NTC

Grâce à leur structure unique, les NTC possèdent des propriétés optiques, électriques, thermiques, mécaniques et chimiques remarquables. D'un point de vue mécanique, ils sont extraordinairement flexibles malgré leur forte rigidité. Les NTC sont également extrêmement résistants : ils sont 100 fois plus résistants mais 6 fois plus légers que l'acier (Chang and Liu, 2010). Ils possèdent des propriétés électriques intéressantes : selon leur structure, ils peuvent se comporter comme des semi-conducteurs ou des métaux. Grâce à leurs propriétés électroniques et leur bonne stabilité à hautes températures, les NTC peuvent supporter de très fortes densités de courant (Yao *et al.*, 2000). Concernant les propriétés thermiques, la faible proportion de défauts structuraux et leur géométrie cylindrique leurs permettent d'avoir une forte conductivité thermique le long de l'axe des tubes (Berber *et al.*, 2000).

Du fait de ces propriétés, le nombre d'applications ne cesse de croitre ; le marché pourrait ainsi dépasser les 8 milliards de dollars américains d'ici 2024 (Global market Insights, 2017). Ils sont employés dans de nombreux secteurs d'applications tels que l'énergie, les matériaux composites ou encore l'électronique (Terrones, 2004). Ils peuvent ainsi être utilisés dans les batteries des équipements mobiles afin d'améliorer le stockage de l'énergie (Zhai et al., 2016). On peut également les retrouver dans la composition des écrans plats afin de permettre une plus faible consommation électrique (Terrones, 2004). Ils sont envisagés pour la réalisation d'écrans flexibles dans lesquels ils remplacent avantageusement le matériau conducteur transparent actuel (Indium Tin Oxide), trop fragile. Ils sont utilisés dans les équipements sportifs comme les raquettes de tennis, les cadres de vélos ou encore les clubs de golf afin de les rendre plus légers tout en étant très résistants. Ils sont aussi dans certains vêtements les rendant plus imperméables et résistants (Vance et al., 2015) tout en leur conférant des nouvelles propriétés (monitoring médical par exemple). Les NTC apparaissent également comme étant une nouvelle opportunité pour les applications biomédicales : même si les premières applications envisagées telles que le transport de molécules thérapeutiques (Bianco et al., 2005) ne sont plus d'actualité (toxicité potentielle), d'autres pour combattre le cancer en limitant la propagation des cellules cancéreuses grâce à leur potentiel redox (Fiorito et al., 2014; García-Hevia et al., 2015) sont toujours en développement. L'agriculture est également un secteur où l'on pourrait utiliser les NTC. En effet, certaines études ont montré qu'ils étaient capables de stimuler la croissance des plantes (Liné et al., 2017). Ils pourraient donc être utilisées en tant que fertilisants dans les champs (Gogos et al., 2012). Grâce à leurs fortes capacités d'absorption, ils pourraient éliminer des contaminants biologiques comme les bactéries ou les virus, ou chimiques (métaux lourds, molécules organiques, etc.). En effet, ces contaminants présentent une forte affinité pour les NTC, ce qui permettrait une élimination plus simple (Upadhyayula et al., 2009). Comme d'autres NM, ils pourraient donc être utilisés dans des secteurs comme la remédiation des sols ou des eaux polluées (Liné and Larue, sous presse).

3. ECOTOXICOLOGIE DES NTC

3.1 Dissémination et détection des NTC dans l'environnement

Tout le long de leur cycle de vie, depuis leur production jusqu'à la destruction des « nanoproduits » en passant par leur utilisation ou encore leur transport, les NTC peuvent se répandre dans l'environnement (Gottschalk *et al.*, 2013b). La dissémination est le plus généralement non intentionnelle. Par exemple, les NTC peuvent être accumulés dans les sols suite à l'usure des pneus contenant des NTC sur les routes (Nowack *et al.*, 2013). Une étude a également mis en évidence des NTC dans les poumons d'enfants Parisiens, une hypothèse avancée par les auteurs de l'étude étant qu'ils viendraient des pots catalytiques des voitures (Kolosnjaj-Tabi *et al.*, 2015). Il est important de noter que les NTC mis en évidence dans ces travaux ressemblent assez peu au niveau de leur morphologie aux NTC synthétisés de manière intentionnelle. Ils se retrouvent également concentrés dans les boues de station d'épuration (STEP), ce qui représente un vecteur fort vers les sols agricoles puisqu'un des principaux débouchés pour ces boues est l'épandage en agriculture (Sun et al., 2016b). Les NTC pourraient être également dispersés dans l'environnement de manière intentionnelle si par exemple ils sont utilisés dans la remédiation des sols et des eaux pollués, ou encore en comme fertilisants en agriculture (Upadhyayula *et al.*, 2009).

A ce jour, la concentration des NTC dans l'environnement ne peut être mesurée directement car nous ne disposons pas des moyens techniques nécessaires. Seules des études de modélisation permettent d'estimer les concentrations présentes dans les différents compartiments environnementaux. Sun et al. (2016b) ont suggéré que la concentration en NTC dans les eaux de surface en 2014 en Europe était de 0,36 ng/kg, de 6,74 µg/kg dans les sédiments, de 35 ng/kg dans les sols urbains et naturels, de 11,7 µg/kg dans les sols traités avec des boues de station d'épuration et enfin de 0,02 ng/m³ dans l'atmosphère.

La détection et la quantification des NTC dans des échantillons biologiques sont très complexes. En effet, il est difficile de détecter une forme spécifique de carbone dans une matrice elle-même essentiellement carbonée. Certaines techniques sont tout de même utilisées mais, mis à part le marquage isotopique au ¹⁴C, il est généralement très difficile d'analyser à la fois qualitativement et quantitativement les NTC dans des matrices biologiques et/ou environnementales (Czarny *et al.*, 2014). Parmi ces techniques, on note la microscopie électronique à transmission (MET), la spectroscopie Raman, les analyses thermogravimétriques (ATG), les mesures microondes ou encore l'imagerie hyper spectrale (Herrero-Latorre *et al.*, 2015). D'autres méthodes basées sur la photoluminescence des NTC ne sont valables que pour certains types de NTC (les mono parois) (Lefebvre *et al.*, 2004) et ne sont donc pas généralisables.

La mobilité des NTC dans les sols a été peu étudiée dans la littérature, mais quelques tendances peuvent être dégagées. Leur biodisponibilité est plus forte dans des sols riches en matière organique et semble faible dans le cas contraire (Cornelis *et al.*, 2014). Au niveau des végétaux, il a été rapporté de nombreuses fois que les NTC sont capables de pénétrer les graines (Khodakovskaya *et al.*, 2009; Hao *et al.*, 2016; Martínez-Ballesta *et al.*, 2016; Mohamed H. Lahiani *et al.*, 2016) mais aussi les racines des plantes (Tan *et al.*, 2009; Miralles *et al.*, 2012; Zhang *et al.*, 2014). Une fois dans la plante, les NTC peuvent être transportés jusque dans les feuilles en empruntant le système vasculaire de la plante. Ils ont ainsi été détectés dans les tiges, les feuilles mais également jusque dans les fruits (Begum and Fugetsu, 2012a; Smirnova *et al.*, 2012; De La Torre-Roche *et al.*, 2013; Hao *et al.*, 2016).

3.2 Impacts des NTC sur les plantes

Avec l'utilisation croissante des NTC et donc une augmentation potentielle de leur concentration dans l'environnement, le nombre d'études sur leurs impacts (éco)toxicologiques s'est multiplié, montrant en particulier que les NTC présentent des risques potentiels pour l'Homme notamment après une exposition par les voies aériennes. Les effets sanitaires des NTC sont d'autant plus préoccupants qu'ils peuvent être comparés à l'amiante du fait de leur morphologie fibreuse et de leur relative biopersistance (Donaldson *et al.*, 2013).

Du fait de leur possible future utilisation dans des domaines comme l'agriculture ou encore la remédiation des sols, il est indispensable de déterminer le comportement et les impacts des NTC sur les écosystèmes terrestres, et plus particulièrement sur les plantes d'intérêt agricole. Ce comportement peut en particulier dépendre des propriétés physicochimiques des NTC (pureté, longueur, diamètre, fonctionnalisation) mais également des conditions environnementales (caractéristiques du sol, conditions météorologiques) (Jackson *et al.*, 2013).

Les impacts des NTC sur les plantes sont à ce jour controversés (Liné, Larue and Flahaut, 2017). Il a été montré à de nombreuses reprises qu'ils peuvent améliorer la croissance des plantes. En effet, ils ont permis l'augmentation de la germination de graines (Khodakovskaya et al., 2009; Oleszczuk et al., 2011; Lahiani et al., 2015a; Lahiani et al., 2016), de la production de biomasse, de la production de fleurs ou encore de l'élongation racinaire (Oleszczuk et al., 2011; Smirnova et al., 2012; Tiwari et al., 2014; Martínez-Ballesta et al., 2016; Lahiani et al., 2016). D'autres études ont montré, au contraire, que les NTC peuvent avoir des impacts plutôt « négatifs » sur les plantes. Ils ont ainsi réduit la croissance de certaines plantes ou encore provoqué une augmentation de la teneur en espèces réactives de l'oxygène (ERO) (Tan et al., 2009). Il a été également prouvé que les NTC peuvent causer des lésions nécrotiques sur les tissus foliaires et même modifier la morphologie des feuilles et des racines (Begum and Fugetsu, 2012a). Pour finir, certaines études n'ont pas mis en évidence d'effet des NTC sur les plantes (Lin and Xing, 2007; Larue et al., 2012; Hamdi et al., 2015). Cette grande disparité dans les résultats peut être expliquée par de nombreux paramètres : la nature des NTC mis en jeu, les conditions d'exposition utilisées pour mener l'étude (en conditions hydroponiques ou en sol) ou encore le type de plante utilisé. De plus, l'évaluation de l'impact des NTC peut se faire selon différents marqueurs de l'état de santé de la plante (morphologiques, physiologiques ou encore biochimiques). Le manque de normalisation des conditions d'étude ainsi que la multitude de margueurs disponibles ne permettent pas une évaluation standardisée de l'impact des NTC sur les plantes. Il existe également une multitude de NTC ayant différents paramètres physicochimiques qui vont conditionner leur comportement. En comprenant les effets de ces paramètres, il sera possible d'utiliser une approche «safe(r) by design» lors de la synthèse des NTC. Cette approche consiste à

synthétiser des matériaux plus sûrs pour l'environnement en modulant leurs propriétés physicochimiques mais tout aussi efficace pour l'industrie.

4. PRESENTATION DE L'ETUDE

Dans l'ensemble, les effets et le comportement des NTC chez les plantes sont encore mal compris et très controversés. La réponse de la plante peut être influencée par plusieurs paramètres qui diffèrent d'une étude à l'autre.

Question de recherche n°1 : La phytotoxicité est-elle liée à l'internalisation des NTC dans les feuilles des plantes ?

De nos jours, la détection des NTC dans les plantes est encore un défi, mais il a été démontré que les NTC peuvent être internalisés. Pour répondre à cette question, nous avons analysé plusieurs techniques de détection des NTC chez les plantes en utilisant des concombres (Cucumis sativus) cultivés dans des conditions hydroponiques comme modèle expérimental. Les résultats sont détaillés au chapitre 4.

Question de recherche n°2 : Les NTC ont-ils des impacts différents selon les espèces végétales ?

Dans la littérature, peu d'expériences analysant différentes espèces de plantes ont été menées. Ils tendent à démontrer que les impacts des NMs peuvent dépendre des espèces végétales. Notre hypothèse de recherche est que la famille de plantes (dicotylédones vs monocotylédones) régit la réponse des plantes. Pour étudier cette hypothèse, nous avons évalué les impacts des NTC sur 4 plantes différentes : 3 dicotylédones : canola (Brassica napus), tomate (Solanum lycopersicum) et concombre (Cucumis sativus) et 1 monocotyledone : maïs (Zea mays) (chapitre 6). Cette expérience a permis de déterminer les espèces les plus sensibles.

Question de recherche n°3 : Dans quelle mesure les caractéristiques des NTC influencent-elles la réponse des plantes ?

Des travaux antérieurs réalisés au laboratoire ont mis en évidence que les diamètres des TiO₂-NP influençaient l'absorption de NM et leurs effets sur les plantes. Avec cette expérience, nous avons voulu vérifier cette hypothèse sur les NTC. Tout d'abord, nous avons étudié les impacts de deux NM différents : TiO₂-NPs et des NTC sur la réponse des plants de tomate (Solanum lycopersicum) (chapitre 5). Pour se faire, nous avons développé la spectroscopie infrarouge à transformée de Fourier (FTIR). Nous nous attendons à ce que la chimie et la forme des NM conduisent à des modifications biomacromoléculaires différentes.

Ensuite, nous émettons l'hypothèse que les paramètres physico-chimiques des NTC (c.-à-d. diamètre, longueur, fonctionnalisation, dispersion) influencent leurs impacts sur les plants de colza (Brassica napus). Pour cela, 5 NTC ont été utilisés (NTC double parois, NTC double parois fonctionnalisés, NTC multi parois, NTC multi parois fonctionnalisés et NTC multi parois courts) (chapitre 7).

Question de recherche n°4 : L'application d'un stress combiné à l'exposition aux NTC entraîne-t-elle une toxicité différente ?

Certaines études ont rapporté qu'un stress combiné entraîne une toxicité plus élevée des NM. Dans le contexte du changement climatique, nous évaluons l'impact du stress thermique sur la toxicité des NTC du colza (Brassica napus). Les effets ont été comparés à la toxicité des NTC sur les plantes poussant dans des conditions de croissance optimales pour vérifier l'augmentation de la sensibilité (chapitre 7).

CHAPTER 1: INTRODUCTION

– Publication –

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Carbon nanotubes: Impacts and behaviour in the terrestrial ecosystem - A review



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1. CARBON NANOTUBES: IMPACTS AND BEHAVIOUR IN THE TERRESTRIAL

ECOSYSTEM - A REVIEW

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1.1. Abstract

For more than twenty years, nanotechnologies have arisen a huge interest and are used in numerous fields. Carbon nanotubes (CNTs) are one of the most used nanomaterials thanks to their excellent optical, mechanical, electrical and thermal properties. All along their lifecycle, CNTs may be spread in the environment during production, use, destruction, reuse or potential accidents in production units or during transportation. For this reason, it is essential to evaluate their behaviour and potential impacts on ecosystems and particularly on the terrestrial ecosystem. After a brief summary of CNT properties, synthesis methods, and applications as well as detection and characterisation techniques, this review will focus on impacts of CNTs on the terrestrial ecosystem, discussing their behaviour in soil, plants and interactions with other pollutants as well as their impacts on soil microbiota, macrobiota and plants.

1.2. Introduction

For more than a decade, nanotechnologies are more and more investigated by industrials and scientists and used worldwide for applications thanks to their remarkable properties. The European Commission defined in 2011 a nanomaterial as "A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions are in the size range 1 nm - 100 nm." (Commission Européenne, 2016). However, nanomaterial definition is different according to countries and to the field in which they are used. All definitions agree about the nanoscale dimensions but definitions differ on size distribution for example. This lack of global consensus is a serious challenge because it leads to legal uncertainty and differing regulatory for the same nanomaterial. The nanotechnology consumer products inventory (CPI) listed officially in 2014 more than 1800 consumer products containing nanoparticles worldwide. In less than ten years, the number of products containing nanoparticles increased by more than 3,000 % (54 products in 2005) (Vance *et al.*, 2015).

Carbon-based nanomaterials are among the most used (Vance *et al.*, 2015). There are different types of carbon nano- objects such as fullerenes (3 dimensions < 100 nm), carbon nanotubes (2 dimensions < 100 nm, CNTs) and graphene and related materials (1 dimension < 100 nm). Since their discovery in 1991 by lijima, they arose an extraordinary enthusiasm (lijima, 1991; Dresselhaus *et al.*, 2003). CNTs can be described as graphene sheets rolled over themselves to form (concentric) cylinders with a nanometric diameter. We can define three kinds of CNTs: single wall CNTs (SWCNTs), double wall CNTs (DWCNTs) with two concentric tubes and multi wall CNTs (MWCNTs) with more than two concentric tubes. CNT diameter varies from a few nanometers for SWCNTs to several tens of nanometers for MWCNTs. Their length is usually of a few micrometers. CNTs have remarkable optical, electrical, thermal, mechanical and chemical properties. They are used in numerous fields such as plastic additives, in batteries or some sporting goods (Terrones, 2004).

It is essential to regulate production and uses of nanomaterials for a safe and sustainable future. So far there is no international agreement to supervise the production, use and commercialisation of nanomaterials. However, few countries started to monitor nanomaterials commercialised in their territories by using registers. In Europe, there is the European regulation for the recording, evaluation, authorization and restrictions about chemical substances (REACh). The recording and the authorization are compulsory for produced or imported nanomaterials with a volume of more than 100 tons. A new authorization protocol will be apply in 2018 for volumes between 1 and 100 tons, without toxicological data required. In theory, nanomaterials are covered by this regulation but practically they are often brought to the market without preliminary recording or monitoring. The first reason is that producers and distributors produce or import very rarely more than one ton per year, the threshold below which it is not compulsory to make a REACh recording. The second reason is that even if there is more than one ton per year, REACh does not oblige to record nanomaterials as new substances. Consequently, the recording gets an extension and the terms and conditions are simplified excluding for example ecotoxicological data. In

France, a precursor in this domain, since January, 1st 2013, industrials and researchers have to declare annually the quantity, the properties and the uses of nanomaterials they produce or import in the R-Nano database handled by the ANSES (French Agency for Food, Environmental and Occupational Health & Safety) (L. 523-1 and L 523-3 of "Code de l'environnement"(*R-Nano.fr*, 2016). In Norway, since 2013, the national public agency of climate and pollution asks for identification of nanomaterials in the chemical product register. In Denmark, producers and importers have to record nanomaterials and products containing or releasing nanomaterials since 2014. Finally, in Belgium, since 2016 there is a royal decree concerning the placing on the market of manufactured nanomaterials.

In the USA, regulations for nanomaterials have been established by numerous organizations including the Environmental Protection Agency (EPA), Food and Drug Administration (FDA), and Consumer Product Safety Commission (CPSC). EPA is controlling nanomaterials by existing regulations of the Toxic Substances Control Act (TSCA) and Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) pursuant Significant New Use Rules (SNUR) of premanufacture notices (PMNs) of 13 chemicals, including CNTs and fullerenes. For nanomaterial manufacture and production, the manufacturers must inform the EPA with information about the nanomaterials within 90 days. For the FIFRA regulation, pesticide products containing nanomaterials must be registered. In Asia, the Japanese Council for Science, Technology and Innovation (CSTI) is paying attention to the new rules implemented in EU and USA. However, there is no legal control related to nanomaterial safety and environment so far. Anyway, Japanese Government is working with ministry of economy, trade and industry (METI) in order to collect information about the industry working with nanomaterials and to evaluate harmful effects of nanomaterials with the ministry of environment. Broadly speaking, scientists, associations and sanitary agencies are worried about the risks associated with nanomaterials and nanotechnology. However, industrials do not want regulatory framework because in the European and international market, nanotechnology is bringing jobs. So far, there is no strict regulation on nanotechnology. However, it is an international problem for environment, safety and health, it is thus essential to roll out international rules for their control.

All along their lifecycle, CNTs may be spread in the environment during production, use, destruction, reuse or potential accidents in production units or during transportation (Gottschalk *et al.*, 2013a). During their release, they can be subjected to physico-chemical modifications which may later modulate their potential toxic effects (Bertrand, 2016). Toxicological studies evidenced that, CNTs present a potential risk for humans upon pulmonary exposure. CNT effects raise concerns because they can be compared to asbestos due to their fibre shape (Donaldson *et al.*, 2013). Asbestos caused a worldwide pandemia of disease in the 20th century such as asbestosis, mesothelomia, bronchogenic carcinoma, etc. (Donaldson *et al.*, 2013). For instance, Kasai *et al.* (Kasai *et al.*, 2015) studied the toxicity of MWCNTs with whole-body inhalation exposure in rats; they found that MWCNTs increased lung weight and inflammatory parameters of the exposed rats.

It is also essential to assess their behaviour and potential impacts on ecosystems. To date, the focus has been mainly on aquatic ecosystems rather than on the terrestrial ecosystems (Kahru and Dubourguier,

2010). This review aims at summarizing the knowledge about behaviour and impacts of CNTs on the terrestrial compartment with a focus on plants. Our survey covered 71 studies on terrestrial ecosystems. The majority of the studies have been realised on plants (65%). Soil microorganisms and macroorganisms have been studied with respectively 14% and 17% of the studies. The less studied domain is the behaviour of CNTs in soil (in laboratory soil column) with only 4% of the mentioned articles. For plants, 46 studies have been published, with different culture conditions (figure 3a): most of the studies were based on plants exposed in a simplified media: hydroponics conditions (35%), filter paper (13%) and jellified medium (17%). Studies using soil exposure, representing the most relevant exposure scenario to mimic real environmental conditions, represent only 17% of the articles (15% in soil, 2% in sediment). The last part of the studies used in vitro tests on plant cells (16%). The exposure time is another parameter to take into account: among the 46 plant studies, 19% focus only on seeds (figure 3b). Most of the studies were realized on seedlings (47%). Long-term exposure with adult plant represents 16% of the studies. Exposure during the entire life cycle, which represents the most realistic scenario, are only 2% of the cases. In total, 84 different plants were studied. 59% were dicotyledons and the rest monocotyledons. Different CNTs have also been studied: SWCNTs, MWCNTs, functionalized or not. MWCNTs are the most used for ecotoxicological studies on plants (more than 84%).



Culture conditions

Figure 3 Literature review of CNT impacts and behaviour on plants (culture conditions and plant stage exposure). Seedling represents plant after germination but still growing. Adult plants are plant which they reach adult height.

Scientists and industrials are getting more and more conscious of nanomaterial effects, at the same time they know the high potential of nanomaterials. Consequently, they are trying to find a compromise between these two aspects for example with the "safe by design approach". In this approach, physico-chemical parameters of nanomaterials are studied. Then, they are trying to find a way to reduce at the maximum the nanomaterial toxicity by playing with the different physico-chemical parameters (Maynard *et al.*, 2006).

In this review, general information on CNTs will be briefly reminded including CNT properties, their synthesis and their different applications. Then the issue of the detection and characterisation will be discussed. The other parts concern the environmental implications of CNTs with their release and potential exposure pathways, their fate and impacts on the soil system and finally the last part will focus on their fate and impacts on plants.

1.3. Carbon nanotube synthesis, properties and applications

High temperature preparation techniques were first used to produce CNTs such as arc discharge or laser ablation. Nowadays, these methods have been replaced by low temperature catalytic chemical vapour deposition (CCVD) techniques (Laurent et al., 1998). With CCVD techniques, the orientation, alignment, length, diameter, purity and density of CNTs may be controlled precisely. Other less common techniques can also be used for CNT synthesis such as liquid pyrolysis and bottom-up organic approaches (Monthioux et al., 2007). Whatever CNT preparation method used, they always contain impurities, most of them corresponding to residual catalyst, but other unwanted carbon species are usually also present to some extent such as disorganised carbon. These impurities have to be chemically treated in order to be eliminated. They can be washed using concentrated acids such as hydrochloric acid or nitric acid. Asproduced CNTs are hydrophobic, and thus obtaining a homogenous suspension of CNTs is challenging. To increase their hydrophilicity, CNTs can be functionalised by modification of the external wall. There are mainly two types of functionalization. The first one, and the most used, is the covalent functionalisation using oxidising treatments which damage the outer wall of the CNTs while grafting oxygen-containing chemical groups. Covalent functionalisation implies strong treatments such as heating with acids, which are damaging CNTs. Consequently, functionalised CNTs are shorter than untreated ones. The second functionalisation is non covalent, and based on the adsorption of a surfactant to obtain a more homogeneous suspension of CNTs. Numerous dispersants/surfactants have been used in the literature. In order to work with living organisms it is required to use non-toxic dispersants. A sap exudate called Arabic gum can be used to disperse CNTs in suspension (Landois, 2008; Larue et al., 2012). 0.25 % (w/v) of Arabic gum is able to stabilize a suspension of 1 g/L of CNTs during one month at pH 5.5 (Larue et al., 2012). Humic acid, one of the most important fraction of humus, can also be employed. 0.25 % (w/v) of humic acid is also able to stabilize a suspension of 1 g/L of CNTs during one month at pH 7.6 (Larue et al., 2012). Other dispersants can be used to disperse CNTs such as gallic acid, an aromatic organic compound common in plants (Ratnikova et al., 2015), carboxymethylcellulose or tween 20, a non-ionic surfactant (Landois, 2008). Bile salts have also been used such as sodium cholate (Wenseleers *et al.*, 2004) or deoxycholic acid (Datsyuk *et al.*, 2009).

Due to their unique structure, CNTs display remarkable physical properties. From a mechanical point of view, they have an extraordinary flexibility despite their high rigidity (Terrones, 2004). The bending is reversible until a critical angle of 110° for a SWCNT (Yu *et al.*, 2000). CNTs are 100 times more resistant and 6 times lighter than steel (Chang and Liu, 2010). They also have useful electrical properties: depending on their structure, they can behave like semiconductors or like metals. Thanks to their electronic properties and their good stability at high temperatures (up to 250°C in air and > 2,600°C in vacuum), CNTs can withstand extreme current densities (one order of magnitude more than copper) (Yao, Kane and Dekker, 2000). Regarding thermal properties, the low proportion of structural defaults and the cylindrical geometry of CNTs lead to high thermal conductivity along the axis of the nanotube, comparable to that of the isolated graphene sheet or high purity diamond ($\lambda \approx 6,600$ W / m K) (Berber *et al.*, 2000).

CNT uses and applications are increasingly numerous and varied: for example, they can be used as field emission sources for visible light (Bonard et al., 2002) or X-rays (Zejian et al., 2006). Their high rigidity, their nanometric diameter and their flexibility allowed Dai et al. (Dai et al., 1996) to realize a tip for scanning probe microscope by gluing a CNT on the tip of an atomic force microscope (Nguyen, Ye and Meyyappan, 2005). Cheung et al. (Cheung et al., 2000) have grown CNTs directly on the tip of an atomic force microscope allowing high resolution images (Xu, Fang and Dong, 2011). CNTs can also be used in the composition of flat screen TV which allows a lower electricity consumption, a more intense luminosity and a larger range of operating temperatures (Terrones, 2004). They are used in batteries of electronic mobile devices allowing a better energy storage (Zhai et al., 2016). Most of the applications are however related to nanocomposite materials and paints in which CNTs usually bring some electrical conductivity in addition to an increase in the mechanical properties. CNTs are found in sport equipment such as tennis rackets, bicycle frames or golf clubs in order to make them lighter. They are also found in clothes making them more resistant and waterproof (Vance et al., 2015). Alternatively, CNTs appear as a new alternative for biomedical applications, they can be efficient to transport and translocate therapeutic molecules (Bianco et al., 2005), or even to fight cancer (Fiorito et al., 2014; García-Hevia et al., 2015). CNTs may also be used in agriculture. In plants, Serag et al. (Serag, Kaji, Tokeshi, Bianco, et al., 2012) investigated the ability of CNTs to penetrate the plant cell walls and most of the subcellular membranes to deliver payloads to specific cellular organelles in plants with the aim of increasing pesticide efficiency and thus their input in the environment. Wood could be reinforced with CNT fibres in order to increase its strength. Due to numerous studies that report growth increase of plants after CNT application, CNTs are imagined to be used as fertilizers (Gogos et al., 2012). Finally, thanks to their high adsorption capacities, CNTs seem to be able to remove a diverse range of biological contaminants such as bacteria or viruses from water systems. They can also be used for the removal of chemical contaminants such as heavy metals or organics (Upadhyayula et al., 2009). CNTs have been used as a sponge for oil during oil spill; they have great sorption capacity and the absorbed oil can be recovered by squeezing or be converted to heat by burning the oil within sponges (Gui *et al.*, 2011).

1.4. Releases and potential exposure pathways

CNT spreading into the environment can occur following different routes. The release will usually be unintentional, with possible chronic and/or acute contaminations. Chronic dissemination corresponds to the contamination by low doses of CNTs, but over a long period of time. Direct release (chronic dissemination) has been considered as very low for most of the scenarios, except for tires (Nowack *et al.*, 2013). For example, CNTs can be accumulated in soil due to the rubbing of CNT-containing tires on roads (Nowack *et al.*, 2013). CNT have been found in the lungs of Parisian kids and this may be due to a production of such nanomaterials by car catalytic converters (Kolosnjaj-Tabi *et al.*, 2015). For most of the other life cycle stages (production, uses or end of life), releases can be possible but it is difficult to assess the real risk due to the lack of knowledge during and after waste management and recycling operations of nanomaterials (Caballero-Guzman *et al.*, 2015).

Acute contamination corresponds to a high release but during a short period, for example during an accident in a production unit or during transportation (Gottschalk *et al.*, 2015b). Upadhyayula *et al.* (Upadhyayula *et al.*, 2012) studied the life cycle assessment of products containing CNTs. They evidenced that the manufacturing stage of CNT containing products dominates the environmental impacts. Likewise, Nowack *et al.* (Nowack *et al.*, 2013) studied the potential release scenarios for CNTs during nanocomposite production. The authors concluded that release during manufacturing may be possible, but this is also the place where exposure can be best controlled.

It is important to mention here that if CNTs released from a material may be similar to the initial incorporated nanomaterial, either individual or agglomerated, CNTs functionalised by residual coating with the matrix material may also be observed (Schlagenhauf *et al.*, 2012). The interactions between CNTs and their environment are also driven by the interface with the outer wall. The presence of residues of polymers, for example, may modify their wettability / hydrophobicity and thus influences directly their fate in water, soils and organisms.

So far, CNT concentration in the environment (as well as other nanomaterials) cannot be measured directly and the research in this domain can only rely on modelling results. Sun *et al.* (Sun *et al.*, 2016b) modelled the environmental concentrations of engineered nanomaterials including CNTs. In surface water, CNT concentration in 2014 was estimated to be around 0.36 ng/L, 6.74 μ g/kg in sediment, 35 ng/kg in natural and urban soil, 11.7 μ g/kg in sewage sludge treated soil and 0.02 ng/m³ in the atmosphere. Gottschalk *et al.* (Gottschalk *et al.*, 2015b) modelled flows and concentrations of 9 engineered nanomaterials in the Danish environment. Authors calculated that the primary sources of CNTs would be waste incineration plants (<1% of total primary sources), sewage treatment plant effluents and overflow (<1%), sewage treatment plant sludge (<1%) and production, manufacturing and consumption including untreated wastewater (99%). The primary recipients of CNTs were soils (91.2%), marine water (3.5%), freshwater (2.8%) and air (2.5%). According to their models, CNT concentrations in surface water of the Danish environment would be between 0.2 and 15 pg/L, in sediments (freshwater) between 0.1 and 5.6 μ g/kg, between 18 and 75 ng/kg in agricultural soils, between 41 and 220 ng/kg in natural soils, between 71 and 290 ng/kg in urban soils and finally between 0.022 and 0.091 ng/m³ in air.

The release can also be intentional, when for example CNTs are used for depollution (nanoremediation). Indeed, they have the potential to remove bacterial pathogens, natural organic matter and cyanobacterial toxins from water systems (Upadhyayula *et al.*, 2009). CNTs may also be used in plant protection or fertilizer products (Gogos, Knauer and Bucheli, 2012). Numerous studies highlighted positive impacts of CNTs on plants, especially at rather low doses)

1.5. Detection and characterization of carbon nanotubes in environmental matrixes

The detection and quantitative analysis of CNTs in biological samples is very complex because it is difficult to detect a specific form of carbon in a carbon based matrix. Sample preparation is often challenging in complex environments (Bourdiol *et al.*, 2013).

Many methods exist to detect CNTs, but apart from the use of isotopic labelling (Czarny et al., 2014), it is generally difficult to analyse them both qualitatively and quantitatively. However, this technic presents several constraints. It is expensive to synthetize CNTs with isotopic labelling like carbon 14 and authorization and adapted installations and equipment to work with carbon 14 are required. Labelling with carbon 13 is another alternative but it is not widespread. Microscopy techniques can be used such as scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM) to determine the length, diameter and number of walls. TEM and SEM are also extensively used to localize CNTs in biological samples looking for fibre shaped structures (Figure 4), however this technique does not provide a formal proof that the fibre is indeed a CNT. The specific surface area of a particle (m²/g) is among the most important parameters to measure. It is even more crucial in ecotoxicology since Mottier et al. (2016) evidenced that the surface area of carbon based nanomaterials is a dose metric more realistic than the size or the number of particles. There are different methods to measure the specific surface area of a particle but the most common is the Brunauer-Emmett-Teller (BET) method. This method is based on the Langmuir theory of physical adsorption of a gas monolayer on a solid (Fagerlund, 1973). However it can be used only in a nanomaterial powder (elimination of the bio-matrix). To analyse the chemical purity or the corona form around CNT, inductively coupled plasma (ICP) techniques are mainly used after a proper acidic digestion.



Figure 4 TEM image of MWCNTs in wheat's roots; roots of wheat (*Triticum aestivum*) exposed to 100 mg/L of MWCNTs dispersed in gallic acid for 7 days; CNT is indicated by arrow; (C.W) cell wall; (P) plaste; (V) vacuole. b. Raman spectra of the CNT suspension, the control plant and the same exposed plant as the TEM image.

Herrero-Latorre et al. (Herrero-Latorre et al., 2015) also reviewed the different analytical methods for detection and characterization of CNTs in environmental and biological samples. Raman spectroscopy can be used to give both qualitative and semi-quantitative information. Two bands in particular, the D one corresponding to sp³-like carbon and the G one corresponding to sp² carbon, are mainly used. The band intensity (especially for the G band) can give information about the concentration and the orientation (polarization effects) of CNTs. The band surface gives indications about the quantity and can thus be used to estimate concentrations. The ratio (intensity or area) between the D and G bands allows measuring the proportion of defectuous carbon present in the sample (Barbillat et al., 1999). There are several other techniques to characterize and detect CNTs such as atomic force microscopy (AFM) (Lamprecht et al., 2009), dynamic diffusion of light, although this may not be well-suited for elongated and flexible nanomaterials such as CNTs (Alimohammadi et al., 2011a), infrared spectroscopy (Mondal et al., 2011) or photoluminescence (Jena et al., 2016). Lutsyk et al. (Lutsyk et al., 2016) recently proposed a new method using selective photoluminescent probes based on ionic complexes with organic dyes. Thermogravimetric analysis (TGA) may also be used; this is a thermal analysis technique measuring the mass variation of a sample vs. the applied temperature, in a controlled atmosphere. This technique is especially relevant in the context of the quantitative assessment of CNTs in complex environmental samples when coupled to other instruments such as mass-spectrometry as well as thermal optical transmittance/reflectance in order to differentiate organic and elemental carbon (Doudrick et al., 2012). Microwave measurements have also been shown to be very sensitive for the specific quantification of CNTs in biological samples (Irin et al., 2012; Bourdiol et al., 2015). Conventional mass spectrometers have troubles to detect CNTs due to

their large molecular weight. Chen *et al.* (Chen *et al.*, 2015) overcome this problem by using the intrinsic carbon cluster fingerprint signal of the nanomaterials.

Smith *et al.* (Smith *et al.*, 2014) used dark-field and hyperspectral imaging (HSI) to obtain spectral image of CNTs in monocytes. These technics are used for medicine purposes so far but it is possible to use them in ecotoxicology for the detection in environmental samples. Photothermal/photoacoustic imaging can also help to localize CNTs in plant leaves. Khodakovskaya *et al.* (Khodakovskaya *et al.*, 2011) used this method to analyse interactions between plants and CNTs.

Herrero-Latorre *et al.* (Herrero-Latorre *et al.*, 2015) concluded that the characterization of CNTs requires a wide range of analytical techniques because all the information usually cannot be obtained using one technique alone. Moreover there is a lack of standardized characterization protocols which makes difficult the comparison of CNTs between studies. Nowadays the most common techniques are TEM, SEM, and Raman. The determination of CNTs in biological and environmental samples still constitutes one of the main challenges in the field.

1.6. Fate and impacts of carbon nanotubes on soil and related organisms

Depending on their length, diameter, functionalization and on environmental conditions, CNTs may have a different behaviour in natural conditions (Jackson *et al.*, 2013).

Behaviour of CNTs in soils was little studied in the literature. However, this is essential to evaluate their potential impacts on terrestrial organisms. Jaisi and Elimelech (Jaisi and Elimelech, 2009) investigated the behaviour of carboxyl-functionalized SWCNTs in a column packed with natural agricultural soil (fine sandy loam soil). They demonstrated that the deposition rate of SWCNTs was relatively high over a wide range of monovalent and divalent cation concentrations added to the soil solution (0.03 to 100 mM). Authors concluded that SWCNTs would not exhibit substantial transport and infiltration in soils because of effective retention by the soil matrix. Kasel et al. (Kasel et al., 2013) studied the behaviour of ¹⁴C-labeled MWCNTs in two different types of natural soils. There was a stronger sorption of CNTs on the silty loam soil compared to the loamy sand but the overall conclusion was that MWCNTs remained in the soil: more than 85% of the applied radioactivity was recovered in the soil fraction. Lu et al. (Lu et al., 2014) studied the behaviour of MWCNTs in 3 types of soils: positively charged MWCNTs were entirely retained in soils, while negatively charged CNTs broke through the soil column and were found in the outlet. They also demonstrated that soil texture, rather than organic matter, controlled MWCNT mobility. Cornelis et al. (Cornelis et al., 2014) reviewed the fate and bioavailability of engineered nanomaterials in soils. They concluded that some general trends can be deducted. Engineered nanomaterial bioavailability is higher in saturated, coarsely textured soil with high content of organic matter than in other soils. In unsaturated, finely textured soils with low organic matter content, nanomaterial bioavailability is expected to be low. CNT behaviour in soil media is dominated by the shape, structure and agglomeration state of CNTs in aqueous soil suspension, but also by the heterogeneity, particle size, porosity, nature and permeability of the soil. The agglomeration of CNTs with soil components and other micro and macroorganisms determine

their impacts. In comparison with the aquatic compartment, CNTs in soil are more prone to heteroagglomeration phenomena (Hiroi *et al.*, 2016). In water, CNTs are more likely to form homo-agglomerates but in sediments they seem to act like in soil (Bouchard *et al.*, 2015). Overall, most of CNTs seem to be retained in the soil fraction except in particular cases when negatively charged MWCNTs have been seen to leak out from the soil matrix.

CNTs may also interact with other pollutants present in the environment. Their large specific surface area can favour the adsorption of other pollutants (ionic species, organic molecules) and thus may influence the behaviour and the toxicity of CNTs and/or of co-pollutants (Jackson *et al.*, 2013). Numerous authors studied the interaction between CNTs and other contaminants in aqueous solution (Jackson *et al.*, 2013), but here, we will focus on CNT interactions in soil. Shrestha *et al.* (Shrestha *et al.*, 2015) studied the influence of MWCNTs on polycyclic aromatic hydrocarbons (PAH) bioavailability and toxicity to soil microbial community in alfalfa rhizosphere. They concluded that MWCNT influence on PAH varied according to the different soil types: in a soil with high organic matter content, MWCNTs increased the pyrene degradation (Shrestha *et al.*, 2015). MWCNTs generally minimized toxicity of highly bioavailable PAHs on microbial community. De la Torre-Roche *et al.* (De La Torre-Roche *et al.*, 2013) studied the impacts of MWCNTs and C₆₀ fullerenes on pesticide accumulation in agricultural plants while C₆₀ fullerenes completely suppressed DDx uptake but increased chlordane accumulation. There is a lack of information and understanding about CNT behaviour in soil and with other pollutants, more studies are needed.

CNT effects on soil microbial activity is controversial (table 1) and was only little studied (12 articles in 10 years). However, the majority of these studies seem to conclude that CNTs decreased soil microbial activity (Chung et al., 2011a; Jin et al., 2013, 2014; Ge et al., 2016). Enzymatic activities of soil bacteria were repressed by both MWCNTs and SWCNTs: MWCNTs decreased enzymatic activities of two natural soils (sandy loam and loamy sand soils) at 500 mg/kg (Chung et al., 2011a). Likewise Jin et al. (Jin et al., 2013) found that SWCNTs lowered significantly enzyme activities of a natural sandy loam soil at concentrations between 30 and 300 mg/kg. In another study, bacterial soil community was affected by the presence of SWCNTs with a major impact after 3 days but bacteria recovered completely after 14 days (Rodrigues, Jaisi and Elimelech, 2013). Interestingly, Shan et al. (Shan et al., 2015b, p. 201) found that MWCNTs at low concentration (0.2 mg/kg) stimulated mineralization of an agricultural soil by bacteria. Ge et al. (Ge et al., 2016) made an interesting work about effects of MWCNTs compared to natural or industrial carbonaceous materials on soil microbial communities using long-term studies in dry soil. They found that MWCNTs reduced soil DNA diversity and altered bacterial communities after one year of exposure. These effects are similar to those observed for natural and industrial carbonaceous materials. There are not enough studies available so far to conclude about a possibly different impact between functionalized and unfunctionalized CNTs on soil microbial activities.

To date few studies (only 10) are available on the effects of CNTs on soil macroorganisms (table 2). All of them focused on earthworms exposed in soil (natural or artificial) (Petersen, Huang and Weber, 2008;

Petersen *et al.*, 2009, 2011). Some studies reported effects on whole organism endpoints such as reproduction or mortality (Scott-Fordsmand *et al.*, 2008; Hu *et al.*, 2013) and two focused on sub-organism endpoints (Hu *et al.*, 2013; Calisi *et al.*, 2016). All studies agreed that CNT uptake by earthworms was rather low. CNTs can enter in earthworms by ingestion and phagocytosis through tissues but earthworms can also eliminate accumulated CNTs (Petersen *et al.*, 2011; Li *et al.*, 2013; Eom *et al.*, 2015). Consequently, their toxicity of CNTs was limited. No mortality was found in soil contaminated with MWCNTs even at high concentration (1,000 mg/kg) but DNA damages and other sub-organism endpoint alterations were evidenced in earthworms at lower concentration (50 mg/kg). Finally, earthworms reproduction was affected by DWCNTs at concentrations between 50 and 500 mg/kg (Zhang *et al.*, 2014).

As soil is expected to be the main sink for CNTs, ecotoxicological risks of CNTs in terrestrial environment is of great concern. More studies focussing on CNT behaviour and impacts soil micro and macroorganisms are thus urgently needed.

Article	Soils / bacteria	Culture conditions	CNT used*	Concentrations	CNT characterization	Effects
Chung <i>et al.,</i> 2011	Sandy loam soil from a landscaped site with grass and a loamy sand soil from a landscaped site with coniferous trees	CNT solutions added to the soil and incubated at 25°C during 11 days	MWCNTs (specific surface area 237.1 m ² /g, specific volume 0.86 cm ³ /g, diameter 15.1 ± 1.2, length 10- 20 μm)	50; 500 and 5,000 mg/kg	BET method, TGA, Raman, TEM	Enzyme activities showed a tendency to be repressed at medium CNT concentration. Enzymatic activities and microbial biomass C and N were significantly lowered at high CNT concentration.
Ge et al., 2016	Grassland soil from a natural reserve (sandy clay loam texture weakly acidic)	Soils incubated at room temperature for one year with CNT contamination	MWCNTs-1 (diameter of 23.3 ± 5.5 nm, specific surface area 72 m ² /g), MWCNTs-2 (diameter of 7.4 ± 1.9 nm , specific surface area of 500 m ² /g), MWCNTs-3 (diameter of 13.6 ± 4.6 nm, specific surface area of 200 m ² /g)	1,000 mg/kg	SEM, TGA	The three types of CNTs reduced soil DNA and altered bacterial communities.
Jin <i>et al.,</i> 2013	Sandy loam soil from a landscaped site dominated by grasses	Soils incubated with CNT powder and suspended forms of CNTs during 23 days	SWCNTs (average length of 1.02 µm, average diameter of 1.0 nm, purity > 90%, specific surface area of 1125.3 m ² /g), MWCNTs (specific surface area of 237.1 m ² /g)	30; 100; 300; 600 and 1,000 mg/kg	TGA, BET method	SWCNTs significantly lowered activities of most enzymes and microbial biomass. MWCNTs showed similar effects but at higher concentration.
(Jin <i>et al.,</i> 2013)Jin <i>et al.,</i> 2014	Sandy loam soil from a grass dominated landscaped site	Soils incubated with CNT powder and suspended forms of CNTs during 25 days	SWCNTs (specific surface area of 1125.3 m²/g, purity > 90%)	30; 100; 300; 600 and 1,000 mg/kg	TGA, BET method	Biomass of major microbial groups showed a significant decrease with CNTs. CNTs altered significantly microbial community composition.
Khodakovskaya <i>et</i> <i>al.,</i> 2013a	Mix of soils (N.I)	Soils contaminated with CNT suspension	MWCNTs (diameter 25 nm, length of few microns)	50 mL of 50 or 200 mg/L of CNTs	TEM, RF-CVD	Bacteroidetes and Firmicutes were found to increase with CNT contamination. Proteobacteria and Verrumicorbia decreased with increasing CNT concentration.

Table 1 Studies on CNT impacts and behavior on soil microorganisms

Liu <i>et al.,</i> 2009	E.coli, P.aeruginosa, B. subtilis and S.auresin cultured in standard growth medium with and without CNTs	Bacteria incubated with CNTs overnight at 30 or 37°C	SWCNTs (average diameter of 0.83 μm)	5; 10; 20; 40 and 80 mg/L	Raman spectroscopy, AFM, TEM, SEM and TGA	Individually dispersed CNTs were more toxic than aggregated CNTs. Inhibiting cell growth and oxidative stress were not the major causes responsible for the death cells.
Rodrigues, Jaisi and Elimelech, 2013	Sandy loam soil from a turf grass field	Soil samples exposed with CNTs during 14 days	SWCNTs (diameter from 0.9 to 1.44 nm)	250 and 500 mg/kg	Raman, TGA, SEM-EDX	Bacterial soil community was affected by CNT presence with major impact after 3 days of exposure but bacteria recovered completely after 14 days.
Shan <i>et al.,</i> 2015	Soil from agricultural field with sand, silt and clay content of 12.9%, 76.1% and 11.0%	Soil incubated with CNTs during 60 days	SWCNTs (diameter <2nm) and MWCNTs (diameter 10-20 nm)	0.2; 20 and 2,000 mg/kg	-	SWCNTs at high concentration reduced mineralization. MWCNTs at low concentration stimulated mineralization
Shrestha <i>et al.,</i> 2013	Sandy loam soil from a field site	Soils incubated with CNT suspension for 28 days	MWCNTs (diameter 30 to 50 nm, length 10 to 20 μm, purity > 95 %)	10; 100; 1,000 and 10,000 mg/kg	TEM, SEM, TGA	No effect on soil respiration, enzymatic activities and microbial community respiration at concentration lower than 10,000 mg/kg. At the highest treatment, abundance of some bacteria genera decreased.
Simon-Deckers <i>et</i> al., 2009	Cupriavididus metallidurans and Escherichia coli	Bacteria exposed to CNTs diluted in water at room temperature under gentle stirring during 24 hours	MWCNTs (specific surface area 42 m²/g, diameter 44 nm, length 1.5 μm)	10 and 100 mg/L	BET method, TEM CNT detection: TEM and STEM	CNTs accumulated on both bacterial strains.
Tong <i>et al.,</i> 2012	Drummer soil (fine- silty) from continous corn no-till plots and a Tracy soil (coarse- loamy) from agricultural fields	Soils treated with CNTs weekly during six weeks	SWCNTs non functionalized, SWCNTs functionalized with polyethyleneglycol and SWCNTs functionalized with m- polyaminobenzene	60; 300 and 6,000 mg/kg	-	Repeated application of SWCNTs can affect microbial activity and induce minor changes in soil metabolic activity. Functionalized CNTs seemed to be less toxic.
Zheng <i>et al.,</i> 2014	Paracoccus denitrificans	Bacteria incubated in mineral medium contaminated by CNTs for 24h	SWCNTs (average diameter of 1-2 nm, length of 0.5-2 μm)	10 and 50 mg/L	SEM, TEM	CNTs were present outside and inside the bacteria. The final nitrate concentration was higher with high concentration of CNTs. CNTs led to the transcriptional activation of the genes encoding ribonucleotide reductase in response to DNA damage and decreased the gene expressions genes involved in glucose metabolism and energy production

					CNT	
Reference	Species	Growth conditions	CNT used*	Concentrations	characterization	Effects
Calisi <i>et al.,</i> 2016	Earthworms (<i>Eisenia fetida</i>)	Earthworms incubated in soil contaminated by CNTs during 14 days	MWCNTs	30 and 300 mg/g	-	Immune cells morphometric alterations, lysosomal membrane destabilization, acetylcholinestrase inhibition and metallothionein tissue concentration changes are highly sensitive to MWCNTs.
Eom <i>et al.,</i> 2015	Eartworms (Caenorhabditis elegans)	Earthworms incubated on nematode growth medium with and without CNTs during 72h	MWCNTs	500 mg/L	-	Phagocytosis could be a potential mechanism of uptake of CNTs and oxidative stress a potential mechanism of toxicity.
Hu <i>et al.,</i> 2013	Earthworms (<i>Eisenia fetida</i>)	Earthworms grown on artificial soil contaminated with CNTs during 7 days	MWCNTs (diameter of 10 nm and length 9-20 μm, specific surface area of 500 m²/g)	100 and 1,000 mg/kg of dry soil	BET method, TEM	MWCNTs absorbed nonylphenol caused much more adverse effects to the earthworms than each chemical alone.
Hu <i>et al.,</i> 2014	Earthworms (<i>Eisenia fetida</i>)	Earthworms grown on artificial soil contaminated with CNTs during 14 days	MWCNTs (purity >99.5%, average inner diameter of 10 nm and length of 10 μm, specific surface area of 500 m²/g)	50; 500 and 1,000 mg/kg of dry soil	BET method, TEM, X- ray diffraction, Raman	No mortality was found in soil contaminated with CNTs even at the highest concentration. DNA damages were found in earthworms at relatively low concentration of CNTs in the medium.
Li <i>et al.,</i> 2013	Earthworms (<i>Eisenia fetida</i>)	Earthworms grown in sandy loam soil spiked with CNTs during 28 days	MWCNTs (diameter from 30 to 50nm, length from 10 to 20 μm, purity >95%)	3,000 mg/kg of soil	TEM	Low bioaccumulation factor of CNTs in earthworms.
Petersen, Huang and Weber, 2008	Earthworms (<i>Eisenia fetida</i>)	Soils from field sites were spiked with CNTs, earthworms were added and stayed for 14 days	¹⁴ C-MWCNTs (diameter from 30 to 70 nm, purity > 99%), ¹⁴ C- SWCNTs (diameter from 1 to 2 nm, purity > 91 %)	30 and 300 mg/kg	TEM, TGA Raman	Adsorption of CNTs on the tissues of earthworms was minimal.

Table 2 Studies on CNT impacts and behavior on soil macroorganisms

Petersen <i>et al.,</i> 2009	Earthworms (Eisenia fetida)	Earthworms grown in soil spiked with CNTs and pyrene for 28 days	MWCNTs (purity 99%, diameter 30-70 nm), SWCNTs (purity 91%, diameter 1-2 nm)	30 and 300 mg/kg	TGA, Raman, TEM	Both CNTs at the highest concentration decreased pyrene bioaccumulation. Presence of CNTs enhanced pyrene elimination rates.
Petersen <i>et al.,</i> 2011	Earthworms (<i>Eisenia veneta</i>)	Earthworms cultured three types of soils (organic carbon fractions 5.7%, 1.6% and 3.9%) contaminated by CNTs during 28 days	MW ¹⁴ CNTs (diameter between 30 and 70 nm)	500 mg/kg of dry soil	TEM, SEM, TGA, electrophoretic mobilities (Malvern Zetasizer Nano ZS)	Limited absorption of CNTs into organism tissues. Earthworms can easily eliminate accumulated CNTs.
Scott-Fordsmand <i>et al.,</i> 2008	Earthworms (<i>Eisenia veneta</i>)	Earthworms in loamy sand soil were fed with foods contaminated by CNTs during 21 days	DWCNTs (diameter of 10-30 nm, length of 5- 15 μm, specific surface area of 1,255,637 nm ² , purity of 99.5 %)	50; 100; 300 and 495 mg/kg of dry food	-	Reproduction of the studied earthworms was affected by CNTs. The most sensitive toxicological parameter was reproduction (cocoon production), with no effect on hatchability, survival or mortality.
Zhang <i>et al.,</i> 2014	Earthworms (<i>Eisenia fetida</i>)	Earthworms on artificial soil contaminated by CNTs during 14 days	MWCNTs (purity > 95%, average length of 10 μm, specific surface area of 500 m²/g)	1,000 mg/kg of dry soil	TGA, TEM, BET method	CNTs induced slight toxicity compared to sodium pentachlorophenate. Expression of enzymatic biomarkers was different with PCP-Na and CNTs at the same time than PCP-Na or CNTs alone.

1.7. Fate and impacts of carbon nanotubes on plants

CNTs can penetrate into the seeds of cabbage (Brassica oleacera) (Martínez-Ballesta et al., 2016), rice (Oryza sativa) (Hao et al., 2016), tomato (Solanum lycopersicum cv Micro-Tom) (Khodakovskaya et al., 2009; Lahiani et al., 2016), barley (Hordeum vulgare hybrid Robust), soybean (Glycine max hybrid S42-T4) (Lahiani et al., 2013) and maize (Zea mays hybrid N79Z 300GT) in hydroponic conditions (Tiwari et al., 2014) (table 3). Functionalized CNTs penetrated directly into the cells, not entering by phagocytosis mechanism (Khodakovskaya et al., 2013; G. Chen et al., 2015; Mohamed H Lahiani et al., 2016). When contamination occurred through root exposure, both functionalized and non-functionalized CNTs have been reported to penetrate (figure 5) (C. Lin et al., 2009; Miralles et al., 2012; Zhang et al., 2014). Then, CNTs are translocated to the upper part of plants by sharing the vascular system with water and nutrients and they can be transported via transpiration (figure 5). CNTs are most of the time detected in stems, shoots, leaves and fruits of the plants although in low concentration (Begum et al., 2012a; Larue et al., 2012; Smirnova et al., 2012; De La Torre-Roche et al., 2013; Zhai et al., 2015a; Cano et al., 2016; Hao et al., 2016). Larue et al. (Larue et al., 2012) established that less than 0.05‰ of the applied MWCNT dose was translocated to the leaves of wheat (Triticum aestivum) and rapeseed (Brassica napus) using ¹⁴C labeled MWCNTs. CNT seem to penetrate plant roots by osmotic pressure, capillarity forces, cell pores or symplastically (figure 5) (Lin et al., 2009; Miralles et al., 2012; Zhang et al., 2014). Lin et al. (Lin et al., 2009) studied the intergenerational transfer of carbon nanomaterials (carbon nanoparticles C₇₀ and MWCNTs 40-70 nm diameter) in rice (Oryza sativa L. ssp. japonica, cv Taipei 309). They concluded that carbon nanomaterials can pass to the progeny through seeds. Khodakovskaya et al. (Khodakovskaya et al., 2011) found that CNTs could penetrate in chloroplasts through the lipid bilayer by lipid exchange. Serag et al. (Serag et al., 2011) proposed that MWCNTs can be taken up in plant protoplasts by endosome-escaping (figure 5). Moreover, short MWCNTs (<100 nm) were targeted to specific cellular sub-structures such as nucleus, plastids and vacuoles. Serag et al. (Serag et al., 2013) also reviewed that CNTs can penetrate plant cell walls, target specific organelles, probe protein-carrier activity and induce organelle recycling in plant cells. According to the different studies that were identified, functionalized CNTs seem to enter more easily in plants compared to non-functionalized CNTs. It is important to precise that most of the studies on plants have been conducted in hydroponics conditions or only at the seed stage. Soil studies, more representative of environmental conditions, are a negligible part of the literature. However, no differences were found between CNT impacts in hydroponics or in soil.



Figure 5 Uptake and distribution of carbon nanotubes in plants. CNTs have been enlarged for better visibility. In the cell, in light blue: vacuole, in green: chloroplasts, in purple: nucleus with the grainy endoplasmic reticulum, in orange: smooth endoplasmic reticulum, and in dark blue the plasmode (for colors: referred to the online version).

- 1. CNTs can enter plant roots through osmostic pressures, capillary forces, pores on cell walls, intercellular plasmadesmata or through direct penetration by root hair or through the primary root cell wall.
 - 2. CNTs can traverse through both cell wall and cell membrane through endocytosis.
- 3. CNTs may share the vascular system with water and nutrients and may be transported via transpiration via the upper part of plants.
- 4. CNTs are found in the upper part of plants. In leaves, they have been seen accumulated in the leaf xylem and in the edge of leaves.
- 5. In cells, CNTs were mainly detected in cytoplasm, cell wall, cell membrane, chloroplast, mitochondria and plasmodes.

Concerning impacts of CNTs on plants (table 3): CNTs increased seed germination on a large range of concentrations (*i.e*: 40; 50; 100 and 500 mg/L) (Khodakovskaya *et al.*, 2009; Oleszczuk, Jośko and Xing, 2011; Lahiani *et al.*, 2015a; Ratnikova *et al.*, 2015; Mohamed H Lahiani *et al.*, 2016). They can increase plant growth with a higher biomass production, higher flower production, or enhanced root elongation (Tripathi *et al.*, 2011; Villagarcia *et al.*, 2012; Khodakovskaya *et al.*, 2013b; Zhang *et al.*, 2015; Taha *et al.*, 2016; Oleszczuk, Jośko and Xing, 2011; Miralles *et al.*, 2012; Smirnova *et al.*, 2012; Tiwari *et al.*, 2014; Lahiani *et al.*, 2015a; Martínez-Ballesta *et al.*, 2016; Lahiani *et al.*, 2016). At the cellular level, CNTs were found to increase cell growth: MWCNTs enhanced growth of tobacco cell culture over a wide range of concentrations (0.005-0.5 mg/mL) (Alimohammadi *et al.*, 2011a; Lahiani *et al.*, 2016).

On the other hand, in some studies CNTs were found to decrease plant growth: MWCNTs induced growth reduction and toxicity related to an increased generation of reactive oxygen species (ROS) in spinach at high concentration (125-1,000 mg/L). They also caused necrotic lesions of leaf cells/tissues and changes of root and leaf morphology (Begum and Fugetsu, 2012b). MWCNTs (10 mg/L) decreased cell dry weight, viability, chlorophyll content and superoxide dismutase (SOD) activity of *Arabidopsis thaliana* cell suspension (Lin *et al.*, 2009). SWCNTs had adverse effects on protoplasts and leaves through oxidative stress, leading to a certain amount of programmed cell death in *Arabidopsis thaliana* (Shen *et al.*, 2010).

CNTs can also have no observed effect on plants, as reported in numerous studies. For example, Hamdi *et al.* (Hamdi *et al.*, 2015) found no effect of MWCNTs functionalized and non-functionalized on seed germination of lettuce (*Lactuca sativa*). Lin and Xing (Lin and Xing, 2007) evidenced no effect of MWCNTs on seed germination and root length of several plants (radish, rape, ryegrass, lettuce, corn, and cucumber) like Larue *et al.* (Larue *et al.*, 2012) in wheat and rapeseed.

Looking at the gene level, CNTs seem to up-regulate genes involved in cell division/cell wall formation in tomato plants (Khodakovskaya *et al.*, 2012). SWCNTs promoted rice root growth through the regulation of expression of the root growth related genes (*Nt*LRX1 and CyCB) (Yan *et al.*, 2016). MWCNTs were also observed stimulating the expression of water channel genes (aquaporins) (Villagarcia *et al.*, 2012; Lahiani *et al.*, 2013; Lahiani *et al.*, 2016). Aquaporins are central components in water-plant relationships, as they are essential for root water uptake, seed germination, cell elongation, reproduction and photosynthesis (Khodakovskaya *et al.*, 2012). The overexpression of aquaporin genes can contribute to cell growth leading to overall plant growth. They also up-regulate genes involved in response to pathogens meaning that CNTs could be sensed by plants as a stress similar to herbivore attack (Khodakovskaya *et al.*, 2011). Other authors also found that CNTs provoke repression of pathogen-activated genes and salicylic acid-mediated pathways in *Arabidopsis thaliana* (García-Sánchez *et al.*, 2015). Same authors demonstrated that there is a greater similarity in the plant response to nanoparticles of different chemical nature, than there is with other environmental stress (salinity, biotic stress...).

CNT impacts on plants can be different according to the types of CNT used (functionalized or not, number of walls) as shown in figure 6.



Figure 6 Root length of wheat (*Triticum aestivum*) exposed in hydroponics to different types of CNTs (DWCNTs non-functionalized (DWCNTs nf), DWCNTs functionalized (DWCNTs f) and MWCNTs (Larue *et al.* unpublished data). Stars indicate significant difference (p<0.05).

Phytotoxicity varied between CNTs non-covalently functionalized with poly-3-aminobenzenesulfonic acid (PABS) and non-functionalized CNTs (Cañas *et al.*, 2008); the first ones affecting more root length than the second ones. In another study, non-functionalized CNTs inhibited root elongation in tomato but enhanced it in onion and cucumber while functionalized CNTs inhibited root elongation in lettuce (Cañas *et al.*, 2008). Toxicity of MWCNTs can increase sharply as the diameter of the agglomerates decreased (Lin *et al.*, 2009), suggesting that a better dispersion could enhance the toxicity. Functionalized CNTs are usually better dispersed but the literature does not always describe them as more toxic, this point is still in debate. Moreover, functionalisation of CNTs induced strong treatments which are reducing the CNT length. It is thus difficult to determine if functionalisation or CNT matters most for the toxicity. Serag *et al.* (Serag *et al.*, 2011) reports that MWCNTs larger than 200 nm accumulated in subcellular organelles while shortest ones (30-100 nm) were found into vacuoles, nucleus and plastids. However, it is the only paper that report this difference between short and long MWCNTs. It is not possible to compare the effect of CNT length in between different papers because experiment conditions and CNTs were different.

Controversial effects of CNTs have been evidenced in plants. It is important to standardize evaluation methods to better understand the results and to allow a better comparison between studies.

Reference	Plant used	Culture conditions	CNT used*	Concentrations	Detection and characterization techniques	Effects
Alimohammadi <i>et</i> <i>al.,</i> 2011	Tomato (Solanum lycopersicum)	Seeds germinated on agar medium with and without CNTs for 2 months	SWCNTs functionalized with QDs and non- functionalized	50 mg/L	CNT characterization: UV- <i>vis</i> -NIR, TEM CNT detection: Raman, UV light radiation	Addition of QDs to CNTs dramatically changed the biological variability by accelerating leaf senescence and inhibiting root formation. CNTs only induced "positive" effects (increase of the chlorophyll content and total weight of the root system).
Martínez-Ballesta <i>et</i> <i>al.,</i> 2016	Cabbage (Brassica oleacera)	One-week-old germinated seed grown in medium with and without CNTs and with and without NaCl	MWCNTs (diameter 6-9 nm, length 5 μm, purity 95%)	10; 20; 40 and 60 mg/L	CNT characterization: TEM	CNTs entered in cells with higher accumulation under salt stress. CNTs had positive effect on growth in NaCl- treated plants. CNTs induced changed in the lipid composition, rigidity and permeability of the root plasma membranes relative to salt stressed plants. CNTs enhanced aquaporin transduction.
Begum and Fugetsu, 2012a	Spinach (Amaranthus tricolor)	Seeds immersed in CNTs suspension for one night and placed in filter paper until germination, then transferred to plastic pots for hydroponic culture with and without CNTs for 15 days	MWCNTs (diameter around 11 nm, length < 1 μm)	125; 250; 500 and 1,000 mg/L	CNT characterization: AFM, SEM, TEM CNT detection: Raman, SEM, TEM	CNTs induced growth reduction and toxicity due to the ROS. CNTs caused necrotic lesions of leaf cells/tissues and changed of root and leaf morphology. CNTs were found in leaves.
Begum <i>et al.,</i> 2012b	Lettuce (Lactuca sativa), rice (Oryza sativa), cucumber (Cucumis sativus), red spinach (Amaranthus tricolor),	Seedlings transferred in medium with and without CNTs and growth for 15 days	MWCNTs (diameter around 13 nm, length around 1 μm)	20; 200; 1,000 and 2,000 mg/L	CNT characterization: SEM, TEM	CNTs reduced root and shoot length. CNTs increased cell death and electrolyte leakage. Very little or no toxic effects were found for chili, lady's finger and soybean. Red spinach and lettuce were more sensitive to CNTs.

Table 3 Studies	on CNT	impacts	and	behavior	on	plants

	lady's finger (Abelmoschus esculentus), chili (Capsicum anuum), soybean (Glycine max)					
Cañas <i>et al.,</i> 2008	Cabbage (Brassica oleracea), carrot (Daucus carota), cucumber (Cucumis sativus), onion (Allium cepal), tomato (Lycopersicon esculentum) and lettuce (Lactuca sativa)	Seeds exposed to CNTs during 24 and 48h	SWCNTs functionalized and non-functionalized (diameter 8 nm, length of few microns)	28; 160; 900 and 5,000 mg/L	CNT characterization: SEM	CNTs and fCNTs inhibit root elongation of four crop species (cucumber, inion, lettuce and tomato). Phytotoxicity varied between CNTs and fCNTs, with CNTs affecting more species. Tomato was the most sensitive species. Microscopy images showed the presence of NTCs on the root surface.
Cano <i>et al.,</i> 2016	Corn (Zea mays)	Germinated seeds cultivated in soil with and without CNTs for 40 days	OH-functionalized SWCNTs, COOH- functionalized SWCNTs and non- functionalized SWCNTs (diameter 1-4 nm, length 5- 30 μm, purity >90% wt%)	10 and 100 mg/kg (wt/dry wt)	CNT characterization: TEM, microwave induced heating method	CNTs accumulated mostly in roots, with minimal accumulation in stems and leaves.
De La Torre-Roche, <i>et al.,</i> 2013	Zucchini (Cucurbita pepo), tomato (Solanum lycopersicum), soybean (Glycine max), corn (Zea mays)	3 to 7 day-old seedlings (depending of the species) grown in soil contaminated with CNTs and pesticides during 28 days	MWCNTs (95 % purity, diameter 13-18 nm, length 10-30 μm)	500; 1,000 and 5,000 mg/kg	CNT characterization: GC- MS and GS-ECD	CNTs suppressed in a dose-dependent fashion the bioaccumulation of weathered chlordane and DDx. CNTs were found in root and shoot tissues.

Deng <i>et al.,</i> 2017	Cabbage (Brassica oleracea)	Seedlings were grown in nutrient solution or in soil with carbamazepine and CNTs	Pristine CNTs and carboxyl- functionalized CNTs (purity 95%, diameter <8 nm, length 10-30 μm, specific surface area 500 m²/g)	50 mg/L (hydroponic experiments), 50 mg/kg (soil experiments)	-	Biomass enhancement was observed on plants grown with CNTs. Co-exposure with CNTs suppressed carbamazepine accumulation. Functionalized CNTs enhanced carbamazepine translocation potential.
Hamdi <i>et al.,</i> 2015	Lettuce (<i>Lactuca sativa</i>)	Seeds germinated on medium contaminated with and without CNTs, with and without humic acid, after 10 days pesticides were added, growth for 19 days in total	MWCNTs functionalized (diameter <8 nm, length 10-30 μm, purity 95%), MWCNTs non- functionalized (diameter 13-18 nm, length 3-30 μm, purity > 99%)	1,000 mg/L	-	CNTs did not influence seed germination. CNT presence and type significantly influenced pesticide availability.
Hao <i>et al.,</i> 2016	Rice (Oryza sativa)	5-day-old seedlings tranplanted in tubes with nutrient solution with CNTs during 15 days	Hollow MWCNTs, Fe-filled CNTs, Fe- Co-filled CNTs (typical diameters of dozens of nm)	0; 10; 50 and 300 mg/L	CNT characterization: TEM, EDS	The three types of CNTs had toxic effects on rice seedlings, and inhibited the growth and development of roots and shoots. The C:N ratio in rice roots significantly increased after treatments with CNTs, and all three types of CNTs had the same effect. CNTs penetrate cell wall and cell membrane, they could be transported to shoots.
Hatami, Hadian and Ghorbanpour, 2017	Henbane (Hyoscyamus niger)	Seeds exposed to different concentrations of CNTs during 14 days under drought stress	SWCNTs (outer and inner diameter of 1-3 and 0.9-2 nm and length of 5-30 µm)	50–800 mg/L	CNT characterization: TEM, SEM, Raman, TGA, BET and X-ray diffraction	SWCNTs at low concentrations induced tolerance in seedlings against low to moderate level of drought by enhancing water uptake and activating plant defense system.
Khodakovskaya <i>et</i> <i>al.,</i> 2009	Tomato (Solanum lycopersicum)	Seeds placed on MS medium without or with CNTs for 3, 12 and 20 days	MWCNTs (purity higher than 98%)	10; 20 and 40 mg/L	CNT characterization: SEM, TEM, TGA, Raman CNT detection: TEM	MWCNTs can penetrate thick seed coat and support water uptake inside cells. Positive effects of MWCNTs on seed germination.

Khodakovskaya <i>et</i> <i>al.,</i> 2011	Tomato (Solanum lycopersicum)	Seeds exposed to CNTs during 10 days	MWCNTs functionalized	50 mg/L	CNT characterization: TEM CNT detection: microarray analysis, real time QPCR, integrated PA/PT scanning cytometry, Raman	MWCNTs induce previously unknown changes in gene expression in tomato leaves and roots, particularly, up-regulation of the stress- related genes. Detection of MWCNTs in roots, leaves, and fruits down to the single nanoparticle and cell level.
Khodakovskaya <i>et</i> <i>al.,</i> 2012	Tobacco cells (Nicotiana tabacum)	Cells grown on MS medium without and with CNTs for 30 days	MWCNTs (diameter 20 nm, length from 500 nm to 1 μm)	0.1; 5; 100 and 500 mg/L	CNT characterization: TEM and Raman	Enhance the growth of tobacco cell culture in a wide range of concentrations (5-500 µg/mL). Correlation between the activation of cell growth exposed to MWCNTs and the upregulation of genes involved in cell division/cell wall formation and water transport.
Khodakovskaya <i>et</i> <i>al.,</i> 2013a	Tomato (Solanum lycopersicum)	Plants grown in soil supplemented with CNTs during 10 days	MWCNTs (diameter 25 nm, length of few microns)	50 and 200 mg/L	CNT characterization: TEM CNT detection: TEM, Raman	Plants grown in soil supplemented with CNTs produce two times more flowers and fruits compared to plants grown in control soil.
Lahiani <i>et al.,</i> 2013	Barley hybrid Robust (<i>Hordeum</i> <i>vulgare</i>), corn hybrid N79Z 300GT (<i>Zea</i> <i>mays</i>) and soybean hybrid S42-T4 (<i>Glycine max</i>)	CNTs deposited on seed surface by airspray techniques or added in growth medium of seeds, 10 days of exposure	MWCNTs functionalized (diameter from 15 to 40 nm, length of several μm)	50; 100 and 200 mg/L	CNT characterization: TEM and Raman	MWCNTs for both deposit technics penetrate seed coats of all tested species and activate germination of MWCNT-exposed seeds. Application of CNTs to the seeds of the three studied species can stimulate expression of water channel genes (aquaporin).
Lahiani <i>et al.,</i> 2015	Soybean hybrid S42- T4 (<i>Glycine maxl</i>), barley hybrid Robust (<i>Hordeum vulgare</i>), corn hybrid N79Z 300GT (<i>Zea mays</i>), tomato (<i>Solanum</i> <i>lycopersicum</i>), switch grass (<i>Panicum virgatum</i>), rice (<i>Oryza sativa</i>), tobacco cell culture (<i>Nicotiana tabacum</i>)	Seeds germinated on medium contaminated with and without CNTs for 10 days (corn), 11 days (barley and soybean), 12 days (rice) and 20 days (tomato and switch grass)	SWCNHs (nanohorns)	25; 50 and 100 mg/L	CNT characterisation: SEM, TEM, TGA, Raman CNT detection: TEM, microwave induced heating technique	CNHs activated seed germination and enhanced growth of different organs of corn, tomato, rice and soybean. CNHs increased growth of tobacco cells. CNHs were found inside cells. CNHs affected expression of a number of tomato genes involved in stress responses, cellular responses and metabolic processes.

Mohamed H Lahiani <i>et al.,</i> 2016	Tomato (Solanum lycopersicum), tobacco callus cells (Nicotiana tabacum)	Callus cells exposed to growth medium with and without CNTs, seeds grown in medium without and with CNTs	COOH- functionalized MWCNTs (diameter 13-18 nm, length 1-12 μm), COOH- functionalized MWCNTs (diameter < 7 nm, length 0.5-2 μm), helical MWCNTs (diameter 100-200 nm, length 1-10 μm)	50 and 100 mg/L	CNT characterization: TEM, Raman	CNTs activated cell growth, germination and plant growth. CNTs were found inside seeds. Helical CNTs affected a number of genes involved in cellular and metabolic processes and response to stress factors. CNTs upregulated expression of the tomato water channel gene.
Larue <i>et al.,</i> 2012	Wheat (Triticum aestivum), rapeseed (Brassica napus)	15-day-old seeds in CNTs suspension for 7 days	MWCNTs (diameter 41.2 nm, specific area 42 ± 2 m²/g)	100 mg/L dispersed with arabic gum or humic acid	CNT characterization: TEM CNT detection: TEM, Raman	Less than 0.005 ‰ of the applied CNT dose was taken up by plant roots and translocated to the leaves. This accumulation does not impact plant development and physiology. It does not induce any modification in photosynthetic activity or cause oxidative stress in plant leaves.
Lin and Xing, 2007	Rape (Brassica napus), radish (Raphanus sativus), ryegrass (Lolium perenne), lettuce (Lactuca sativa), corn (Zea mays) and cucumber (Cucumis sativus)	Seeds exposed to CNTs during 5 days	MWCNTs (diameter 10-20 nm, length 1-2 μm, purity > 95%, surface area 126 m²/g)	20; 200; 2,000 mg/L	CNT characterization: BET	CNTs did not impact seed germination and root length.
Lin <i>et al.,</i> 2009	Thale cress T87 suspension cells (Arabidopsis thaliana)	72-hour-old cell cultivation exposed to CNTs in the cell suspension Cells exposed to CNTs for 2, 3, 4, 5, 6 and 7 days	MWCNTs (average diameter 9.5 nm, average length 1.5 μm, surface area 250-300 m²/g)	10; 60; 100 and 600 mg/L	CNT characterization: TEM, BET, ICP-MS CNT detection: TEM	CNTs decreased cell dry weights, cell viabilities, cell chlorophyll contents and superoxide dismutase activities. Toxicity of CNTs increased sharply as the diameters of the agglomerates of MWCNTs become smaller.

Martínez-Ballesta <i>et</i> <i>al.,</i> 2016	Cabbage (Brassica oleacera)	5-day-old seeds placed in containers with continously-aerated Hoagland nutrient, exposure for 7 days	MWCNTs (diameter between 6 and 9 nm, length of 0.1 to 0.5 μm)	Exp 1 : 10; 20; 40 and 60 mg/L Exp 2 : 10 mg/L with NaCl	CNT characterization: TEM	"Positive" effect on the growth under both saline and non-saline conditions. Increase Na concentrations in roots of Na-Cl treated plants.
Miralles <i>et al.,</i> 2012	Alfalfa (Medicago sativa), wheat (Triticum aestivum)	Seeds cultivated in medium contaminated by CNTs during 6 days	MWCNTs functionalized with Fe3O4 (67.2% purity, 10.9 ± 1.9 nm, 116.1 m²/g)	40; 80; 160; 320; 640; 1,280; 2,560 mg/L	CNT characterization: TGA, TEM, Raman, N2 adsorption/desorption isotherms CNT detection: TEM, Raman	CNTs did not impact germination of both species. CNTs enhanced root elongation. CNTs were absorbed onto the root surfaces without significant uptake or translocation.
Moll <i>et al.,</i> 2016	Red clover (Trifolium pratense)	5-day-old seeds transferred in agricultural soil (brown earth with a sandy loamy to loamy fine fraction) with and without CNTs and growth during 14 weeks	MWCNTs (diameter 20-30 nm, length 10-30 μm, purity >95%)	10; 100 and 1,000 mg/kg	-	CNTs did not affect plant biomass and arbuscular mycorrhizal fungi root colonization. CNTs decreased the number of flowers. CNTs increased nitrogen fixation.
Mondal <i>et al.,</i> 2011	Mustard (<i>Brassica juncea</i>)	Seeds germinated on petri dishes with and without CNT suspension until complete germination (radicle attained a length of 1 mm)	MWCNTs (purity >60%, diameter around 30 nm)	2.3; 6.9; 23 and 46 mg/mL	CNT characterization: SEM, FTIR, X-ray diffraction CNT detection: FTIR, SEM	CNTs increased moisture content of seeds and enhance water absorption machinery of root tissues. CNTs can be transported through the plant vascular cylinder.
Oleszczuk, Jośko and Xing, 2011	Cress (Lepidum sativum), sorgo (Sorghum saccharatum), tomato (Solanum lycopersicon), radish (Raphanus sativus), cucumber (Cucumis sativus)	Seeds germinated on four different sewage sludges spiked with CNTs with a storage during 7 and 31 days for aging	MWCNTs (diameter <10 nm, surface area 357 m ² /g, purity >95%), MWCNTs (diameter 40-60 nm, surface area 73 m ² /g, purity >95%)	0.1; 1 and 5 g/kg	-	CNT influence on sludge toxicity varied with respect to CNTs' outer diameter, type of sewage sludge and plants tested. CNTs had positive effects on seed germination and root growth of two sewage sludge.

Park and Ahn, 2016	Carrot (Daucus carota)	Seeds exposed to CNTs during 5 days in petri- dishes with and without CNTs and AgNPs during 5 days	MWCNTs (median diameter 6.6 nm, length of 5 μm)	10; 100; 200; 500; 1,000 and 2,000 mg/L	-	CNTs did not significantly affect seed germination and seedling growth. CNTs decreased H ₂ O ₂ levels. CNTs reduced levels of a seed protein, DcHsp17.7, during seed germination and increased chlorophyll content.
Ratnikova <i>et al.,</i> 2015	Tomato (Solanum lycopersicum)	Seeds sonicated in suspension with and without CNTs, and seeds germinated in petri dishes until germination (until root (radicle) is visible)	CBNMs (hydrophilic fullerols and hydrophobic MWNTs)	50 mg/L with gallic acid	CNT characterization: TEM, SEM and microRaman	CNTs did not penetrate seed coat. CNTs enhanced germination and seedling length and weight.
Serag <i>et al.,</i> 2011	Periwinkle (Caranthus roseus)	Perwinkle cell suspension culture incubated with CNT suspension during 3h at 25°C or 4°C	MWCNTs (purity 95%, average outer diameter 20- 30 nm, length 0.5- 2 μm)	10; 20; 40; 60 and 80 mg/L	CNT detection: TEM, confocal microscopy imaging	CNTs are entering passively through the cell membrane and it's not associated with the endosomal route. Isolated CNTs were observed inside cells as a result of a direct penetration of the plasma membrane. No CNTs found in any organelles associated with endocytosis cycle. CNT distribution followed a size distribution of short CNTs (30-100 nm) inside organelles, while long CNTs (>200 nm) were found inside subcellular structures
Serag, Kaji, Tokeshi and Baba, 2012	Thale cress (Arabidopsis thaliana)	Incubation of Thale cress cells with suspension of CNTs	Cup-staked CNTs (average outer diameter 60-100 nm, length 1-100 µm)	-	CNT detection: confocal microscopy imaging, AFM	CNTs participate in cell biochemical reactions. CNTs were detected into the structure of tracheid and showed such mutual and parallel arrangement with a lignin polymer.
Serag, Kaji, Tokeshi, Bianco, <i>et al.</i> , 2012	Thale cress (Arabidopsis thaliana)	Incubation of Thale cress cells with CNTs for 3h	Cellulase- immobilized cup- staked CNTs	-	CNT characterization: AFM CNT detection: AFM, epifluorescence microscopy	Cellulase-imobilized CNTs penetrated the thick cellulosic cell wall and they are transported into the cell

Song <i>et al.,</i> 2017	Mung bean (Phaseolus radiatus) Radish (Raphanus sativus)	Seeds germinated on sediments (organic carbon content 1.58%, 47.6 % of clay, 28.87 % of silt and 23.53% of sand) spike with CNTs, phenanthrene and cadmium during 72h	Three MWCNTs (purity>95%) with different outer diameter (10–20 nm, 30–50 nm, >50 98 nm) and specific surface area of respectively 134 m ² g ⁻¹ , 103 m ² 206 g ⁻¹ , and 70.1 m ² g ⁻¹	0.5%, 1.0%, or 1.5% (w/w)	CNT characterization: SEM, FTIR, and BET method	MWCNTs showed a better adsorption performance with phenanthrene and cadmium (II) compared with sediments. MWCNTs did not inhibit significantly the germination but root growth was more sensitive than biomass production to the changes of contaminant concentration.
Shen <i>et al.,</i> 2010	Thale cress (Arabidopsis thaliana), rice (Oryza sativa)	Protoplasts cultured in CNTs Injection of CNTs into intact leaves	SWCNTs (diameter 1-2 nm, length 5- 30 μm, purity 90%)	5; 25; 100 and 250 mg/L	CNT characterization: Fluorescence, TEM	CNTs had adverse effects on protoplasts and leaves through oxidative stress, leading to a certain amount of programmed cell death.
Smirnova <i>et al.,</i> 2012	Sainfoin (Onobrychis arenaria)	Seeds germinated on petri-dishes with and without Taunit suspension (CNTs) for 10 days	Taunit suspension: loose black powder composed of grainy agglomerates containing MWCNTs (diameter 5-10 nm, length of at least 2 µm, purity 98%)	100 and 1,000 mg/L	CNT characterization: TEM and light electron microscopy	CNTs stimulated the growth of roots and stems, and enhanced the peroxidase activity in these part of plants. CNTs were found in leaves and stems tissues.
Stampoulis, Sinha and White, 2009	Zucchini (Cucurbita pepo)	Seeds exposed to CNTs for 5 and 12 days 4-day-old seeds in CNT suspension during 15 days	MWCNTs (purity >99%, number of walls from 3 to 15)	1000 mg/L	-	CNTs did not impact seed germination and root length. CNTs reduced biomass of plants of the 15 day hydroponic trial.

Taha <i>et al.,</i> 2016	Date palm (Phoenix dactylifera)	8-month-old callus cells subcultured four times with 6 weeks intervals on CNT media	MWCNTs (diameter 11-170 nm, length 5-9 μm)	0.05; 0.1 mg/L	CNT characterization: TEM	Low concentrations of CNTs promoted callus fresh weight, increased number of germinated embryos, shoot length and leaf number and enhanced root number, root length, plantlet length and hairy roots. CNTs can penetrate plant tissues and enter its cells. CNTs can facilitate the adsorption or transportation of nutrients into plant tissues.
Tan and Fugetsu, 2007	Rice (Oryza sativa L.)	Callus cells of three- month-old plants transferred to a cell suspension culture, after 10 days add of CNTs for 4 days	MWCNTs (diameter 20-40 nm, length 0.5-50 μm, surface area 3.14 x 10 ⁻² - 6.28 μm ²)	0.05 and 0.1 g/L	CNT characterization: SEM	CNTs decreased cell density, possibly indicating a self-defense response. CNTs interacted directly with rice cells and may had a detrimental effect on rice growth.
Tan, Lin and Fugetsu, 2009	Rice (Oryza sativa L.)	6-day-old cell culture exposed to CNTs during 6 days	MWCNTs (diameter 10-30 nm, length 5-15 μm, surface area 86 m²/g, purity 95%)	20 mg/L	CNT detection: TEM	CNTs increased ROS content and decreased cell viability. Individual tubes found in contact with cell walls.
Tiwari <i>et al.,</i> 2014	Corn (Zea mays)	Seeds germinated on medium with and without CNTs for 7 days	MWCNTs (diameter 6-9 nm, length 5 μm, purity >95%)	20 mg/L	CNT characterization: SEM	CNTs enhanced germinative growth at low concentration but depressed it at higher concentration. CNTs improved water absorption, plant biomass and concentration of the essential Ca, Fe nutrients. CNTs perforated the black-layer seed-coat while in presence of FeCl ₂ /FeCl ₃ they didn't perforate
Tripathi, Sonkar and Sarkar, 2011	Common gram (Cicer arietinum)	One-day-old seeds exposed to CNTs	MWCNTs (diameter 10-30 nm)	6 mg/L	CNT characterization: EDX, TEM, Raman CNT detection: SEM, TEM, fluorescence	CNTs increased growth rate of roots, shoots and branching. CNTs enhanced water absorption

Villagarcia <i>et al.,</i> 2012	Tomato (Solanum lycopersicum)	Seeds cultivated in medium contaminated by CNTs during 28 days	MWCNTs (diameter 8-35 nm, several micrometers in length, 94 % purity), MWCNTs purified by HCl washing and sonification (98% purity), MWCNTs further oxidized and decorated with carboxylic groups, MWCNTs sonicated in acetone, MWCNTs coated with PEG	40 mg/L	CNT characterization: TEM, SEM, TGA, Raman, zeta potential	Highest increase in plant growth was observed for plants exposed to well disperse MWCNTs and MWCNTs functionalized with strong negative groups. Production of tomato water channel protein was activated in plants exposed to MWCNTs functionalized with various groups.
Zaytseva, Wang and Neumann, 2017	Soybean (Glycine max)	Seeds cultivated in petri dishes with filter paper contaminated by CNTs during 10 days; seeds cultivated in CNTs suspension during 36 hours	MWCNTs (purity above 98%, outer diameter of 20–70 nm, inner diameter of 5–10 nm and length of >2 μm)	1000 mg/L	-	MWCNTs induced oxidative stress in radicle tips which coincided with MWCNTs accumulation. MWCNTs reduced Zn translocation from the cotyledons to the seedlings. MWCNTs exhibited adsorption potential for Zn and Cu.
Zhao <i>et al.,</i> 2017	Thale cress (Arabidopsis thaliana), soybean (Glycine max), rice (Oryza sativa), maize (Zea mays)	Plants in hydroponic conditions with semisolid medium (MS basal medium with vitamins and sucrose) CNTs and/or SPAOMs during 25 days	14C MWCNTs (specific surface area of 111 m ² /g, specific radioactivity of 0.1 mCi/g, surface oxygen content of 8.6%, diameter 36.5 ± 12,7nm, length 350 nm)	0.45; 0.9; 2.25 and 4.5 mg/L	CNT characterization: X- ray photoelectron spectroscopy, thermal gravimetric, SEM CNT detection: liquid scintillation counting	Changes in biochemical parameters were much more sensitive than physiological parameters. CNTs could alleviate the toxicity of SPOAMs to <i>Arabidopsis</i> . Hydrodynamic diameter did not significantly affect CNTs uptake

Zhai <i>et al.,</i> 2015	Corn (Zea mays), soybean (Glycine max)	7-day-old germinated seeds were added in medium contaminated with and without CNTs during 18 days in hydroponic conditions	pristine-MWCNTs, amine (NH2)- functionalized MWCNTs, carboxylate (COOH)- functionalized MWCNTs (diameter 20-30 nm, length 0.05 - 2.0 μm)	10; 20 and 50 mg/L	CNT characterization: TEM	The three types of CNTs were directly taken up and translocated to roots, stems and leaves. CNTs accumulated in phloem and xylem cells within specific intracellular sites like the cytoplasm, cell wall, cell membrane, chloroplast and mitochondria. CNTs stimulated maize growth and inhibited soybean growth.
Zhang <i>et al.,</i> 2014	Rice (<i>Oryza sativa</i>)	Germination of seeds during 7 days in Hoagland medium with and without CNTs, then transplanted to the normal Hoagland medium	SWCNTs (diameter 1-2 nm, length around 30 μm), MWCNTs (diameter 20-40 nm, length 10-30 μm)	5; 20 mg/L	CNT characterization: TEM	SWCNTs located in the intercellular space while MWCNTs penetrated cell walls in roots. CNTs promoted rice root growth through the regulation of expression of the root growth related genes. CNTs caused a similar histone acetylation and methylation statuses across the local promoter region of the Cullin-RING ligases 1 (CRL1) gene and increased micrococcal nuclease accessibility of this region, which enhanced this gene expression.
Zhang <i>et al.,</i> 2017	Corn (<i>Zea mays</i>)	Plants cultivated in soil contaminated with CNTs, pyrene and methypyrene during 26 days	MWCNTs (inner and outer diameter of the MW ranged in 5-15 nm and 50- 80 nm, respectively, with the length ranging in 10-20 mm)	50 and 3000 mg/kg	-	Concentrations of both pyrene and 1-CH ₃ - pyrene decreased with increasing amendment level of MWCNTs, indicating an increasing suppression their bioaccumulation and translocation in plants.

1.8. Conclusion

CNTs represent a large group of carbon-based nanomaterials which can differ in many ways such as diameter, length, number of layers, impurities or surface modification. In the literature, a variety of different CNTs have been used, with different suspension media and various suspension protocols. Despite the large range of CNTs, general conclusions about behaviour and impacts of CNTs on the terrestrial ecosystem can be drawn from the reviewed studies. First, changes in surface properties or adsorption of other compounds (cocktail effect) determine CNT environmental behaviour. Indeed, non-functionalized CNTs are hydrophobic, and thus difficult to disperse, they agglomerate rapidly. Functionalization of CNTs makes them more hydrophilic. CNTs have strong adsorption properties, which can be used intentionally in remediation applications to remove pollutants but may also lead to the binding of compounds present in the environment such as natural organic matter or contaminants with a Trojan horse effect. In general, CNTs will remain in soil and will not reach aquifers. Soil macroorganisms, and earthworms in particular, have a low bioaccumulation of CNTs due to an efficient depuration system. In plants, CNTs seem to penetrate in both seeds and roots and are subsequently translocated into the upper part of plants to edible parts. Very low concentrations were found in plants.

CNT impacts on terrestrial ecosystem are divided in 3 categories. Some studies agreed that CNTs can increase plant growth and soil microbial activity but also the development of soil macroorganisms. Other studies reported opposite effects. Finally, a number of other studies concluded that CNTs had no influence. Obviously, CNT toxicity varied according to their intrinsic characteristics, the medium type and the dispersion method. CNTs could be perceived as an environmental stress. Organisms will react differently to defend themselves against this stress, for example by the overexpression of some genes. This could contribute to cell growth and in turn to organism growth. The impact that one could qualify as "positive" due to the growth increase may be a simple stress response to an environmental factor but further investigations in more environmentally relevant conditions would be needed to conclude. There is a gap between the high concentration range tested on organisms in the literature so far and the prediction of expected concentration of CNTs in soils. There is a lack of studies on CNT impacts and at realistic concentrations.

Detection of CNTs in carbonaceous matrices still constitutes one of the main challenges of this field of research. The development of quantitative techniques for accurate measurement of CNTs in biological and environmental samples will help a lot understanding the transfer of CNTs, their fate and impact in complex soil-based ecosystems.

There is also a lack of standardized methods, leading to controversial results on CNT impacts, making difficult the comparison and analysis of earlier works. It is important to make the connection between exposure conditions and effect, this will help to understand the controversial results.

Conclusion on toxicity and behaviour of CNTs is difficult to reach due to the different points highlighted earlier in the text. In toxicology, more studies are available and authors hypothesised that short CNTs

(30 – 100 nm) circulate more easily due to their size and are less toxic because they are eliminated easily. Longer CNTs were compared to asbestos. This category may be defined as the most toxic because CNTs can enter into organisms but they cannot be eliminated so they may have toxic effects (Jaurand *et al.*, 2009; Donaldson *et al.*, 2013). However, in ecotoxicology, this conclusion is not so obvious.

As the CNT production and uses are expected to be still increasing, their spreading into the environment will keep expanding. It is thus essential to better evaluate CNT behaviour and impacts on ecosystems. More studies are urgently needed to understand mechanistic pathways of penetration and biodistribution of CNTs in plants, microorganisms and macroorganisms in order to allow, if possible, a safe use of CNTs. It is also essential to assess the influence of physico-chemical parameters of CNTs on their impacts. Knowing the effects of these parameters will allow creating CNTs "safer by design".

2. LITERATURE UPDATE

Since the review publication (end of 2017), a focus has been made in the literature about the use of CNTs in agriculture. According to PubMed database, publications about CNTs and agriculture increased these last few years (57 in 2016, 79 in 2017, 84 in 2018 and already 39 for 2019 at the time of writing this manuscript). Indeed, since the discovery of CNT "positive" effects on plant growth, many researchers are interested in the use of CNTs as growth regulators.

Several articles have confirmed the "positive effects" of CNTs on plants. For instance, Yuan et al. (2017) demonstrated that CNTs increased the number of nodules and enhanced the nitrogenase activity in a rhizobium legume system resulting in a better growth and development of the plants in comparison to the control. Lahiani et al. (2018) conducted a long-term exposure on agricultural crops (barley, soybean and corn) in hydroponics conditions. They highlighted no toxic effect on plant development and an enhancement of photosynthesis. Joshi et al. (2018) looked at the impacts of CNTs on wheat by priming the seeds. Only "positive" effects were reported such as enhancement in grain number, biomass, stomatal density, xylem-phloem size and water uptake. Finally, McGehee et al. (2017) reported that CNTs can increase fruit production of tomato plants exposed in hydroponics conditions.

Among these studies, some stated that CNTs can be found inside plants (Lahiani *et al.*, 2018) and even in the fruits of exposed plants (McGehee *et al.*, 2017). This highlights the potential risk for food safety and a transfer to consumers via the food chain. However, so far only few authors have evaluated the possible toxicity of plants containing CNTs on upper trophic levels. Lahiani et al. (2019) demonstrated that tomato fruits after plant exposure to CNTs did not affect human intestinal microbiota. Joshi et al. (2018) reported no toxic effect on intestinal human cell lines of wheat grains harvested from plants primed with CNTs. These studies strengthen the will to use CNTs in agriculture.

However, CNT impacts are still controversial. Indeed, several studies also reported phytotoxicity symptoms after CNT exposure. Hao et al. (2018) showed that CNTs decreased shoot height and root length of rice but also decreased root cortical cell diameter and increased antioxidant enzymes SOD and POD activities. Basiuk et al. (2019) reported phytotoxic effects of CNTs on cactaceae plants after 40 weeks of growth.

Despite the negative effects highlighted in their review, Verma et al. (2019) established that carbon based NM "*have a future in agricultural nanotechnology*". They concluded that it is important that agricultural scientists and field supervisors identify and implement the correct dose, application method and duration of the application in order to reap the benefits of the use of carbon based NMs.

The use of CNTs as growth regulator is not the only potential application foreseen in the field of agriculture/environment. Plant genetic engineering is an important tool in crop development and CNTs may be good candidate as a molecule carrier. CNTs have been used to deliver plasmid DNA to chloroplasts of different plant species without external biolistic or chemical aid (Kwak *et al.*, 2019).

The adsorption capacities of CNTs can also be used in order to remove contaminants such as organic compounds in wastewater treatment plants for instance (Sophia and Lima, 2018). CNTs also showed high removal capacities on pharmaceutical compounds such as carbamazepine, dorzolamide and ciprofloxacin (Álvarez-Torrellas *et al.*, 2017; Ncibi and Sillanpää, 2017).

The detection of CNTs in carbonaceous matrices has not evolved much since the review publication. A research group has worked on the development of a digestion method of plants to minimize matrix background signals that can interfere with the detection of CNTs. They showed that a nitric acid digestion in conjunction with Raman analysis is an effective approach for detecting CNTs in plants (Das et al., 2018). Using this method, they obtained a detection limit of 25 mg/g dry weight in lettuce spiked with CNTs. They were also able to detect CNTs in lettuce grown hydroponically with CNTs (5, 10 and 20 mg/L) during 18 days. They used the same digestion protocol to detect CNTs using programmed thermal analysis (Das et al., 2018b) allowing to reach a lower detection limit (0.065 mg/g of plant tissues). In their last study, they applied this same digestion protocol for UV-VIS spectroscopy analysis (Das et al., 2019), they obtained detection limits of 0.10-0.12 mg/g for leaves, 0.070-0.081 for stem and 0.019-0.180 for roots of lettuce plants. In the literature, special attention has been paid on microwave techniques to quantify CNTs in environmental matrices. He et al. (2019) worked on the development of a two-step technique using microwave induced heating. The first step consisted of a pre-treatment of the samples in order to disperse bundled CNTs within the sample. Then samples were analysed using microwave induced heating. This technique showed good results with soil spiked with CNTs. However, the mass of CNT to detect was high (between 0.1 and 0.6 mg of CNT per sample corresponding to 10 mg/g of soil), this method may be difficult to apply to environmental samples with low CNT concentration.

This literature update showed that limited progresses were made concerning CNT behaviour, impacts and detection in plants. In contrast, many studies looked at the use of CNTs in agriculture showing interesting results such as boost of plant development and "*no toxicity*" for fruits containing CNTs.

It is thus extremely important and urgent to identify behaviour and potential effects of CNTs before their massive and voluntary introduction into the environment.

3. THESIS OUTLINE

Overall, effects and behaviour of CNTs in plants are still not well understood and very controversial. The plant response can be influenced by several parameters that differ among studies (Figure 7).

Research question n°1: Is phytotoxicity related to CNT internalization in plant leaves?

Nowadays, CNT detection in plants is still a challenge but it has been demonstrated that CNTs can be internalized. To answer this answer question we analyzed several techniques to detect CNTs in plants using cucumber plants (*Cucumis sativus*) grown in hydroponics conditions as the experimental model. Results are detailed in Chapter 4.

Research question n°2: Do CNTs have differential impacts according to plant species?

In the literature, few plant screening experiments have been conducted. They tend to demonstrate that impacts of NMs may be dependent on plant species. Our research hypothesis is that plant family (dicots *vs* monocots) governs plant response. To investigate this hypothesis, we evaluated CNT impacts on 4 different plants: 3 dicots canola (*Brassica napus*), tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*) and 1 monocot maize (*Zea mays*) in chapter 6. This experiment allowed to determine the most sensitive species.

Research question n°3: How much CNT characteristics influence plant response?

Previous works carried out in the laboratory have highlighted that TiO₂-NP diameters influenced NM uptake and effects on plants. With this experiment, we aimed to check this hypothesis on CNTs. First, we investigated the impacts of two different NMs using TiO₂-NPs and CNTs on tomato plant (*Solanum lycopersicum*) response (chapter 5). For this purpose, we developed Fourier-Transformed InfraRed spectroscopy (FTIR). We expect that chemistry and shape of the NMs will lead to different biomacromolecule modifications.

Then we hypothesize that the physicochemical parameters of CNTs (*i.e.* diameter, length, functionalization, dispersion) influence their impacts on canola plants (*Brassica napus*). For this purpose, 5 CNTs were used (DWCNTs, functionalized DWCNTs, MWCNTs, functionalized MWCNTs and short MWCNTs) (chapter 7).

Research question n°4: Does applying a combined stress to CNT exposure lead to a different toxicity?

Some studies reported that a combined stress results in higher toxicity of NMs. In the context of climate change, we assess the impact of heat stress on CNT toxicity on canola plants (*Brassica napus*). The effects were compared with CNT toxicity on plants growing under optimal growth conditions to verify the increase in sensitivity (chapter 7).


Figure 7 Graphical scheme of the different parts of my PhD project with the corresponding chapter in yellow circles: (3) CNT characterization, (4) Detection of CNTs in plants, (5) Assessing plant response to 2 NMs using FTIR spectroscopy, (6) Assessing plant response of 4 types of plants to DWCNTs and (7) Evaluation of the influence of the physicochemical parameters of CNTs on plant response under optimal conditions or heat stress.

CHAPTER 2: MATERIALS AND METHODS

1. CARBON NANOTUBE PREPARATION

One of the main objectives of this work was to establish correlations between the physicochemical characteristics of CNTs and their impacts on plants. Different CNTs were used varying in their number of walls, diameter, length, functionalization, and ¹³C or ¹⁴C enrichment. To facilitate the reading, abbreviations for the different CNTs will be used in all the manuscript. The raw DWCNTs synthesized at CIRIMAT will be written DWCNTs; the functionalized DWCNTs synthesized at CIRIMAT, DWCNTs f; the raw MWCNTs from Cheaptubes, MWCNTs; the functionalized MWCNTs from Cheaptubes, MWCNTs f; the short MWCNTs from NanoGrafi, short MWCNTs; the DWCNTs from Service de Chimie Bioorganique et de Marquage (SCBM) laboratory (CEA Saclay) enriched with ¹⁴C, DW¹⁴CNTs and finally the ¹³C-enriched MWCNTs synthetized at CIRIMAT, MW¹³CNTs. There are 2 different MWCNTs ¹³C because two different enrichment ratio in ¹³C were realized. One was enriched at 2% of ¹³C and the other at 50%. In the manuscript, they are respectively named MW¹³CNTs 2% and MW¹³CNTs 50%. Due to the expensive price of these two CNTs, most of the characterization was performed on MWCNTs synthetized following the very same protocol but with a ¹²C precursor except for the Raman analysis allowing the calculation of ¹³C enrichment. DW¹⁴CNTs were also used during this study. They were prepared in collaboration with F. Taran (CEA Saclay) by grafting of ¹⁴C onto the surface of the DWCNTs synthetized at the CIRIMAT. Due to the cost of the DW¹⁴CNTs and difficulties related to handling of radioactive samples, no characterization was done on this specific sample. However, they were expected to have really close morphological characteristics than the primary DWCNTs.

Among the 8 CNTs used during this research, 5 were synthetized in the CIRIMAT laboratory using catalytic chemical vapor deposition (CCVD): the 3 DWCNTs and the 2 MW¹³CNTs.

This section presents the different methods used to prepare the catalyst powders and the different processes used for synthesis and purification of the CNTs produced at the CIRIMAT from different carbon sources (*i.e.* methane (CH₄), ethanol (EtOH)) (Figure 8).

The other CNTs were bought from Cheaptubes USA (MWCNTs and MWCNT f) and Nanografi Turkey (short MWCNTs). They were also synthetized using CCVD but with different precursors nickel (Ni) and iron (Fe) for Cheaptubes and iron (Fe) and Cobalt (Co) for Nanografi.



Figure 8 Graphical scheme of the CNT synthesis and functionalization made at the CIRIMAT. Yellow circles relate to the corresponding paragraphs of the chapter 2.

1.1. Catalyst powder synthesis

1.1.1. Catalyst powder synthesis by citric combustion

A catalyst powder of elemental composition $Mg_{0.99}(Co_{3/4}Mo_{1/4})_{0.01}O$ was used for the synthesis of DWCNTs with CH₄. This powder was prepared as reported in Flahaut *et al.*, (2003b) by citric combustion from the following precursors: ammonium heptamolybdate tetrahydrate ((NH₄)₆Mo₇O₂₄, 4H₂O), magnesium nitrate (Mg(NO₃)₂, 6H₂O) and cobalt nitrate (Co(NO₃)₂, 6H₂O).

Briefly, citric acid was dissolved at 50-60°C in deionised water. Then, under stirring, the ammonium tetrahydrate was added followed by the two other precursors. Heating and stirring were stopped after complete dissolution of the precursors. The citric combustion was performed in a muffle furnace at 550°C and 15 minutes were required to complete the exothermal redox reaction and to transform the solution into a spongy/friable solid. Finally, this powder was ground manually in order to reduce the volume. The obtained powder was then ready for calcination.

1.1.2. Catalyst powder preparation by impregnation

This method was used for the preparation of the catalyst for the MW¹³CNT synthesis with EtOH. The method was defined according to the work of P. Landois (Landois, 2008). The target elemental composition was close to the one of the catalyst used for the synthesis of DWCNTs, and the weight composition of Co/Mo/MgO was 1.6/0.8/97.6 %m.

The metal salts were the same as the ones used for the catalyst powder preparation by citric combustion but the preparation method was different: the metal salts were simply dissolved in deionised water at 60°C and the magnesium nitrate was replaced by commercial magnesia (MgO, 325 mesh, 265 m²/g, purity 99.5%). The slurry was stirred for 1 hour in order to maximize the metal impregnation on the magnesia. The suspension was then frozen using liquid nitrogen, freeze-dried and ground for calcination.

1.1.3. Calcination of the catalyst powder

This step is essential after both preparation methods. It allows complete removal of residual carbon produced during the decomposition of citric acid (present in excess), or in the case of the catalyst prepared by impregnation, it allows the decomposition of metal nitrates and the formation of cobalt and molybdenum oxides. It was performed by heating the powders in a muffle furnace at 450°C for 1 hour under air flow. After this step, the powder was ready to be used for CNT synthesis.

1.2. CNT synthesis by CCVD

1.2.1. DWCNT from methane

Between 4 and 5 g of the catalyst powder prepared by citric combustion $Mg_{0.99}(Co_{3/4}Mo_{1/4})_{0.01}O$ were introduced in an alumina boat, this boat was then placed at the center of a quartz reactor. It is inside this reactor placed into a tubular furnace that the synthesis of the DWCNTs took place (Figure 9).



Figure 9 Diagram of the experimental setup used for the synthesis of DWCNT by CCVD from CH₄. An alumina boat with the catalyst powder was introduced in a tubular reactor fed with nitrogen (N₂) or methane and dihydrogen (CH₄ + H₂). Gas flows were regulated using mass flowmeters (MFM). A washing bottle was placed at the exit in order to monitor the gas flow and another to avoid the backflow in the tubular reactor.

The synthesis was composed of three steps:

- (1) Increase in temperature until 1000°C at a speed of 5°C.min⁻¹, under H₂ and CH₄ atmosphere (18% CH₄) with a flow of 15 L.h⁻¹. Under the H₂ flow, the cobalt (II) was reduced selectively and formed cobalt nanoparticles. CH₄, the carbon source, could then be catalytically decomposed on the cobalt/molybdenum nanoparticles. This is how the DWCNTs were formed. The molybdenum helped for the decomposition of the carbon source.
- (2) Cooling down until 120°C at a speed of 5°C.min⁻¹, under H₂ and CH₄ atmosphere with a flow of 15 L.h⁻¹.
- (3) Reactor flushing with nitrogen (N₂) until room temperature during 20 min. This last step allowed removing all traces of H₂ and CH₄ before opening the reactor.

The sample obtained after the synthesis was a compact black powder: it is called nanocomposite powder, which contained around 12 wt. % of CNTs, the remaining 88 wt. % were composed of the magnesia support and carbon-encapsulated metal nanoparticles. The sample was then ground manually using a spatula. Several batches of synthesis were mixed together in order to obtain a unique sample used for all the experiments.

1.2.2. MW¹³CNT synthesis from ethanol

The MWCNTs were also synthetized using CCVD but with ethanol as carbon source (Figure 10). A N₂ flow (3 L/h) was used to carry EtOH vapors by flowing in a flask containing 2 g of ¹³C-enriched. The ¹³C EtOH (Sigma-Aldrich, CAS 14742-23-5, ref Sigma 324523) was enriched at 50% (only one carbon out of the two was a ¹³C). The 50% ¹³C ethanol was used pure or mixed with usual ethanol (¹²C) depending on the desired enrichment ratio. The flask was immerged in a thermostatically controlled oil bath at 25°C in order to avoid interferences from room temperature variations. A heating wire (60°C) was rolled around the hose connecting the EtOH reservoir to the reactor in order to avoid the condensation of EtOH vapors. The vapors were then carried until the reactor where the decomposition occurred at high temperature (850°C).



Figure 10 Diagram of the experimental setup used for the synthesis of MW¹³CNT by CCVD using EtOH. An alumina boat with the catalyst powder was introduced in a tubular reactor fed with nitrogen (N₂) or N₂ + EtOH. Gas flow was controlled using a mass flowmeter (MFM). The liquid ethanol was placed in a flask put in a thermostatically controlled oil bath. The red pipe represents the heating wire set at 60°C. A washing bottle was placed at the exit in order to monitor the gas flow and another to avoid any backflow in the tubular reactor.

1 g of catalyst powder prepared by impregnation was introduced into an alumina boat. The boat was then placed in the tubular reactor, and heated at 850°C (5°C/min) in N₂ atmosphere (3 L/h). During a 30 min dwell time at this temperature, a valve was operated in order to force the flow of N₂ through the washing

bottle containing the ¹³C EtOH. Then, the temperature was decreased to room temperature (5°C/min) in N_2 atmosphere (3 L/h). At the end of the experiment, a black composite powder was obtained.

1.3. Extraction and washing of CNTs

These steps were performed for the CNTs synthetized at the CIRIMAT and were the same for both DWCNTs and MW¹³CNTs. The composite powder was treated using concentrated hydrochloric acid (HCl, 37%) in order to dissolve the catalytic support (MgO, Co and Mo). Briefly, 1 g of nanocomposite powder was introduced in an Erlenmeyer and moistened with 3-4 g of deionised water before adding 15 mL of 37% HCl. The mixture was sonicated in an ultrasonic bath for 10 min. The dissolution of MgO, Co and Mo gave a blue-green coloration to the solution (CoCl₄²⁻ complex formation + Mo ions). The mixture was stirred overnight in order to achieve a complete dissolution of accessible metal nanoparticles. HCl is not oxidizing, so the extraction procedure did not damage the CNT structure (*i.e.* no functionalization of the external layer or opening of the tubes), and did not allow the elimination of the metallic particles encapsulated in carbon shells, or any other form of carbon (disorganized carbon). The slurry was filtered on cellulose nitrate membrane (Merck Millipore, 0.45 μ m). CNTs were then washed several times with deionised water until pH neutrality of the filtrate. After this step, wet CNTs could be directly used for functionalization or freeze-dried for further uses. For the freeze-drying, CNTs were immerged in deionised water and dispersed in an ultrasonic bath for few minutes in order to avoid agglomerate formation which are difficult to grind when CNTs are dry. The yield of the extraction step was 12 wt. % (Flahaut *et al.*, 2003b).

1.4. CNT covalent functionalization using nitric acid

The functionalization was obtained using nitric acid (HNO₃) and was done for both DWCNTs and MWCNTs (Cheaptubes). Briefly, dry or wet CNTs (100 mg dry weight) were introduced into a 250 mL flask. 100 mL of 3M HNO₃ were added. The mixture was sonicated in an ultrasonic bath during 10 min in order to break down agglomerates. The suspension was then heated in reflux conditions at 130°C during 24h, then cooled down to room temperature and filtered through a polypropylene membrane (Merk Millipore, 0.45 μ m). CNTs were washed several times until reaching the pH neutrality of the filtrate. They were dispersed in deionised water, frozen and freeze-dried for further uses. The yield of this process was 82 wt. %.

1.5. CNT dispersion in aqueous medium

1.5.1 Dispersion with ultrasounds

For the preparation of the stock suspensions and to disperse efficiently the CNTs, an ultrasonic probe (Vibra Cell 75042, 20 kHz, 500 W, 12.5 mm diameter rod) was immerged in the suspension during 15 minutes (Figure 11A).

The molecular stirring caused by this equipment provokes a temperature increase which can induce a failure of the equipment (safety). To avoid this, the suspension was placed into an ice bath and the sonication was following a square wave composed of 1s of pulse and 1s of relaxation. It helped to limit the

temperature increase and improved the efficiency of the process. During this operation, CNT damaging could occur (creation of structural defects, shortening by breaking).

An ultrasonic bath (USC300T, VWR, 45 kHz, 80W) was also used to re-disperse the CNTs and homogenize the suspensions just before use (Figure 11B).



Figure 11 A. The ultrasonic probe with the generator, the soundproof box, the microprobe and the crystallizer with ice. B. The ultrasonic bath.

Since the preparation of a suspension of CNTs may modify the physicochemical properties of the nanotubes, all the characterizations were performed on CNTs after suspension preparation by sonication, just before use.

1.5.2. Non-covalent functionalization using carboxymethylcellulose (CMC)

For experiments carried out in hydroponic conditions, stable suspensions of CNTs were required. For this purpose a combination of the physical dispersion method described previously and a non-covalent functionalization using a dispersing agent (carboxymetylcellulose - CMC) was used. CMC is widely used in food industry but also in cosmetics and pharmaceutical applications. It is colorless and non-toxic (Bourdiol *et al.*, 2013). The efficiency of this compound to disperse CNTs was evidenced in previous work (Bourdiol, 2013). The same concentrations of CMC and CNTs were used to prepare the suspension (*i.e.* 100 mg.L⁻¹ of CMC for a suspension of 100 mg.L⁻¹ of CNTs). Figure 12 illustrates the effect of CMC on the suspension stability.



Figure 12 Effect of CMC on DWCNT suspension stability. A. 100 mg.L⁻¹ DWCNT suspension without dispersant just after preparation. B. 100 mg.L⁻¹ DWCNT suspension without dispersant 24h after preparation. C. 100 mg.L⁻¹ DWCNT suspension with CMC (100 mg.L⁻¹) just after preparation. D. 100 mg.L⁻¹ DWCNT suspension with CMC (100 mg.L⁻¹) after 24h.

2. BIOLOGICAL MODELS AND EXPOSURE CONDITIONS

The different plant species used during this thesis as well as the exposure conditions (soil and hydroponics) will be presented in this part (Figure 13).



Figure 13 Graphical scheme of the plant species used and the two exposure conditions. Yellow circles relate to the corresponding part of the Chapter 2.

2.1. Plant species

2.1.1. Tomato

Tomato plant (Solanum lycopersicum) of the variety Red Robin was used in this study (Figure 14).



Figure 14 Botanical drawing of tomato plant (Solanum lycopersicum) (Magnan, Lytchiz' blog)

This plant belongs to the Solanaceae family. It is a family of dicotyledonous regrouping herbaceous plants, bushes, trees and creepers with alternate, simple leaves without stipule. Tomato plant is widely grown worldwide for its fruits (818,808 tons only in France in 2017 (Agreste, 2019)). In research, the plant is used as a model plant especially the Red Robin variety. Indeed, this variety is a miniature tomato plant: it does not exceed 30 cm in height. The growing cycle is very short (around 75 days) which makes the plant very interesting for ecotoxicity test.

2.1.2. Canola

Canola (*Brassica napus*) is an annual plant with yellow flowers belonging to the Brassicaceae family (Figure 15). It is an herbaceous plant of the dicotyledonous family. Canola is widely used worldwide in the cool temperate zones mainly for the oil production for human consumption from its seeds and the remaining for cattle nutrition. It represents an important part of the crop culture in France (1,401,443 ha in France in 2017 (Agreste, 2019)). The Brassicaceae family is well known in research because some plants of the family like *Arabidopsis halleri* are metal hyper accumulators (Tewes *et al.*, 2018).



Figure 15 Botanical drawing of canola (*Brassica napus*) (Müller, 1887).

2.1.3. Cucumber

Cucumber (*Cucumis sativus*) is an herbaceous and creeping vegetable plant belonging to the Cucurbitaceae family (Figure 16).



Figure 16 Botanical drawing of cucumber (*Cucumis sativus*) (Blanco, 2008)

It is a family of dicotyledonous plants from tropical and sub-tropical regions. The cucumber production in France in 2017 was 144,885 tons which represents 1,404 ha (Agreste, 2019). Cucumber can easily grow in hydroponic conditions (Gashgari *et al.*, 2018).

2.1.4. Maize

Maize (*Zea mays*) is an annual herbaceous plant part of the Poaceae family (Figure 17). Poaceae is a family of monocotyledon grasses with the lower part of each leaf enclosing the stem, forming a leaf-sheath. It is widely grown worldwide, more than wheat and rice, for its starch-rich grains but also as a forage plant. In France in 2017, 1,435,699 ha of maize were cultivated which represents more than 14 million of tons (Agreste, 2019). They are doing the so-called C4 photosynthesis (*vs.* C3 for most plants). It differs by its carbon dioxide fixing method. C4 plants have a specific leaf anatomy where chloroplasts exist not only in the mesophyll cells but also in the bundle sheath cells. This allows the minimization of the photorespiration and thus to have a photosynthetic yield much higher than the C3 plants.



(Zea mays).

2.2 Exposure conditions

Seeds were stored at 4°C until use. This preservation allows a stratification of the seeds and a synchronization of the germination in order to have plants of the same age during experiment. Just before experiments, seeds were sterilized using a sodium hypochlorite solution at 2.5% during 5 min and rinsed several times using deionized water.

For the culture conditions at EcoLab (France), plants were grown in a controlled chamber with 10 hours of light per day, 22°C during the night, 24°C during the day and a hygrometry of 85%. For the culture conditions at CAES (United States), plants were grown in a greenhouse.

2.2.1. Hydroponics

Hydroponics was used for cucumber plants growing to investigate the different detection techniques. This exposure condition is not environmentally relevant but it was chosen to maximize the CNT transfer in the aerial parts of the plants. Seeds of cucumber were germinated in soil until the appearance of the cotyledons (7 days). Seedlings were then transferred individually in Falcon tube of 50 mL (Figure 18). Caps of the tube were carefully punched to allow insertion of the plantlet. Tubes were covered with an aluminum foil to protect roots from the light. A needle connected to an air system was also introduced inside the tube in order to oxygenate the nutritive solution. 50 mL of Hoagland media (or 50 mL of DWCNT suspension at 100 mg.L⁻¹ with 100 mg.L⁻¹ of CMC in Hoagland media) were added to the tubes.



Figure 18 Experimental design for cucumber exposure in hydroponics.

The Hoagland medium was composed of MgSO₄.7H₂O (0.5 mM), Ca(NO₃)₂.H₂O (2.5 mM), KH₂PO₄ (0.5 mM), KCl (2.5 mM) and microelements H₃BO₃ (25 μ M), MnSO₄.H₂O (5 μ M), ZnSO₄.H₂O (0.4 μ M), CuSO₄.5H₂O (0.2 μ M), Na₂MoO₄.2H₂O (0.25 μ M) and EDTA Fe-Na (4 μ M) (Larue, 2011). The medium level was completed with water every day. During exposure, we can expect CNTs to have a different behavior in the medium due to the influence of root exudates and a microbial community that progressively develops in the medium (Huang, Zhao and Keller, 2017). Therefore, after 7 days of growth the medium was entirely renewed. This system avoided contamination between the aerial parts of the plant and the CNT suspension. The exposure duration was 15 days.

2.2.2. Soil

For the other experiments, more environmentally relevant exposure conditions were employed by using soil as medium to grow and expose plants. For experiments realized at EcoLab, a standard soil commercialized by LUFA Speyer (Germany) was used. It is an agricultural soil in which the culture and fertilization historic is known, well characterized and widely used by the scientific community. For the experiments at CAES, the soil used was an agricultural soil from CAES fields. Characteristics of the two soils are presented in the following table (Table 4).

Table 4 Soil characteristics for the LUFA soil 2.1 and the agricultural soil used at the CAES (CAES soil). ND = non determined.

	LUFA soil 2.1	CAES soil					
Organic carbon (%)	0.74 ± 0.14	2.5 (Total organic matter)					
рН	5.1 ± 0.5	6.2					
Cationic exchange capacity	4.0 + 1.0	11.5					
(CEC) (meq/100g)							
Water holding capacity	31 8 + 3 0	60.0					
(g/100g)	51.0 ± 5.0	00.0					
Bulk density (g/1000mL)	1430.0 ± 57.0	ND					
Particle size (%)							
< 0.0002 mm	2.8 ± 0.8	ND					
0.002 – 0.006 mm	2.1 ± 0.8	ND					
0.006 – 0.02 mm	3.0 ± 0.7	ND					
0.02 – 0.063 mm	5.8 ± 1.5	ND					
0.063 – 0.2 mm	27.2 ± 3.0	ND					
0.2 mm – 0.63 mm	56.6 ± 3.9	ND					
0.63 – 2.0 mm	2.5 ± 0.6	ND					
Soil type (USA classification)	Silty sand	Fine sandy loam					

For soil contamination, two methods were used:

(i) For one experiment, CNT suspension was added to the dry soil (1 mL of suspension per gram of dry soil). The mixture was then agitated for 3 hours on a stirring table. After mixing, the mixture was filtered through filter paper to remove excess water. Soil was weighted and placed into pots in order to have 150 g of equivalent dry soil in each. This method was designed in order to obtain a contamination as homogenous as possible since the behavior of CNTs in soil is not known. For this experiment, seeds were germinated on a compost soil. After the germination, seedlings were transferred into hydroponics in boxes in order to grow for three weeks to allow the plants to develop more biomass before exposure. Hydroponic conditions rather than soil or sand were chosen for this step in order to avoid damaging the roots while transferring

the plants to soil for the exposure. This could have induced potential ways of internalization at root wounds or would have created more stress for the plants. Finally, plants were transferred into the soil for exposure.

(ii) The second method was used for all the other experiments. It was designed to obtain a homogenous contamination without deconstructing the soil. For this method, the maximum water holding capacity of the soil was measured. The volume of liquid (CNT suspension or water) added to the soil was half of the water capacity. For the contamination, around 100 g of soil was spread out in a tray (maximum 2 cm of thickness). The CNT suspension was then dispersed onto the soil surface using a pipette as homogeneously as possible (Figure 19). Then the soil was introduced into a large container and mixed vigorously. After that, soil was transferred into culture pots in order to have the equivalent of 150 g of dry soil in each. To start the experiment, seeds of the different plants were introduced directly into the soil for exposure and grown for 5 weeks.

In both methods, plants were watered *ad libitum* by filling a cup under the pot every day.

Figure 19 Contamination of the soil with CNTs in a tray.

3. CNT CHARACTERIZATION AND DETECTION

Table 5 Analyses used for CNT characterization and detection in biological matrices with the type of equipment or the method, the purpose, the analysis mode (bulk or for imaging), the location of the analysis and the analyst. NP = information Non Provided

Analysis	Equipment or method	Uses	Bulk or imaging	Location	Analyst
Chemical elemental analysis	Organic micro-analyzers	Mass content of C, O and N		Institut Sciences	NP
	ICP-AES	Metal content	Bulk	Analytiques, CNRS, Lyon	NP
Specific surface area (SSA)	Brunauer, Emmet and Teller Method	SSA measurement	Bulk	CIRIMAT, Toulouse	C. Liné
TEM observations	TEM	Diameter, length, aspects of CNTs	Imaging	Plateforme Raymond Castaing, Toulouse	C. Liné
	TEM and high-resolution TEM	CNT detection in plants	Imaging	CMEAB, Toulouse and CEA Grenoble	C. Liné
Raman analysis	Raman spectroscope	Structural quality of CNTs	Bulk	CIRIMAT, Toulouse	C. Liné
		CNT detection in plants	Bulk	CIRIMAT, Toulouse	C. Liné
Thermogravimetric analysis	Thermogravimetric analyzer	Thermal stability determination	Bulk	CIRIMAT, Toulouse	A. Brahmi
XPS analysis	X-Ray photoelectron spectroscope	Quantitative atomic composition	Bulk	CIRIMAT, Toulouse	J. Esvan
Zeta potential	Laser Doppler electrophoresis (zetasizer)	Particle surface charge	Bulk	CIRIMAT, Toulouse	C. Liné
Two-photon excitation microscopy	Two-photon microscope	CNT detection in plants	Imaging	IPBS, Toulouse	E. Bellard
μXRF	Synchrotron μ X-Ray beamline (ID21, ESRF)	Co detection in plants	Imaging	ESRF, Grenoble	H. Castillo Michel
Broadband microwave biosensor	Broadband microwave biosensor	CNT detection in plants	Bulk	LAAS, Toulouse	K. Grenier D.Dubuc
Micro nuclear reaction analysis	Nuclear microprobe	CNT detection in plants	Imaging	AIFIRA, Bordeaux	S. Sorieul
Isotopic-mass ratio spectrometry analysis	Isotopic-ratio mass spectroscope	Isotopic ratio (¹³ C/ ¹² C) in plants	Bulk	SHIVA, Toulouse	I. Moussa

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Autoradiography	X-ray films	¹⁴ C detection in plants	Imaging	CEA Saclay	A. Sallustreau F. Taran
Hyperspectral imaging	Hyperspectral imaging spectrophotometer	CNT detection in plants	Imaging	CytoViva	T. Larrouy
FTIR	FTIR spectroscope	CNT impacts on plant biomacromolecules	Bulk	ESRF, Grenoble	H. Castillo Michel J. Reyes Herrera

3.1. Chemical elemental analysis of CNTs

3.1.1. Organic micro-analysis

The mass contents of carbon (C), oxygen (O) and nitrogen (N) in CNTs were determined using organic micro-analyzers. Measurements were made at Institut des Sciences Analytiques (UMR 5280) of the CNRS in Lyon.

For C and N quantification, samples underwent a total combustion under He/O flux and under pressure. C, H and N present in the sample were transformed respectively in carbon dioxide, water and various nitrogen oxides. Nitrogen oxides were further reduced in molecular nitrogen which can be separated on chromatographic column from CO₂ and H₂O resulting from the combustion process. CO₂ and N quantities were determined thanks to a thermal conductivity detector. The uncertainty for carbon was 0.50 % (absolute), 0.30 % for nitrogen and 0.20 % for hydrogen.

For O quantification, a total pyrolysis was applied to the sample at 1080°C under nitrogen flux. O from the compounds of the pyrolysis were transformed into carbon monoxide through passage on activated carbon at 1120°C which can be quantified via an infrared detector specific for CO. The uncertainty of the measurement was 0.30 % (absolute).

For the determination of metal content (remaining precursors, depending on samples: Co, Mo, Ni, Fe), samples were wet mineralized (Ayouni-Derouiche *et al.*, 2014) and analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (CREALINS) with an uncertainty of 2 %.

3.1.2. X-Ray Photoelectron Spectroscopy (XPS)

The X-Ray photoelectron spectroscopy (XPS) is a non-destructive analysis allowing determining the quantitative atomic composition and the chemical composition of the surface of a material on a depth from 1 to 5 nm. XPS analysis was used to quantify elements of the CNT sample such as remaining catalysts. The principle of the technique is based on the interaction of a source of photons with atoms from the sample. Electrons orbiting around the nucleus can be ejected due to the photon beam. They are propelled into the matter and travel a specific distance proportional to the kinetic energy and the material. Electrons are then collected and analyzed according to their kinetic energies.

Analyses were made using a XPS Kalpha ThermoScientific (CIRIMAT) with an aluminum source. For sample preparation, CNT powder was deposited on a carbon adhesive tape placed on a metallic holder. The photoelectron emission spectra were recorded using Al-K α radiation (hn=1486.6 eV) from a monochromatized source. The spot size was 400 µm. The pass energy was fixed at 30 eV for narrow scans (and 150 eV for the survey). We used Flood Gun for the charge effects. The spectrometer energy calibration was made using the Au 4f7/2 (83.9 ± 0.2 eV) and Cu 2p3/2 (932.8 ± 0.2 eV) photoelectron lines. XPS spectra were recorded in direct N(Ec). Background signal was removed using the smart method.

First, a survey was done on the sample consisting of a scan of the sample on a large energy range. It allowed identifying the nature of the elements present at the surface. Then, high resolution analysis was done on the different chosen elements by selecting the energy bands of interest. For data treatment, area under the different peaks was calculated; each peak representing a chemical element.

Measurements and data fitting were realized by Jerome Esvan (CIRIMAT).

3.2. BET method

The specific surface area (SSA) is the total surface of an object which includes asperity surface of the particles like the pores. It is expressed in $m^2.g^{-1}$. The SSA was determined using the Brunauer, Emmet and Teller method (BET) (Brunauer, Emmett and Teller, 1938). It is calculated from the nitrogen (N₂) quantity adsorbed on the sample assuming an atomic monolayer, and measured during a sudden desorption. The surface occupied by a single N₂ molecule being known, the measurement of the desorbed gas quantity allowed calculating the real surface (available for the gas) of the powder grains.

CNT powder samples were first degassed at 100°C under N₂ atmosphere during 2 hours in order to remove any contaminant from the surface (adsorbed water, *etc.*). Then samples were cooled down at 77K (liquid nitrogen temperature) for adsorption of a monolayer of N₂ molecules. After heating the samples back to room temperature, the desorption peak was recorded. The N₂ desorption was measured using a Micrometrics Flow Sorb II 2300. A calibration was done by injecting a known quantity of N₂ (in the same conditions as for the sample measurement). The uncertainty of the measurement was 3%. I performed the analyses with the help of Marie Claire Barthélémy at the CIRIMAT.

3.3. Raman spectroscopy analysis

Raman spectroscopy, like infrared spectroscopy, is a scattering technique based on the vibrational state of the analyzed molecules. The sample is illuminated with a monochromatic laser beam. A fraction of the photons of this beam is reflected or absorbed by the sample and at the same time a much lower fraction is diffused in all the directions. Among the diffused photons, most of them have the same frequency (v_0) than the radiation source, this is the Rayleigh diffusion. For only one diffused photon out of one million, a frequency modification is observed: it is the Raman effect. This state is the result of a molecule excitation from its fundamental state through a virtual electronic state induced by the monochromatic light (Siesler, 2002; Larkin, 2018). Raman shifts are measured in wavenumbers in cm⁻¹ (inverse of the wavelength).

For CNTs, four characteristic vibration modes are observed in a typical Raman spectra (Figure 20):

- The radial breathing modes (RBM) are found at low wavenumbers between 100 and 300 cm⁻¹. Each peak location is related directly to the diameter and the chirality (n, m) of the corresponding nanotubes (Costa *et al.*, 2008).
- The D band, situated between 1320 and 1340 cm⁻¹, is proportional to the ratio of structural defects present in the sp² carbon structure (this band is related to the quantity of sp³ carbon present in the carbon network).

- The G band or graphite band, between 1575 and 1590 cm⁻¹, informs about the hexagonal network of sp² carbon. It is specific to graphite, graphene and CNTs (*vs.* organic carbon).
- The G' band or 2D band, between 2620 and 2640 cm⁻¹, corresponds to the second harmonic of the D band. While the D band intensity is related to the default quantity in the material structure, the G' band is not affected by this factor.



Figure 20 Typical Raman signature of carbon nanotubes (DWCNTs) with the different characteristics bands (RBM, D, G and G').

The ratio between the D and the G bands (I_D/I_G) gives supplementary information on the structural quality of the CNTs. The quality is decreasing when the ratio between the two bands increases (Dresselhaus *et al.*, 2005; Costa *et al.*, 2008; Saito *et al.*, 2011).

During this work, Raman spectroscopy was performed on a confocal microscope Raman Labram HR800 Horiba Jobin Yvon. The sample have been exposed to a 633 nm laser radiation with a power of 8 MW and placed under a microscope equipped with an objective 100x which gives a lateral resolution of 0.833 μ m (1.22 x λ / O.N) and an axial resolution of 2.4 μ m (4 x λ / O.N²). For sample preparation, CNTs were sonicated for few minutes in ethanol. Few drops of the suspension were then placed on a glass slide and allowed to dry for a few minutes. After spectrum acquisition and baseline correction (LabSpec software), the intensity of the D and G bands were measured and the intensity ratio was calculated to determine structural default of our CNTs.

Raman spectroscopy was also used to determine the enrichment rate of ¹³C of the MW¹³CNTs synthetized with ¹³C ethanol. Indeed, the presence of ¹³C in the sample causes a shift in the low frequencies of the D, G and G' bands, due to the supplementary neutron per carbon atom. By making the assumptions that all

the carbon atoms of the MW¹²CNTs are ¹²C, the content of ¹²C (x) and ¹³C (x-1) can be determined in relation to total carbon. The followed equation has to be applied (Simon *et al.*, 2005):

$$\frac{dw}{w_{x=1}} = \sqrt{\frac{12}{12x+13(1-x)}} - 1 \tag{V.1}$$

With $dw = w_x - w_{x=1}$, w_x , wavelength value (cm⁻¹) corresponding to the peak of G band of MW¹³CNTs and $w_{x=1}$, wavelength value (cm⁻¹) corresponding to the peak of the G band of MW¹²CNTs.

$$x = \frac{{}^{12}C}{{}^{12}C + {}^{13}C}$$
, ¹²C content of the MWCNTs ¹³C in relation to the total carbon.

$$1 - x = \frac{{}^{13}C}{{}^{12}C + {}^{13}C}$$
, ¹³C content of the MWCNTs ${}^{13}C$ in relation to the total carbon.

$$(V.1) \Leftrightarrow x = 13 - \frac{12}{\left(\frac{dw}{w_{x=1}} + 1\right)^2} \tag{V.2}$$

Observations of biological samples were made using the same equipment as for CNT characterization. For sample preparation, different type of samples were tested: full leaves, and tissue digestions (explained in 3.9. Sample preparation for biological tissues).

I performed the Raman measurements and analysis with the help of Olivier Marsan (CIRIMAT).

3.4 Thermogravimetric analysis

Thermogravimetric analysis (TGA) is obtained by measuring the mass variation of a sample vs temperature in a given atmosphere and permits to determine its thermal stability (in different atmospheres), decomposition profile and volatile content (Coats and Redfern, 1963). Applied to CNTs, TGA in air allows for example to determine both the carbon content and the quantity of remaining catalysts in the sample (Pang, Saxby and Chatfield, 1993).

Measurements were done on a SETARAM TAG 16. The specificity of this equipment is the ultra-sensitive thermobalance with two separated ovens allowing compensating Archimede buoyancy potentially disturbing the measurements. For the analysis, around 3 to 4 mg of CNTs were placed in a platinum crucible.

Measurements were realized by Abdérahmane Brahmi (CIRIMAT).

3.5 Laser Doppler electrophoresis (LED)

The zeta potential provides information about the surface charge of particles. Indirectly, it gives information on the interaction probability among particles in suspension, in other words about the stability of the suspension. The zeta potential is the charge that a particle gains thanks to the ions surrounding it when it is in suspension in a liquid. The potential is measured on the shear plane of the particle *ie*. at the limit between the part of the solution that moves with the particle and the rest of the solution. The zeta

potential is measured by determining the electric potential of the shear plane of a particle away from the particle surface, somewhere in the diffuse layer (Xu, 2008; Bhattacharjee, 2016). The zeta potential of a particle is not set, it can change according to the composition of the solution used. It is highly depending on the pH and the ionic strength.

The zeta potential was measured using the laser Doppler electrophoresis (LED) method with a Zetasizer (Malvern). The suspension placed inside a cell was irradiated with a laser light and an electric field was applied. Due to the electric field, the particles moved and the resulting scattered light was measured and the particle velocity determined from the frequency shift. Mobility was obtained as the ratio of the velocity to the electric field strength. Zeta potential was then deduced using a predefine model (Tucker *et al.*, 2015). The potential was measured in DI water for all the CNTs. For DWCNTs, we also measured it in soil solution.

I performed the measurements at the CIRIMAT with the help of Vincent Baylac.

3.6 Broadband microwave biosensor

This technique is based on the dielectric properties of CNTs and ionic solutions when they are subjected to an external electromagnetic (EM) field. Without external EM field, the permanent dipole orientation of a polar molecule is random while in the presence of an electric field, dipoles tend to orientate according to the field lines. Some time is necessary for the permanent dipoles to orientate themselves and for the dipolar polarization to reach its maximum value. In the presence of an alternative external electric field, dipole rotation is able to follow field variations until a certain frequency (high frequency, HF). When the frequency is too high, the rotation is not done instantly, revealing a relaxation phenomenon. This phenomenon is observable at frequencies between 10⁷ and 10¹⁰ Hz (Bureau, 2016; Capacitor Electronics, 2019).

CNTs are used in many different applications for their important intrinsic conductivity and their high shielding properties against EM interferences (Liu *et al.*, 2010). Indeed, it has been observed several time that CNTs have high microwave permittivity and behave like a dielectric material at HF waves (Wu and Kong, 2004; Dragoman *et al.*, 2006). These properties can be used in order to detect CNTs in environmental matrices.

The team MH2F (Micro et nanosystèmes HyperFréquences Fluidiques) of the LAAS-CNRS (Laboratoire d'analyse et d'architecture des systèmes) has developed a new system for analysis and diagnosis in biological, medical and environmental fields using the association of microsystems HF and microfluidic. It is a HF biosensor allowing using the interaction in the near field of EM waves with biological fluids such as cell suspension in a culture medium (Figure 21).



Figure 21 Concept of the broadband microwave biosensor developed by MH2F LAAS-CNRS. RF = Radio Frequency, EM = Electromagnetic. From Grenier et al., 2010.

The HF EM field emitted by the source spreads on the circuit. A part of the EM waves passes through the microfluidic channel and interacts with the fluid. The result is a modulation of the EM signal according to the characteristics of the fluid. This technique presents several advantages: it is non-invasive and requires a very small volume (microliter range) (Grenier *et al.*, 2010).

We used this microwave biosensor to detect CNTs in plant samples. In order to avoid interferences from the plant matrix, samples were digested (3.9. **Erreur ! Source du renvoi introuvable.**). The signal found came only from the CNTs and not from the plants.

The length of the microfluidic channel is 2 mm, the length is 300 μ m and the thickness is 200 μ m. The sensing area corresponds to a volume of 0.13 μ L. Including the dead volume of the device, the total volume to inject for a single analysis ranged between 0.5 and 1 μ L. The filling, cleaning and refilling of the HF microfluidic channel were manually performed with a syringe and controlled using a microscope equipped with a CCD camera. Two coplanar microprobes were connected to the HF circuit on both sides of the microfluidic channel (Figure 21) and to a vector network analyzer in charge of the microwave parameter measurements. Analyses were made with the help of Lise Rigal, Katia Grenier and David Dubuc (LAAS).

3.7 Isotopic-ratio mass spectrometry (IRMS) analyses

Isotopic-ratio mass spectrometry (IRMS) measures the relative abundance of stable isotopes such as ${}^{2}H/{}^{1}H$, ${}^{13}C/{}^{12}C$, ${}^{15}N/{}^{14}N$ and ${}^{18}O/{}^{16}O$ in a given sample. Isotopes are atoms with the same number of protons but with a different number of neutrons. So these atoms will differ in mass but hardly in chemical behavior. For the analysis, sample consisting of ground solid materials (*e.g.* plant tissues, animal materials, soils, sediments) are converted to simple gases (H₂, CO₂, N₂ or CO) by combustion. Sample components are then

separated in an analytical column based on their varying interactions with the carrier gas and the stationary phase within the column. Gas molecules are ionized by a beam of electrons. The newly formed ions are then focused and accelerated. The trajectories of the ions are determined by a magnetic field, which allows the separation of ions according to their mass. At the end of the pathway, these ions are detected using a Faraday cage. Ions with different mass to charge ratios (m/z) are distinguished from one another and quantified (Chartier, Isnard and Nonell, 2014). For instance, to measure the isotopic difference of carbon atoms, m/z of 44, 45 and 46 are monitored; this corresponds to the three isotopes of carbon: ¹²C, ¹³C and ¹⁴C. The abundance of isotopes are then compared with the isotopic ratio of specific standards (international carbonate standard, Vienna Pee-Dee Belemnite, 13C/12C = 0.0112372) (Tcherkez, Mahé and Hodges, 2011).

Here, MW¹³CNTs were synthetized specifically for this experiment with 2% of enrichment. IRMS was used to measure a possible enrichment of ¹³C in exposed plants in relation to the natural enrichment (around 1.1% (Tcherkez, Mahé and Hodges, 2011)). This measure allows proving and quantifying the presence of CNTs in plants.

All experiments were done on the isotopic platform SHIVA in EcoLab in collaboration with Issam Moussa. Briefly, dry leaf powder was put into a tin capsule and weighted. δ^{13} C values for the samples were determined using Isoprime 100 stable isotope ratio mass spectrometer coupled with an elemental analyzer VarioEl Micro. Stable isotope ratio results were reported as per mil (‰) deviations from the Vienna PeeDee Belemnite (VPDB). The isotope ratio is expressed as the ratio of the heavy to the light isotope relative to a standard. Here for ¹³C, it is: $\delta^{13}C = \{(R_{sample}/R_{standard}) - 1\} \times 1000$, where R is the corresponding ¹³C/¹²C.

3.8 Imaging techniques

3.8.1 Two-photon excitation (TPE) microscopy imaging

The two-photon excitation (TPE) microscope is a powerful tool derived from the confocal microscope. It provides three-dimension imaging of living cells in depth. This technique is based on the principle that an atom is able to absorb simultaneously two photons as opposed to conventional confocal microscopy based on the absorption of a single photon. The TPE microscopy is using a powerful laser emitting a brief and intense laser beam. In order to excite the atom at a certain wavelength ($\lambda_{excitation}$), the two absorbed photons have to have half of the needed energy for the excitation, that is to say a wavelength of 2 times $\lambda_{excitation}$. For this reason, the excitation source is emitting photons in the near infrared allowing a better penetration in tissues. Another advantage of the TPE microscopy is that the excitation is confined to the level of the focal volume. It means that no fluorescence is emitted outside of the focal plan. It is an advantage in comparison to the one photon microscopy permitting to have an increase in the signal/noise ratio and to achieve better axial and radial resolutions (Rubart, 2004; Oheim *et al.*, 2006).

For the TPE microscopy, no sample preparation was needed. Since this technique provides three dimension imaging, it is not compulsory to make cross sections of the sample. It allows avoiding cutting artifacts which makes this technique very interesting.

Observations were realized on a microscope Zeiss FLIM 7MP with an immersion objective and a femtosecond pulsed laser with a pre-compensation camera Coherent Chaméléon Vision II (690-1080 nm) (TRI, IPBS, Toulouse). Plans of 212.5 μ m x 212.5 μ m with a spatial resolution of 0.0854 μ m/pixel and an axial resolution from 1 μ m to 36 μ m depth were realized. I did the observations at IPBS (Toulouse) with the help of Elisabeth Bellard (IPBS).

3.8.2 Autoradiography analysis

Autoradiography is a technique used to study the local distribution of radioactive isotopes. By using X-ray films, it determines the relative positions and intensities of radiolabeled bands.

The radiolabeled sample is introduced into a detection chamber under gaseous atmosphere (98% argon and 2% triethylamine). Every emitted β particle ionizes a gas molecule. This ionization releases electrons which are speeded up by a potential difference between the two faces of the chamber. The successive collisions between electrons and gas molecules generate an electron flux. The obtained signal is then converted into a position of the emitting point using algorithms. The recording of all these positions allows recreating an image with the quantitative distribution of the radioactivity in the sample (Bq/cm²). Using a β -imager, the resolution can reach 60 x 60 μ m² with a high sensitivity. With a μ -imager, the resolution can reach 15 x 15 μ m².

All analyses were performed in collaboration with IBITEC (Institut de Biologie et de Technologie) of the CEA in Saclay with Antoine Sallustreau and Frederic Taran. For this analysis, DW¹⁴CNTs were used. Due to the use of ¹⁴C, plant exposure was done in the laboratory for ¹⁴C labelling. Aerial parts of the plants were

dried in an oven at 40°C before being analyzed using radio-imagers of the Laboratoire de Chimie pour le Vivant (CEA Saclay).

3.8.3 Synchrotron based micro X-Ray fluorescence (µXRF) imaging

Synchrotrons are accelerator-based sources of exceptionally intense, tightly focused beams of X-ray, ultraviolet and infrared radiations allowing researches in various fields (*e.g.* chemistry, material physics, archaeology, structural biology) which are not possible to achieve with conventional laboratory equipments. The source is an electron gun emitting a very thin electron beam. Electrons are accelerated in a linear accelerator until a speed close to the speed of light. The electron beam is then driven into a second circular accelerator, also called booster, where the energy is increased until a few GeV (6 GeV at the European Synchrotron Radiation Facility ESRF). At this level of energy, electrons are injected in the storage ring and turn during several hours. In the storage ring, different devices (dipoles, inverters) divert the electron trajectory. Upon deviation, electrons lose energy under the form of light: it is the synchrotron radiations in the beamline. All the system is placed under high-vacuum (10⁻¹⁰ mbar) in order to avoid electron collision with air molecules. Several techniques can be performed using synchrotron radiations such as X fluorescence (XRF). There are many advantages to use the synchrotron radiation like the very large emission spectra from infrared to X-ray and a brightness unrivaled by other methods (Adams, 2003, 2010).

There are around 50 synchrotron radiation facilities in the world and 2 are in France: ESRF (European Synchrotron Radiation Facility) in Grenoble and SOLEIL (Source Optimisée de Lumière d'Energie Intermédiaire du LURE – Laboratoire d'utilisation du rayonnement énergétique). In this study, all the analyses were performed at ESRF using the beamline ID21.



Figure 22 Bohr atom model illustrating the basic principle of X-Ray fluorescence. A. X-ray excitation leads to the ejection of a core-shell electron from the atom. B. The generated vacancy is filled up by a higher-shell electron, a process that results in the emission of a photon whose energy is equal to the difference in binding energies of the two shelves involved in the transition. From Fahrni et al., 2007.

Micro X-ray fluorescence (μ -XRF) is a non-destructive method allowing elementary mapping of samples. As illustrated by the schematic Bohr atom model (Figure 22), X-ray excitation leads to the ejection of a core-shell electron from the atom. The vacancy is then filled up by a higher-shell electron. This process results in the emission of a photon. The emitted photon energy is characteristic for each element. Those photons are collected by a detector which transforms this signal in a spectrum. In the spectrum, most of the time, each peak approximately corresponds to one element. However, for some elements such as Ca and, K they can overlap and appeared in one peak (Fahrni, 2007).

Synchrotron radiations allow increasing the ratio signal/noise in comparison to a standard source of X-rays in laboratory. This makes the method very sensitive with a high spatial resolution. The detection limit is around 0.1 μ g/g and the spatial resolution on ID21 at ESRF is 0.2 x 0.8 μ m² (Tiwari *et al.*, 2013). Acquired spectra were analyzed with PyMCA software (Solé *et al.*, 2007). The peak deconvolution of the spectra on each analyzed pixel allows obtaining reliable elemental cartographies. On each pixel, the number of counts for each considered element is available. This permits to make an image with a color intensity proportional to the number of counts.

Synchrotron analyses were realized by Hiram Castillo-Michel (ESRF).

3.8.4 Micro Nuclear Reaction Analysis (µNRA)

Ion beam analysis is based on the detection of emitted radiations after the interaction of an ion beam with the atoms of samples. This beam is produced by a particle accelerator. It is then speeded up by a potential difference of several MeV. Ions moved in the beamline composed of several equipment (deflectors to guide the trajectory, object-slits to define the size of the beam and control its position, a Faraday cage to measure the beam intensity, different lenses to focus the beam into a few μ m size spot and finally electrostatic plates that control the scanning of the sample). The analytical chamber, under high-vacuum (10-6 mbar), is composed of several detectors allowing to acquire different signals as described below.

Several analytical techniques can be performed using the nuclear microprobe for example micro particles induced X-ray emission (μ PIXE), Rutherford backscattering spectroscopy (RBS), scanning transmission ion microscopy (STIM) or nuclear reaction analysis (NRA). Here, only the techniques used will be described: μ PIXE and μ NRA.

Analyses were carried out at the Centre d'Etudes Nucléaires de Bordeaux-Gradignan (CENBG) on the AIFIRA (Applications Interdisciplinaires des Faisceaux d'Ions en Région Aquitaine) microprobe in collaboration with Stéphanie Sorieul at Laboratoire d'Etude des Eléments Légers at CEA in Saclay in collaboration with Suzy Surblé and Hicham Kodja.

 μ PIXE analysis is based on the same principle as μ XRF (see § 3.8.3). μ PIXE presents several advantages in comparison to other methods of X-ray fluorescence. For instance, thanks to the high energy of the incident source (in the MeV range), the analyzed depth is between 0.1 and 150 μ m depending on the type of sample. This same high energy allows exciting all elements (except H and He), unlike XRF, if they are

present in sufficient concentrations. Indeed, to be efficient, XRF needs to have incident energy just above the energy required to excite the element of interest and so other elements with higher energy are not excited and thus not detected. Finally, μ PIXE performed on thin samples is free from any matrix effects. However, light elements (*i.e.* C, O, N, *etc.*) can't be detect by the PIXE detector. In order to detect the isotopes of C, NRA has to be used.

 μ NRA analysis is based on the study of gamma electromagnetic rays or emitted particles during nuclear interactions between a high energy ion beam and the nucleus of the studied target. This technique is used for the detection and dosage of light elements (from helium to fluorine) and of their isotopes whatever the nature of the matrix. The analysis is isotopically selective by finely tuning the beam conditions in mass and energy. The detection limits are between 10 and 1,000 ppm depending on the studied element and the nuclear reaction used. The depth of analyze is between 50 Å and 150 μ m, depending on the matrix and the beam conditions (Berger and Revel 2005).

Sample preparation is explained in section 3.9.2.

3.8.5 Transmission electron microscopy (TEM)

The transmission electron microscope (TEM) was developed based on optical microscope to obtain a higher spatial resolution. In optical microscopy, the resolution (around 200 nm) is limited by the wavelength of the photon source (white light). Electrons have a much lower wavelength, TEM thus allows to have a spatial resolution of nanometer-size.



Figure 23 Scheme of a Transmission Electronic Microscope (TEM)

At the top of the column, an electron flow is generated by the electron gun (tungsten filament) (Figure 23). This flow is then accelerated by a potential difference and focused using several magnetic lenses. The beam is transmitted through a thin sample and forms the corresponding image on a fluorescent screen under the sample. The dense areas do not allow a lot of electrons to come through and appear darker on the screen. On the opposite, less dense areas appear lighter. It is the same for heavy elements (high atomic number) which appear darker than the light ones. The image is then recorded with a digital camera (Fultz and Howe, 2013).

For TEM observations, CNT powders were dispersed with an ultrasonic bath in ethanol for few minutes. Few drops of the suspension were then deposited on a TEM copper grid with a carbon coating (Lacey Carbon 400 Mesh Cu). Samples were observed using a TEM JEOL 1400 with an acceleration voltage of 120 kV. I carried out the observations with the help of Armel Descamps-Mandine at UMS Castaing, CNRS (Toulouse).

Sample preparation is detailed in 3.9.1. Biological samples were observed at the Centre de Microscopie Electronique Appliquée à la Biologie (CMEAB) in Toulouse with a TEM HT 7700 Hitachi at 120 kV using a CCD AMT XR41 camera with the help of Isabelle Fourquaux.

3.8.6 Hyperspectral imaging (HSI)

Hyperspectral imaging (HSI) is a technique which combines the advantages of optical spectroscopy with two-dimensional object visualization obtained by optical imaging (Vasefi, MacKinnon and Farkas, 2016). Each pixel of the image contains spectral information. The images are stacked up to form an image cube (Lewis and Lewis, 2014). The size depends on the number of wavelength used. The term "hyperspectral" rather than just "spectral" refers to the range of wavelengths measured, which typically included near-infrared, visible and sometimes near-ultraviolet spectra. Dark-field imaging enhances the effectiveness of the technique. It decreases the background signal and emphasizes materials that scatter the most light.

HSI has been investigated as a technique to detect NMs (Mortimer *et al.*, 2014; Roth *et al.*, 2015). The CytoViva® nanoscale microscope and HSI system mounted on an optical microscope is a new tool that can be used at the nanoscale. This instrument employs a novel dark-field microscopy illuminator which results in images with better contrast and higher signal-to-noise ratio. Spectral information can be obtained upon both the visible and near-infrared (nIR) wavelengths (Roth *et al.*, 2015; CytoViva, no date). CNTs exhibit intrinsically photostable excitonic fluorescence in the nIR region (Roxbury *et al.*, 2015), this property can thus be used to detect CNTs in biological samples.

We used an enhanced darkfield transmission optical microscope (Olympus BX43) equipped with a dual mode fluorescence (DMF) and the HSI spectrophotometer CytoViva[®] to investigate the presence of CNTs in our plant samples. We recorded spectra at nIR wavelengths (400-1000 nm) at a high spectral resolution of 2.5 nm. Sample preparation is described in 3.9.1. Data were collected by Thibaud Larrouy.

3.9 Sample preparation for biological tissues

3.9.1 Sample preparation for TEM and hyperspectral imaging

Biological samples, such as plants, require an important preparation for TEM observation because they are highly hydrated.

Sample preparation was done at the Centre de Microscopie Electronique Appliquée à la Biologie (CMEAB) in Toulouse by Isabelle Fourquaux. Four steps were necessary for the sample preparation:

- (1) Sample fixation. The aim of this step was to fix the structure of the plant tissues by creating inter and intra molecular bonds using chemical coupling agents. Glutaraldehyde (2.5%) was used as coupling agent in a sodium cacodylate buffer (0.4 M, pH 7.4) at 4°C. Two washing baths were then performed using the cacodylate buffer at 4°C. The last step of the sample fixation was to cut the sample into a cube of 1 to 2 mm. The post-fixation was realized using osmium tetroxide at 4%. Osmium reacts mainly with the lipid unsaturated double bounds but also with other groups such as –SH, -C=C-, -SS, -CHO and –OH for a good membrane preservation. The fixation and the post/fixation described here are standard methods for the study of ultrastructures in plants. They allow a good preservation of the tissues (Kuo, 2014).
- (2) Dehydration and substitution. Dehydration allows the penetration of the inclusion resin which is hydrophobic. Samples were immersed in a series of graded ethanol solutions (from 30 to 70%) and finally in a bath of propylene oxide until complete removal of the water. The substitution was then obtained using EmBed 812 resin (Electron Microscopy Sciences) and propylene oxide at room temperature.
- (3) Impregnation and inclusion. During this step, the resin EmBed 812 entered into plant tissues. Then samples were embedded in the same resin. After a polymerization at 60°C, the resin solidified which allows ultrafine sectioning of the sample.
- (4) Sectioning using an ultramicrotome Leica EM UC7. Cross sections of 70 nm were cut and deposited on nickel grids for TEM observations. For hyperspectral imaging, thicker sections were also made of 2 μm.

In this study, we chose to avoid the use of staining agents (such as uranyl acetate) since they can produce artifacts during observation very similar to CNT structures (Schwab *et al.*, 2015).

3.9.2 Sample preparation for μ NRA and μ XRF analyses

Samples of plant tissues (roots or leaves) were cut in small pieces of few millimeters long. Fragments were placed in Tissue-Teck OCT (Optimal Cutting Temperature) resin in an Eppendorf tube (Figure 24A).



Figure 24 A. Fragment of the plant tissue in an Eppendorf tube with OCT resin. B. Cryomicrotome used at EcoLab. C. Orientation of the sample during cutting.

Then, they were cryo-fixed in isopentane cooled down by liquid nitrogen at -165°C. The use of isopentane allows going down to a lower temperature than using only liquid nitrogen and avoid turbulences related to liquid nitrogen use. When samples are immersed in isopentane, they freeze instantly. The blocks of resin were cut using a cryo-microtome Microm HM500 (Figure 24B). The temperature inside the chamber of the cryo-microtome was set to -20°C. Sample fragments were placed perpendicular to the knife blade (Figure 24C). The thickness of the cross sections depended on the type of analysis performed after (between 20 and 50 μ m). Cross sections were then freeze-dried in order to remove all traces of water.

3.9.3 Digestion of plant tissues

For some of the techniques used, digestion of the plant tissues was necessary in order to avoid the fluorescence background due to photosynthetic pigments of the plant leaves. The protocol used was adapted from Das et al. (2018). Plant tissues were dried at 80°C overnight. They were ground into fine powder using a FastPrep system. Concentrated nitric acid (HNO₃, 70%) was added to the powder (1 mL of HNO₃ for 10 mg of tissues) in a Cortex tube. Then the mixture was digested at 62°C for 12 hours. After the digestion, samples were centrifuged at 14,500 g for 2 min. The supernatant was removed and replaced with water. The procedure was repeated several times until reaching pH neutrality of the supernatant.

4. METHODS FOR THE EVALUATION OF CNT TOXICITY IN PLANTS



Figure 25 Graphical scheme of the different methods used for the evaluation of CNT toxicity in plants. Yellow circles relate to the corresponding parts of the Chapter 2.

4.1 Plant development

Several macroscopic parameters were studied: germination rate, plant height, number of leaves, fresh biomass, dry biomass and leaf area.

Plant height and number of leaves were recorded every day during the experiment. Fresh biomass was measured at the end of the experiment. Then tissues were dried overnight at 50°C and dry biomass was weighted. For leaf area measurements, after exposure, leaves were disposed on a white paper and pictures were taken for each plant. The leaf area was determined using ImageJ software.

4.2 Plant physiology

In total six physiological biomarkers were used to assess impacts of CNTs. Two different extractions were needed: a methanol extraction for photosynthetic pigments (chlorophylls a and b as well as carotenoids), total phenolic compounds, tannins and flavonoids and a trichloroacetic acid (TCA) extraction for lipid peroxidation measurement by determining malondialdehyde (MDA). Protocols were optimized during my M2 internship at the LGCgE (ISA Lille) (Pourrut et al., under writting).

After exposure, plant leaves were immediately frozen in liquid nitrogen and kept at -80°C until analysis. Frozen plant leaves were ground in a 2 mL microplate of 96 wells with two glass beads of 4 mm in each well. Wells were filled up with around 10 mg or 50 mg of fresh material for methanol and TCA extractions respectively. Plants were ground in a bead mill 2 times for 1.5 minute.

For pigments and secondary metabolites, the wells containing the powdered plant material were then filled up with 1.5 ml of 95% methanol, mixed for 2 min, covered with an aluminum foil and incubated at

4°C for 24 h in the dark for photosynthetic pigments (as light can alter leaf pigments) or 48 h for secondary metabolites. After incubation, microplates were centrifuged for 5 min at 4500 rpm.

Chlorophyll a and b were measured by transferring 100 μ L of the supernatant to new microplates and measuring the absorbance at 470, 652 and 666 nm (Lichtenthaler, 1987). The concentration was expressed as milligram per gram of fresh weight (mg/g f.wt.) according to standard curves of chlorophylls a and b.

The total phenolic compounds were determined based on the Folin-Ciocalteu assay (Ainsworth and Gillespie, 2007). The reaction mixture of 200 μ L contained 20 μ L of supernatant, 40 μ L of Folin reagent (10% v/v) and 0.10 mmol of sodium hydrogen carbonate (NaHCO₃). The mixture was allowed to stand 2 h at room temperature for color development. Then absorbance was measured at 760 nm. Concentrations of phenolic compounds were calculated using a standard curve of gallic acid. Results were expressed as milligram of gallic acid equivalent per gram of fresh weight (mg GAE/g f.wt.).

The flavonoid concentration was determined by aluminum chloride method using catechine as a reference compound (Settharaksa *et al.*, 2014). Briefly, the reaction mixture (final volume 200 μ L) contained 25 μ L of methanolic extract, 7.25 μ M of sodium nitrite (NaNO₂), 0.11 μ M of aluminum chloride (AlCl₃) and 0.02 mM of sodium hydroxide (NaOH). The mixture was homogenized during 1 minute and absorbance was measured at 595 nm. Concentrations of flavonoids were calculated using a standard curve of catechine. Results were expressed as milligram catechin equivalent (CE) per gram of fresh weight (mg CE/g f.wt.).

For tannins, reaction mixture (final volume 100 μ L) contained 50 μ L of methanolic extract and 6.57 μ mol of vanilline (El Euch, Bouajila and Bouzouita, 2015). The mixture was left in the dark for 15 min and absorbance was measured at 500 nm. Tannin concentrations were calculated using a standard curve of catechine. Results were expressed as milligram catechin equivalent per gram of fresh weight (mg CE/g f.wt.).

For lipid peroxidation measurement, 1 mL of TCA at 1% was added to the wells for extraction and microplates were then homogenized for 2 min at 15 Hz. Thereafter plates were centrifuged during 10 min at 3000 g. 300 μ L of supernatant were added to a mixture containing 0.37 mmol of TCA and 0.14 μ mol of butylated hydroxytoluene (BHT) (final volume 200 μ L). In another plate, 300 μ L of supernatant were mixed in a solution containing 0.37 mmol of TCA, 0.14 μ mol of BHT and 13.53 μ mol of thiobarbituric acid (TBA) for a final volume of 600 μ L. The two plates were heated at 95°C during 25 min. Then the reaction was stopped by placing the plates on ice. Plates were centrifuged during 10 min at 3000 g. Absorbance of the two supernatants were read at 440, 532 and 600 nm. Lipid peroxidation was calculated using the following equations (Hodges *et al.*, 1999):

1) [(Abs 532+TBA) - (Abs 600+TBA) – (Abs 532-TBA – Abs 600 – TBA)] = A

2) [(Abs 440 + TBA – Abs 600 + TBA) * 0.0571] = B

3) MDA equivalents (nmol/ml) = (A – B / 157000) * 106
Results were expressed as nmol per gram of fresh weight (nmol/g f.wt.).

4.3 Plant biomacromolecules with Fourier Transformed InfraRed (FTIR) spectroscopy

Like Raman spectroscopy, Fourier transformed infrared (FTIR) spectroscopy is based on the vibrational theory of molecules. Difference between Raman and IR spectroscopy is that Raman is a scattering technique while IR spectroscopy is an absorption technique. IR operates with a polychromatic source unlike Raman using a monochromatic source. From the source, the sample absorbs specific frequencies corresponding to its molecular vibrational transitions. Several sources can be used for FTIR spectroscopy (*e.g.* synchrotron, conventional thermal or alternative source such as quantum-cascade lasers) (Baker *et al.*, 2014). The beam enters the sample compartment where it is transmitted through or reflected by the surface of the sample (Figure 26A).



Figure 26 Scheme of a FTIR spectrometer. B. Schematic representation of the three main sampling modes for FTIR spectroscopy. Both images are from Baker et al., 2014.

Molecules will absorb infrared radiation with a vibrational frequency depending on the strength and polarity of the vibrating bonds. The signal is also influenced by intra- and intermolecular effects (Türker-Kaya and Huck, 2017). The beam finally passes through the detector for final measurement. The measured signal is sent to the computer where the Fourier transformation is calculated. The Fourier transformation allows decomposing the signal into its constituent frequencies. A background spectrum is measured to set up a relative scale for the absorption intensity (Siesler, 2002).

There are several sampling modes for FTIR spectroscopy (Figure 26B). In transmission mode, the sample is illuminated by the source and the detector is placed behind the sample. It is more suitable for very thin samples (<10 μ m). In transflection (or reflection) mode, the sample is placed on the same side as the detector. The detector records the signal reflected by the sample (Siesler, 2002; Türker-Kaya and Huck, 2017). Finally, the last sampling mode is the attenuated total reflection (ATR). With ATR, the sample is placed on a crystal with an index of refraction larger than the one of the sample. The source is coupled into the crystal and directed towards the sample surface. The detector records the measuring light that leaves the sample after one or several reflections. At the interface between the sample and the crystal, light penetrates into the sample: it is called the evanescent wave. It may be absorbed by the sample and thus the light reaching the detector carries the information about the IR spectrum of the sample (Barth, 2007).



Figure 27 Typical biological spectrum showing the main biomolecular peaks from 3,000 to 800 cm⁻¹, where v = stretching vibrations, δ = bending vibrations, s = symmetric vibrations and as = asymmetric vibrations. Image from Baker et al., 2014.

Applied to the analysis of biological materials, FTIR gives semi-quantitative and qualitative analysis of the biomolecules of the sample. FTIR presents several advantages for the analysis of biological tissues like providing direct and non-destructive examination while maintaining native compositions of samples without the need of extraction, purification and separation steps. In plant tissues, the most important spectral regions are typically the regions between 3,500 and 2,550 cm⁻¹ associated to the lipids, the amide I and II regions (1,700 and 1,500 cm⁻¹) and between 1,450 and 600 cm⁻¹, (Baker *et al.*, 2014) (Figure 27).

For the analysis, leaves were dried overnight at 50°C and about 20 mg were ground using a FastPrep grinding machine (2 x 15 sec at maximum speed). Each powdered sample was analyzed in ATR-mode using a diamond crystal (Thermo Nicolet, Nexus, Smart Orbit) in (technical) triplicates and one spectrum was the

sum of 64 scans. The infrared spectra were collected from 4000 cm⁻¹ to 400 cm⁻¹. Technical detailed are presented in Table 6. OMNIC software was used to export experimental spectra (ThermoFisher Scientific).

Detector	Source	Range	Gain	Optical velocity	Aperture	Nb of scans	Resolution	Data spacing	Final format
DTGS KBr	IR	4,000 - 400 cm ⁻¹	8	0.6329	100	64	4 cm ⁻¹	0.964 cm ⁻¹	Absorbance

Table 6 Technical parameters used for the FTIR spectroscopy analysis at ESRF.

FTIR data acquisition generates a huge amount of data. This makes the data treatment complicated. That is why in this study, a special attention has been paid to FTIR data processing. A principal component analysis (PCA) followed by a linear discriminant analysis (LDA) was first applied to the data in order to identify differences among conditions. Then if differences were detected, a linear regression was performed in order to identify where the differences could be found in the spectra. All data processing was performed using Orange Software (Demšar *et al.*, 2013). Data acquisitions have been done at ESRF in Grenoble in collaboration with Hiram Castillo-Michel and Juan Reyes-Herrera (Chapter 5: Assessing plant response to 2 NMs using FTIR spectroscopy).

CHAPTER 3: CNT CHARACTERIZATION

Table 7 Summary of the physicochemical characteristics of the different CNTs using TEM, micro-analyzers, ICP-AES, BET method, Raman analysis, TGA and zeta potential analysis. * Median from Flahaut et al., 2003. ** data from providers

	DWCNTs	DWCNTs f	MWCNTs	MWCNTs f	Short MWCNTs	MW ¹³ CNTs
Catalysts used	Co/Mo	o-MgO	Fe,	/Ni	Fe/Co	Co/Mo-MgO
Inner diameter (nm)	1.35*	1.35*	9.4 ± 2.6	8.2 ± 2.9	5.6 ± 1.9	7.3 ± 3.2
Outer diameter (nm)	2.1 ± 0.7	2.0 ± 0.8	24.2 ± 8.6	23.4 ± 10.8	12.3 ± 4.0	16.8 ± 6.7
Length (microns)	1 to 100	1 to 100	1 to 20**	1 to 20**	<0.5**	1 to 20
Specific Surface Area (m²/g)	985	240	90	90	60	293
Elemental analysis (CNOS)	C 89.75 % O 2.13%	C 76.93% O 17.25% N 0.30%	C 94.88% O 0.56%	C 93.16% O 1.92% N 0.10%	C 87.65% O 2.18% N 0.22% S <0.20%	C 85.96% O 3.27%
Elemental analysis (Metals from catalysts)	Co 3.99% Mo 0.96%	Co 0.99% Mo 0.12%	Fe 0.21% Ni 1.90%	Fe 0.32% Ni 1.28%	Fe 0.18% Co 0.66%	Co 3.69% Mo 0.41%
I_D/I_G intensity ratio	0.23 ± 0.00	0.22 ± 0.00	1.28 ± 0.00	1.18 ± 0.00	1.77 ± 0.02	1.17 ± 0.05
Zeta potential in DI water (mV)	-27.5	-40.4	-17.8	-41.5	-18.7	-29.3

1. CHEMICAL ELEMENTAL ANALYSIS

1.1. Bulk analysis

C, O, S and N were the four analyzed elements (Table 8). Obviously, C was the main element in the CNT samples: 89.75 wt. % for DWCNTs, 94.88 wt. % for MWCNTs, 76.93 wt. % for DWCNTs f, 93.16 wt. % for MWCNTs f, 87.65 wt. % for short MWCNTs and 85.96 wt. % for MW¹³CNTs. Then O was found in the different CNTs: 2.13 wt. % for DWCNTs, 0.56 wt. % for MWCNTs, 17.25 wt. % for DWCNTs f, 1.92 wt. % for MWCNTs f, 2.18 wt. % for short MWCNTs and 3.27 wt. % for MW¹³CNTs. Functionalized CNTs had a higher oxygen content due to the carboxylic groups (-COOH) attached during the oxidation process than the raw CNTs. In minor quantities, N was detected in DWCNTs f (0.30%) and in short MWCNTs (0.22 wt. %) as well as in MWCNTs f (0.10 wt. %). The presence of N in functionalized samples may be explained by the use of nitric acid for their oxidation. The S content is negligible with less than 0.20 wt. % for the short MWCNTs and below the detection limit for all other samples.

Table 8 Elemental analysis (C, O, N and S) of the five CNT samples used (in wt. %). For orange boxes, analysis was not done because those elements were not expected. UDL = under detection limit.

CNTs	C (wt. %)	O (wt. %)	N (wt. %)	S (wt. %)
DWCNTs	89.75	2.13	UDL	
DWCNTs f	76.93	17.25	0.30	
MWCNTs	94.88	0.56	UDL	
MWCNTs f	93.16	1.92	0.10	
Short MWCNTs	87.65	2.18	0.22	<0.20
MW ¹³ CNTs	85.96	3.27		

The metal content was also analyzed: only the catalysts known to have been used for the synthesis were controlled (Table 9). For DWCNTs, results indicated 3.99 wt. % and 0.96 wt. % of Co content and 0.99 wt. % and 0.12 wt. % for Mo content for raw and functionalized CNT respectively. The decrease in content for both metals in the functionalized tubes is explained by the dissolution during the oxidation process. For the MWCNTs from Cheaptubes, 0.21 wt. % of Fe and 1.90 wt. % of Ni were found for raw MWCNTs, to be compared to 0.32 wt. % of Fe and 1.28% of Ni for functionalized MWCNTs. For the short MWCNTs from Nanografi, 0.18 wt. % of Fe content and 0.66 wt. % of Co content were quantified. Finally, the MW¹³CNTs contained 3.69 wt. % of Co and 0.41 wt. % of Mo.

CNTs	Co (wt. %)	Mo (wt. %)	Fe (wt. %)	Ni (wt. %)
DWCNTs	3.99	0.99		
DWCNTs f	0.96	0.12		
MWCNTs			0.21	0.19
MWCNTs f			0.32	1.28
Short MWCNTs	0.66		0.18	
MW ¹³ CNTs	3.69	0.41		

Table 9 Elementary metal analysis (Co, Mo, Fe and Ni contents) of the five CNT samples used (in wt. %). For the green boxes, elements were not quantified. Quantification was done only on elements that were expected according to the catalyst used.

The presence of metals in the CNT samples is due to the reduction of catalyst precursors during the synthesis, producing nanometer-sized metal particles acting as the actual catalyst for the growth of CNTs. However, generally during the cooling down step, some of these catalytic metal nanoparticles end-up coated by graphitic shells (Flahaut *et al.*, 2002). The presence of these impurities may influence the properties, behavior and toxicity of CNT samples (Ge *et al.*, 2012). For example, it has been proven that the amount of metal impurities in CNTs influenced their redox properties (Pumera and Miyahara, 2009; Pumera, Ambrosi and Chng, 2012). Despite their apparent encapsulation, metals may be released and directly interact with the surrounding environment. Preparation protocols of CNTs can mobilize those metals and make them bioavailable (Lin Guo *et al.*, 2007). Hull et al. (2009) showed that carbon NM released metal impurities which increased their aquatic toxicity. Catalysts of the NM used were Cu and Fe. In plants, Miralles et al. (2012) have demonstrated that the impurities and not solely the CNTs impacted the plants by enhancing germination and seedling growth. Fe was the catalyst used for the synthesis. Fe was present at 5.3 wt%.

In our study, remaining metal catalysts were present in all the CNTs tested and may affect their toxicity on plants. In the case of DWCNTs, there are some evidences that the encapsulated Co present in the samples is not oxidized in air even when stored in air at room temperature, which supports the tight encapsulation and makes the release in water rather unlikely (Flahaut *et al.*, 2002).

1.2. Surface atomic composition using X-Ray Photoelectron Spectroscopy (XPS)

To begin, a survey was performed on each sample in order to identify what were the detectable elements. After element identification, a high resolution analysis was carried out for each element (Table 10). For DWCNTs f, MWCNTs, MWCNTs f, short MWCNTs and MW¹³CNTs only C and O were found. For DWCNTs, C, O but also Cl, Co and Mo were detected. As expected, for all the CNTs, C is a major element: 98% for DWCNTs, 90% for DWCNTs f, 99% for MWCNTs, 93% for MWCNTs f, 98% for short MWCNTs and 87% for MW¹³CNTs. O is the second element for all the CNTs: 2% for DWCNTs, 10% for DWCNTs f, 1% for MWCNTs, 7% for MWCNTs f, 2% for short MWCNTs and 7% for MW¹³CNTs. O content was higher for the functionalized CNTs compared to the raw equivalents, as expected. Minor elements (less than 1%) for

DWCNTs were found such as Co, Cl and Mo. Co and Mo corresponds to remaining catalysts. A possible explanation for traces of Cl could be the processing of the CCVD composite powder by HCl, used to dissolve the catalytic support. Traces of Cl could come from a tiny amount of Cl ions still adsorbed after the washing of the samples.

Mass percentages were calculated from the atomic percentages and compared with the values obtained with the chemical analysis. XPS analysis is a technique that quantifies the atomic composition of a sample but only at the surface of the material (depth from 1 to 5 nm) while the chemical analysis with microanalyzers and ICP-AES quantify the composition of the whole sample. In this study, both analyses are interesting. Indeed, the surface of the CNT is in direct contact with the soil and the roots of the plants and if CNTs can penetrate into the plants, the whole CNT will interact with the plant constitutive elements. Most of the time mass percentages obtained with the XPS analysis were higher. Remaining catalysts were found only in DWCNTs using XPS while remaining catalysts were found in all the samples using ICP-AES. It implies that remaining catalysts were present deeper in the material, which makes them probably not directly available for plants. The experimental errors related to XPS analysis are much higher than usual chemical elemental analysis, and this also has to be considered.

Table 10 XPS results for the different CNTs with the peak energy of the different elements (C, O, Co, Cl and Mo), the area under the peak, the atomic percentage, the mass percentage corresponding and the mass percentage from the chemical elemental analysis.

CNTc	Flomonts	Peak energy	Area under the	Atomic	Mass	Elemental Analysis
CIVIS	Liements	(eV)	peak	%	%	(Mass %)
	С	284.34	60959.44	97.68	95.49	89.75
	0	532.48	2801.53	1.83	2.39	2.13
DWCNTs	Со	778.28	1566.21	0.19	0.91	3.99
	Cl	199.55	348.27	0.23	0.67	
	Мо	231.95	401.17	0.07	0.55	0.99
DWCNTs f	С	284.27	51151.53	90.40	87.59	72.25
	0	532.32	13346.63	9.60	12.41	17.25
MWCNTs	С	284.29	68135.46	98.79	98.39	94.88
	0	532.10	2043.21	1.21	1.61	0.56
MWCNTs f	С	284.16	47883.33	93.38	91.36	93.16
	0	532.48	8337.85	6.62	8.64	1.92
Short	С	284.16	59830.24	97.71	96.67	87.55
MWCNTs	0	532.46	3443.64	2.29	3.03	2.10
MW ¹³ CNTs	C	284.21	42246.13	86.88	90.80	85.96
	0	532.48	8337.85	6.62	9.20	3.27

2. CNT MORPHOLOGY



Figure 28 TEM pictures of the different CNTs. A and B for DWCNTs, C and D for DWCNTs f, E and F for MWCNTs, G and H for MWCNTs f, I and J for short MWCNTs and K and L for MW¹³CNTs

Pictures of the different CNTs were taken using TEM (Figure 28). Inner and outer diameters were determined by measuring 150 individual tubes. The DWCNTs had a mean outer diameter of 2.1 ± 0.7 nm and the DWCNTs f of 2.0 ± 0.8 nm with for both most of the tubes with a diameter between 1 and 2 nm (Figure 29A and 29B). Due to their small diameter, it was not possible to determine the size of the inner diameter with the TEM used. The MWCNTs had a mean outer diameter of 24.2 ± 8.6 nm with 63% of the tubes with an outer diameter between 15 and 30 nm (Figure 29C). They had a mean inner diameter of 9.4 ± 2.6 nm with most of tubes with an inner diameter between 5 to 15 nm (Figure 29C). The MWCNTs f had a mean outer diameter of 23.4 ± 10.8 nm with most of tubes with an outer diameter between 15 and 30 nm (Figure 29D). They had an inner diameter of 8.2 ± 2.9 nm with most of tubes with an inner diameter between 5 to 15 nm (Figure 29D). They had an inner diameter of 8.2 ± 2.9 nm with most of tubes with an inner diameter between 5 to 15 nm (Figure 29D). They had an inner diameter of 8.2 ± 2.9 nm with most of tubes with an inner diameter between 5 to 15 nm (Figure 29D). The short MWCNTs had a mean outer diameter of 12.3 ± 4.0 nm and a mean inner diameter of 5.6 ± 1.9 nm. Most of the tubes had an inner and an outer diameter between 5 and 15 nm (Figure 29E). Finally, MW¹³CNTs had an outer diameter of 16.8 ± 6.7 nm (Figure 29F). They had an inner diameter of 7.3 ± 3.2 nm with most of the tubes between 5 and 15 nm (Figure 29F).





>30

10 0

<5

5-15

15-30

>30

10

0

<5

5-15

15-30

It was not possible to estimate the mean length of most of the CNTs using TEM because they were either too entangled or overlapping with the copper grid. The length was taken from the producer characteristics for MWCNTs and MWCNTs f (length indicated between 1 to 20 μ m). The length of DWCNTs and DWCNTs f was taken from earlier studies (1 to 100 μ m, Flahaut et al. 2003). The short MWCNTs were bought for

their claimed short length (<500 nm according to NanoGrafi). With TEM, it was possible to measure only the length of the CNTs smaller than 2000 nm (Figure 30).



Length of short MWCNTs

40% of the CNTs had a length between 500 and 1000 nm. However, 16% of the CNTs had a length longer than 2000 nm. In conclusion, short MWCNTs were significantly longer than what the producer announced, but most of them were still smaller than the other CNTs used in this work.

From the TEM images, it was possible to see some remaining catalysts (red arrows in Figure 28A). Raw DWCNTs had more remaining catalysts than the functionalized DWCNTs. It is consistent with the data from the elemental analysis: 3.99% of Co for the DWCNTs and 0.99% for the DWCNTS f. Indeed, the functionalization process allows the elimination of catalysts. The DWCNTs looked very long with a regular surface (Figure 28A, B, C and D) while the MWCNTs did not have a regular surface (Figure 28E, F, G and H). Their diameter was more heterogeneous (standard deviation of 8.6 nm for the outer diameter of the MWCNTs and 10.6 nm for the MWCNTs f). For the MWCNTs f, many tubes looked broken. It is reported in the literature that suspension preparation such as the use of ultrasonic probes can influence CNT structure (Kennedy *et al.*, 2009). In order to check the effects of the suspension preparation process, MWCNTs before and after the suspension preparation were observed using TEM (Figure 31).

Figure 30 Distribution diagram of the length for the MWCNTs shorts.



Figure 31 TEM pictures of the MWCNTs before (A and C) and after the suspension preparation (B and D).

After the suspension preparation (sonication with an ultrasonic probe and an ultrasonic bath), tubes were more fragmented. There is thus an important impact of the sonication on the tube structure. This was particularly the case for the MWCNTs but not so much for the other CNTs. This highlights the importance of carrying out the characterization of CNTs after the suspension preparation as we did in this work.

3. SPECIFIC SURFACE AREA (SSA)

The SSA of the DWCNTs was the highest and reached 985 m²/g (Table 11). The SSA of DWCNTs f was lower: 240 m²/g. For the MWCNTs and the MWCNTs f, the SSA was the same: 90 m²/g. For the short MWCNTs, we obtained 60 m²/g. Finally, for the MW¹³CNTs, the SSA was 293 m²/g.

CNTs	SSA (m²/g)
DWCNTs	985
DWCNTs f	240
MWCNTs	90
MWCNTs f	90
Short MWCNTs	60
MW ¹³ CNTs	293

Table 11 Specific Surface Area (SSA) in m²/g of the five CNT samples used.

The SSA is influenced mainly by the number of walls/diameter, bundling and agglomeration, impurities, and the surface functionalization (Birch *et al.*, 2013). DWCNTs had the highest SSA due to their small diameter and low number of walls. The SSA decreased when the surface of the tubes was functionalized. Indeed, when tubes are functionalized, they strongly agglomerate upon drying and this is likely to prevent the access of nitrogen to the surface, leading to a lower SSA. Lower SSA was found for the MWCNTs due to their larger diameter and higher number of walls. The SSA of the MW¹³CNTs was higher than any other MWCNT sample, probably due a favorable combination of outer diameter and number of walls (unfortunately, the number of walls was not possible to measure with the used TEM).

The SSA could be a parameter influencing the toxicity of CNTs: indeed, a CNT with a higher SSA should be more reactive than a CNT with a smaller one because the ratio between the number of atoms at the surface and in the bulk increases exponentially with decreasing particle size, making the reactivity more and more important (Auffan *et al.*, 2009). It is also good to keep in mind that the more walls a CNT has, the heavier it is, meaning that for a given weight concentration, the actual number of nanotubes present in the exposure suspension is larger when the SSA is higher (Peigney *et al.*, 2001). For these reasons, it is expected that the number of DWCNTs is higher than the number of MWCNTs for the same weight concentration. In aquatic ecotoxicology, the SSA was proven to be the best dose metric for a more realistic assessment (Mottier *et al.*, 2016). Authors compared the toxicity of different carbon NMs including CNTs on the growth of *Xenopus laevis* after *in vivo* exposure. They tested several dose metrics such as mass concentration (mg/L), number concentration (number of particles/L) and SSA concentration (m²/L). They established that the most relevant descriptor factor of toxicity was the SSA concentration. Likewise, in human toxicity, similar conclusions have been reached (Stoeger *et al.*, 2006; Hull *et al.*, 2012).

4. STRUCTURAL DEFECTS AND ¹³C ENRICHMENT DETERMINATION

Raman spectroscopy allows characterizing CNTs by looking at the structural defects and the ${}^{13}C/{}^{12}C$ isotopic enrichment ratio. Analyses were performed on the MWCNTs theoretically enriched with 2 % wt. of ${}^{13}C$ and 50 % wt. of ${}^{13}C$ and the MWCNTs with ${}^{12}C$ with a 633 nm laser. Figure 32 displays representative spectra of the different types of CNT used (between 50 and 3,000 cm ${}^{-1}$).



Figure 32 Raman spectra of the different CNT used (DWCNTs, MWCNTs, short MWCNTs, MWCNTs ¹²C, MWCNTs 2% ¹³C and MWCNTs 50% ¹³C) between 50 and 3,000 cm⁻¹ using a 633 nm laser.

Table 12 Raman shift of the D and G bands with the ratio between the intensities of the D and G bands (Ig/Ig).	
Values are mean of 10 spectra.	

	D Raman shift (cm ⁻¹)	G Raman shift (cm ⁻¹)	I _D /I _G
DWCNTs	1316 ± 2	1584 ± 0	0.23 ± 0.00
DWCNTs f	1314 ± 1	1584 ± 0	0.22 ± 0.00
MWCNTs	1328 ± 1	1579 ± 1	1.28 ± 0.00
MWCNTs f	1330 ± 0	1581 ± 0	1.18 ± 0.00
Short MWCNTs	1328 ± 0	1603 ± 3	1.77 ± 0.02
MW ¹² CNTs	1321 ± 3	1582 ± 4	1.17 ± 0.05
MW ¹³ CNTs 2%	1323 ± 1	1580 ± 6	1.21 ± 0.07
MW ¹³ CNTs 50%	1301 ± 0	1566 ± 6	1.20 ± 0.06

Raman spectra of the DWCNTs were specific with a D band around 1315 cm⁻¹ and a G band around 1583 cm⁻¹ with a much higher intensity than the other CNTs. The intensity ratio between the D and the G bands was *ca*. 0.22. For the MWCNTs, the D and G bands were located respectively at *ca*. 1328 cm⁻¹ and 1579 cm⁻¹, respectively. The intensity ratio between the bands was close to 1.28. For the short MWCNTs, the D band was measured at 1328 cm⁻¹ and the G band around 1603 cm⁻¹. The intensity ratio was *ca*. 1.77.

The ratio of intensities between the D and the G bands is a good indicator of a sample quality (Costa *et al.*, 2008). Similar intensities between these two bands indicate a high ratio of structural defects. For the DWCNTs, ratios were lower in comparison to the other CNTs (17% of the value measured for MWCNTs). DWCNTs thus had much less structural defects than the other samples. In comparison, MWCNTs f with a ratio close to one were the CNTs presenting the more structural defects. In general, due to their multiple graphene layers MWCNTs are more prone to this type of defects than the DWCNTs and the SWCNTs (Costa *et al.*, 2008).

Those structural defects can affect CNT properties (He and Pan, 2009; Domínguez-Rodríguez *et al.*, 2016). Indeed, it has been shown that when structural defects are present, CNTs tend to interact more with adsorbates nearby such as hydrogen. The same is true for ions as well. Structural defects offer preferential adsorption sites (lower surface energy). Thus, CNT reactivity can change with the presence of structural defects, modifying their behavior in the soil and potentially their effects on plants.

For the CNTs synthetized with ethanol (MW¹²CNTs, 50% MW¹³CNTs and 2% MW¹³CNTs), the position of the G band was used to determine the ¹³C enrichment. Enriched samples were synthesized at CIRIMAT using the CVD method with ¹³C-enriched ethanol. The theoretical enrichments of these two samples were 2% and 50% (according to the amount of ¹³C-enriched ethanol used for their synthesis). Equations presented in chapter 2 were used to determine the effective enrichment rate. It was not possible to determine the enrichment of the 2% ¹³C because of the high standard deviation found for the position of the G band. The standard deviation was 6.44 cm⁻¹ which represents almost 10% of enrichment. The calculation for the 50% ¹³C sample was performed as follows:

$$x = 13 - \frac{12}{\left(\frac{dw}{w_{x=1}} + 1\right)^2}$$
$$x = 13 - \frac{12}{\left(\frac{1565 - 1582}{1582} + 1\right)^2} = 0.7376$$

 $13C \ enrichment = 1 - x = 1 - 0.7376 = 0.2624$

We determined a 13 C enrichment of 26 ± 9% for the MWCNTs 50% 13 C.

5. THERMAL STABILITY

After dry-freezing, the different CNTs were analyzed by TGA (Figure 33). A thermogram is a superposition of mass loss due to the oxidation of carbon into gaseous carbon dioxide and the mass gain due to the oxidation of residual metal catalyst into solid oxides (Stefov, Najdoski and Bogoeva-gaceva, 2015).



Figure 33 Thermograms of the different CNTs showing the mass variation Δm (%) and the derivative mass dTG (%/min).

CNTs	Final mass loss Δm (%)	Temperature mass loss (°C)
DWCNTs	-93.3	421
DWCNTs f	-97.6	449
MWCNTs	-95.1	560
MWCNTs f	-96.4	562
Short MWCNTs	-92.9	527
MW ¹² CNTs	-29.9 and -95.1	380 and 483

Table 13 Comparison of the thermogravimetric results of the different CNTs with the final mass loss (%) and the temperature mass loss (°C).

Both DWCNTs were thermally stable up to ca. 310°C. After that, the process of decomposition was a relatively fast. The temperature of maximum rate of decomposition of the DWCNTs was 421°C and for the DWCNTs f it was 449°C. The higher temperature found for the DWCNTs f was due to the purification of the CNTs which takes place during the oxidation: metals and metals oxides are known to catalyze the combustion of carbon and their partial removal during the oxidation step thus improves their thermal stability. The weight loss before 300°C was more important in the case of DWCNTs f because they lost oxygen-containing functions present on their surface (thermal decomposition). A final mass loss of 93.3% and 97.6% was found respectively for DWCNTs and DWCNTs f. Because DWCNTs f contained less residual metals, they also contained less ashes in the end. Both MWCNTs were thermally stable up to around 420°C. The temperature of maximum rate of decomposition was also higher for the functionalized compared to the raw but the difference was much lower for the MWCNTs in comparison to the DWCNTs (-27°C for the DWCNTs and -2°C only for the MWCNTs. The short MWCNTs were found stable up to around 420°C like the other MWCNTs but the temperature of maximum rate of decomposition happened at lower temperature (527°C). The final mass loss was the lowest in comparison to the other CNTs (92.9%). And finally for the MW¹³CNTs, a first mass loss was identified at 380°C with a mass loss of 29.9% and a second mass loss was found at 483°C with 95.1% of mass loss. This is likely to correspond to 2 categories of carbon species within this sample, but TEM observations did not give any clue about this.

6. ZETA POTENTIAL

The zeta potential was obtained with CNTs in suspension in DI water at pH 6.7 (Table 14). The zeta potential of DWCNTs was -27.5 mV while it was -40.4 mV for the DWCNTs f. For the MWCNTs, it was -17.8 mV and for the MWCNTs f, -41.5 mV. For the short MWCNTs, the zeta potential was -18.7 mV. Finally, for the MW¹³CNTs, it was -29.3 mV. In order to assess the influence of the dispersion medium, the zeta potential was also measured for DWCNTs in Hoagland medium containing 100 mg/L of CMC (exposure medium for hydroponic experiments). We found a zeta potential of -59.5 mV. The zeta potential of DWCNTs was also measured in a LUFA soil solution in order to have an idea about the behavior of CNTs in the soil. A zeta potential of -32.1 mV was measured.

CNTs	Zeta potential in DI water (mV) pH 6.7	Zeta potential in Hoagland with CMC 100 mg/L (mV) pH 6.7	Zeta potential in LUFA soil solution (mV) pH 6.11
DWCNTs	-27.5	-59.5	-32.1
DWCNTs f	-40.4		
MWCNTs	-17.8		
MWCNTs f	-41.5		
Short MWCNTs	-18.7		
MW ¹³ CNTs	-29.3		

Table 14 Zeta potential values in mV in DI water, in Hoagland medium containing 100 mg/L of CMC and in a LUFA soil solution

Zeta potentials of functionalized CNTs were lower than for the raw CNTs. A higher absolute value is associated with a higher stability of the particles in suspension due to better electrostatic repulsion. Particles with a zeta potential of more than +30 mV or less than -30 mV are usually considered as stable (Gupta and Trivedi, 2018). In this study, functionalized CNTs (both DW and MW) had a zeta potential lower than -30 mV. It has been shown that the functionalization of CNTs makes them more hydrophilic which makes them more stable in suspension (Ernst *et al.*, 2017). MW¹³CNTs were found to have a zeta potential close to -30 mV. They were indeed more stable in suspension than the other raw CNTs but still much less stable than the functionalized CNTs. The highest zeta potential was found for the DWCNTs in the Hoagland medium with the addition of CMC. This evidences that the CMC allows the CNTs to be stable in suspension. The zeta potential of the DWCNTs in soil solution was found close to -30 mV.

CHAPTER 4: DETECTION OF CNTS IN PLANT MATRIX

The detection and quantitative analysis of CNTs in biological samples is very complex because it is difficult to detect a specific form of carbon in a carbon based matrix. In the literature, many different techniques are used to detect CNTs in plants (*e.g.* Raman spectroscopy, hyperspectral imaging, *etc.*). However, for each technique, only a limited number of studies is available and the robustness of the technique is thus rather unknown. Testing and calibration procedures are usually not described, and replication of approaches proposed in earlier studies are not available. Furthermore, they often used plant tissues with high CNT concentrations which is not representative of what we can find in the environment. In this article, we aimed to investigate different techniques to detect CNTs in plants. We choose to study only leaves of the plant in order to have more realistic concentrations of CNTs. We used several techniques: Raman spectroscopy, transmission electronic microscopy, broadband microwave biosensor, two photon excitation microscopy, hyperspectral imaging, synchrotron micro X-Ray fluorescence, isotope ratio mass spectroscopy, micro ion beam analysis and autoradiography. For that, cucumber plants were grown in hydroponic conditions at 100 mg.L⁻¹ of DWCNTs or MWCNTs during 15 days.



Figure 34 Graphical scheme resuming the experiments done for the CNT detection

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ARTICLE: CNT DETECTION IN PLANT MATRIX

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1. INTRODUCTION

CNT market is still expected to grow to reach 9 billion dollars by 2023 (MarketsandMarkets[™], 2019). Since their use is spreading, their concentration in the environment is therefore expected to increase. In addition to that, CNTs may be intentionally release into the environment since one of their foreseen applications could be as growth regulator in agriculture. Furthermore, there are some evidences that CNTs may persist in the environment (Bjorkl, Tobias and Petersen, 2017). Their detection and quantification in the environmental and biological matrices is an important question and still represent a major technical bottleneck (Petersen *et al.*, 2016). This is due to diverse technical reasons: (i) it is complicated to detect one specific form of carbon (CNTs) in a carbonaceous background, (ii) there is a huge diversity of CNTs (different lengths, diameters, number of layers, presence of functional groups or not, different agglomeration states), and it is therefore tricky to develop one universal analytical method for all types of CNTs (Petersen *et al.*, 2016; Clarisse Liné, Larue and Flahaut, 2017); (iii) finally, within a given batch of CNTs, nanotubes also usually have different lengths, diameters and/or number of layers.

Most of the time, in studies detecting CNTs, authors are using nonrealistic exposure conditions. Experiments are mostly conducted in hydroponics with high CNT concentration (up to 5 g/L). Many analytical techniques have been tested to detect CNTs in complex environmental media (Petersen *et al.*, 2016, 2019; Clarisse Liné, Larue and Flahaut, 2017). However, for each technique, only a limited number of studies is available and the robustness of the technique is thus rather unknown. Testing and calibration procedures are usually not described, and replication of approaches proposed in earlier studies are not available. We have reviewed here the detection methods for CNTs in plants proposed in the literature. Over 33 publications, 17 different techniques were investigated. The two most used techniques were Raman spectroscopy and TEM (Figure 35A). TEM is a technique which is nowadays highly available for laboratories. Sample preparation can be challenging for tissues but data acquisition and treatment are very simple. Raman spectroscopy is widely used for CNT characterization. Furthermore, the sample preparation is minimal and the technique is compatible with *in vitro* and *in vivo* samples. Other less widespread techniques such as microwave-induced heating, fluorescence, IR and FTIR spectroscopies were described.



Figure 35 Literature review (33 publications from 2008 to 2018) of the different techniques used to detect CNTs in plants (A) and the different studied plant organs (B). TEM = transmission electronic microscopy, SEM = scanning electron microscopy, MIH = microwave induced heating, IR = infrared spectroscopy, FTIR = Fourier transformed infrared spectroscopy.

On average, between 1 or 2 organs were studied in this set of 33 articles. 21 studies analyzed only 1 organ, and 5 analyzed 3 parts of the plant. The most analyzed organs were roots and then leaves (Figure 35B). When exposures are made by roots, CNT concentration is higher in the roots compared to the other plant organs. CNT detection is thus easier in roots. Furthermore, in roots, there is less background signal than in the other plant organs presenting chlorophylls which make the analysis easier with spectroscopic techniques for example.

The aim of this chapter was to review some of the main techniques used for the detection of CNTs in plants, and to address the associated bias, limitations and robustness. We chose to perform all the experiments in hydroponic conditions to relatively high CNT concentration (100 mg/L) to be in "optimal" conditions for CNT uptake. This exposure type is often used in nanoecotoxicology research since it is less complex than exposure in soil and usually ensures a greater availability of NMs to plants (Petersen *et al.*, 2019).

An issue faced during these experiments was how to maintain a constant CNT concentration for plants, since suspensions were not stable in most cases. CNTs agglomerated and settled down very quickly in deionized water, and even faster in Hoagland medium, quickly limiting the exposure to CNTs. In order to overcome this problem, a dispersant was added to stabilize the CNT suspensions: carboxymethylcellulose (CMC) as already described in chapter 2 (1.5.2). CMC is nontoxic and allowed the suspension to be stable for few days (Bourdiol *et al.*, 2013).

We have deliberately chosen to focus on the detection of CNTs in the upper part of the plants. Indeed, during hydroponic exposure, CNTs are in direct contact with the roots, where they can massively attach and thus contaminate root inner tissues during sectioning (Deng, White and Xing, 2014). In aboveground tissues, there is no such problem since the tissues are not in direct contact with the contaminant; the results reflect then the reliable quantity of CNT transfer to the leaves.Briefly, for the experimental set-up,

cucumber seedlings were exposed in hydroponics to 100 mg/L of CNTs (+ 100 mg/L CMC) during 15 days. Three different CNTs were used and all synthesized at CIRIMAT (as detailed in chapter 2): (i) raw DWCNTs; (ii) raw DWCNTs, with external grafting of ¹⁴C; (iii) MWCNTs with different enrichment rate of ¹³C (theoretically 2 or 50%). Before presenting the results of the different techniques used, the morphological impacts of these two kinds of CNTs (DWCNT (outer diameter of 2.1 nm) *vs* MWCNT (outer diameter of 16.8 nm)) are presented.

2. MORPHOLOGICAL IMPACTS

The experimental set-up used for this chapter was already described in chapter 2. Briefly, cucumber seedlings were exposed in hydroponics to 100 mg/L of CNTs during 15 days. Three different CNTs were used and all synthesized at CIRIMAT (as detailed in chapter 2): (i) raw DWCNTs; (ii) raw DWCNTs, with external grafting of ¹⁴C; (iii) MWCNTs with different enrichment rate of ¹³C (theoretically 2 or 50%). Before detailing the results of the different techniques used, the morphological impacts of these two kinds of CNTs (DWCNT (outer diameter of 2.1 nm) *vs* MWCNT (outer diameter of 16.8 nm)) will be presented.

2.1. Effects of DWCNTs

After 15 days of exposure, morphological parameters such as height of the plant, root system length and fresh and dry biomasses were measured (Figure 36).

No significant difference was observed between the control and the exposed plants for any of the investigated parameters. At the end of the experiment, all plants had 2 leaves. The height of the cucumber plant was on average 2.96 cm for the control and 3.22 cm for the exposed plants. The total fresh biomass was around 2 g for both conditions with a similar water content of 93%. The ratio between the dry root biomass and the dry leaf biomass was 0.73 for the control plants and 0.63 for the exposed plants.



Figure 36 Morphological parameters (height, root size, fresh and dry biomass (leaves, roots and total), water content and ratio roots/leaves) of the control cucumber plants and the cucumber plants grown in the DWCNT suspension at 100 mg/L during 15 days. Results were the average of 6 experiments (with 3 replicates per condition, per experiment *i*.e. 18 biological replicates). No significant difference was found, student test, p-value < 0.05.

2.2. Effects of MWCNTs





Figure 37 Morphological parameters (height, root size, fresh and dry biomasses (leaves, roots and total), water content and ratio roots/leaves) of the control cucumber plants and the cucumber plants grown in MW¹³CNT suspension at 100 mg/L during 15 days. Results are an average of 3 experiments with 3 replicates per condition for each experiment. No significant difference was found, student test, p-value < 0.05.

Similarly to DWCNT exposure, no significant difference was identified between the control and the exposed plants for any of the different morphological parameters. On average, plants in both conditions measured 3.23 cm. Control plants had a total fresh biomass of 1.97 g and the exposed plants of 2.32 g.

The dry biomass was on average 145 mg for both conditions and the water content of the entire plant was on average 93.15%. The ratio between roots and leaves was on average 0.43 for the control plants and 0.48 for the plants exposed to CNTs.

At the end of the exposure, we observed a slight depigmentation of the oldest leaf in the plants exposed to MW¹³CNTs in comparison to the control plants (Figure 38). We did not observe this depigmentation on the experiments with DWCNTs. Further chlorophyll quantification was not possible since those leaves were used for CNT detection experiments.



Figure 38 Pictures of the oldest cucumber leaf of the control plants (A and B) and the plants exposed to MW¹³CNTs at 100 mg/L during 15 days (C and D).



Figure 39 Leaf area of the oldest (F1) and the youngest (F2) leaves as well as the total leaf area of control plants and plants exposed to MW¹³CNTs at 100 mg/L during 15 days. No significant difference was found, student test, p-value < 0.05.

No significant difference was identified for the different leaf area (p-value = 0.7841 for F1, p-value = 0.4099 for F2 and p-value = 0.3172 for the total leaf area). However, there was a trend of having bigger leaf surface area for the plants exposed to the MW¹³CNTs (22% of increase for the total leaf area).

2.3. Discussion

No significant impact of the DWCNTs and MW¹³CNTs was observed on plant height, fresh/dry biomasses and water content of the plants. However, MW¹³CNTs lead to a leaf depigmentation after 15 days.

Stampoulis, Jet and Haven (2009) observed a reduction by 60% of zucchini biomass after an exposure to 1000 mg/L of MWCNTs during a hydroponic test. Begum *et al.* (2012) detected adverse effects on root and leaf morphology as well as growth inhibition and cell death on red spinach, lettuce and cucumber after a hydroponic exposure to MWCNTs up to 1000 mg/L during 15 days. Begum and Fugetsu (2012) observed a leaf color changing when red spinach were exposed to MWCNTs at 1000 mg/L. Ten times lower concentrations were used during our hydroponic assay which can explained why no significant impact was identified on the morphological parameters of the plants. Only visual impacts on the oldest leaf were observed. Also no study using DWCNTs in hydroponic conditions was found. However, the difference of impacts between the DWCNTs and the MWCNTs may be explained by the smaller length of MWCNTs. Indeed, DWCNT length was established between 1 to 100 μ m while MWCNTs had smaller length between 1 to 20 μ m. Shorter CNTs may be more internalized and translocated to the plants which could cause more damages (Liné, Larue and Flahaut, 2017).

3. CNT DETECTION

3.1. Raman spectroscopy

Raman spectroscopy is a widely used technique for the detection of CNTs in plants (S. Lin *et al.*, 2009; Khodakovskaya, Dervishi, Mahmood, Xu, Li, Watanabe and Alexandru S. Biris, 2009; Alimohammadi *et al.*, 2011a; Tripathi, Sonkar and Sarkar, 2011; Begum and Fugetsu, 2012b; Miralles *et al.*, 2012; Villagarcia *et al.*, 2012; Khodakovskaya *et al.*, 2012, 2013b; Larue *et al.*, 2012; Giraldo *et al.*, 2014; Ratnikova *et al.*, 2015; G. Chen *et al.*, 2015; Lahiani *et al.*, 2015a; Mohamed H Lahiani *et al.*, 2016; Das, You, *et al.*, 2018). Indeed, the Raman signature of CNTs is unique and usually rather intense. The fewer the number of walls, the higher the Raman signal, so DWCNTs are much easier to detect than MWCNTs. This technique presents several advantages. It gives access to qualitative information but could also be quantitative if a calibration is performed. It works with both *in vivo* and *in vitro* samples (Petersen *et al.*, 2016). In the studied literature, roots were the most analyzed organ using Raman spectroscopy followed closely by leaves and then seeds. The lateral resolution achieved is quite good and depends on the laser used.

Sample preparation is a key aspect of any experimental design. One of the major advantages of Raman spectroscopy is the ability to obtain precise spectral information with minimal sample preparation. However, it is essential to assess constraints related to sample preparation that can have an impact on the signal quality (e.q. type of substrates, sample flatness, sample stability) (Butler et al., 2016). Several sample preparation protocols can be used for the analysis of biological samples especially that Raman spectroscopy can apply both to bulk and 2D samples. Tissues can be analyzed fresh, in suspension or freeze-dried. According to the literature, fresh samples appear to be the best sample preparation for Raman analysis (Lyng, Gazi and Gardner, 2011; Butler et al., 2016) while freeze-drying could lead to a decrease in intensity (Lyng, Gazi and Gardner, 2011). However, many challenges may arise from the use of 2D samples (fresh and freeze-dried). The first one is the fluorescence signal which can be important with samples containing pigments such as chlorophylls. The fluorescence overlaps with the signal of analyzed compounds. The second problem can be the difficulty to obtain a flat sample. Indeed, the sample that would not be flat will lead to focusing problems and thus modifies the signal quality. Analyzing liquid samples permits to overcome the two last challenges but lead to the loss of 2D information. Other problems could also be faced such as the stability of the suspension. Das et al. 2018 developed a protocol for a digestion-Raman analysis approach to study MWCNTs in lettuce plants. The proposed protocol required a preliminary digestion of the plant tissues in nitric acid in order to minimize the fluorescence background.

In this work, we compared the two most promising sample preparations: fresh leaves and digested leaves.

The second issue that we had to face was the **laser** choice. In the literature, several lasers with different wavelengths were used to detect CNTs in plants: 514.5 nm, 532 nm, 633 nm, 785 nm or 1064 nm. The choice of the laser was not justified in any of the reviewed papers. There was also no obvious reason to select one wavelength over another. There are advantages and disadvantages to each laser wavelength

that have to be taken into consideration according to the study goal (Byrne *et al.*, 2015). A fluorescence background can be expected from biological samples, but fluorescence could also originate from the substrate or the microscope objective. This can be sorted out by using a laser that does not excite in these particular wavelength regions (between 400 and 700 nm) *i.e.* 1064 nm laser. Furthermore, due to the intense laser power used and the fact that biological samples are very fragile/sensitive, local thermal decomposition may occur (Butler *et al.*, 2016). This decomposition is more intense when using UV or visible lasers. To overcome these two challenges, a photobleaching can also be realized. The underlying mechanism is not well understood, but when the exposure to excitation light is prolonged it often results in a decrease in autofluorescence (Yang, Akkus and Creasey, 2017). A laser with a high excitation wavelength can also be used to decrease fluorescence such as the 1064 nm laser. However, when using this kind of laser, a decrease in intensity of the spectrum is likely to occur. Acquisition time has to be longer in order to compensate in comparison to a lower laser excitation wavelength. This may contribute to add noise in the Raman spectrum and can limit applicability (Yang, Akkus and Creasey, 2017). In this work, we compared two different lasers: 633 nm and 1064 nm.

3.1.1. Fresh leaf analysis

First, a suspension of DWCNTs was prepared at 100 mg/L in deionized water using the same sonication procedure as described in the chapter 2 (ultrasonic probe followed by ultrasonic bath) to be used as a reference sample to optimize the laser choice. This suspension was analyzed using the two lasers: 633 and 1064 nm (Figure 40A). Using the 633 nm laser, we obtained the classical Raman signature of DWCNTs with the D band around 1315 cm⁻¹ and the G band around 1580 cm⁻¹. With the laser 1064 nm, the G band was located at the same position, but the D band was observed around 1290 cm⁻¹ which represents a 25 cm⁻¹ shift. The D band position is known to vary according to the laser excitation wavelength (Dresselhaus *et al.*, 2002).

Then, a control cucumber leaf (no exposure to CNTs) was analyzed using the 633 nm laser (Figure 40B) showing a very strong fluorescence background (between 5000 and 15000 counts). Few drops of DWCNT suspension were added on the leaf surface and allowed to dry. Even if the amount of DWCNTs was locally very high (visible black spots), it was almost impossible to identify the signal arising from the CNTs (only a small hump is visible at 1590 cm⁻¹ in Figure 40B). Expectedly, it was not be possible to detect the presence of CNTs using Raman spectroscopy with this laser wavelength (633 nm) in the leaves of a plant grown in contaminated medium. Indeed, the concentration of CNTs should be much lower (0.005‰ of the applied CNT (Larue *et al.*, 2012) so here around 0.005 μ g/kg dry leaves).





Figure 40 Raman spectra of the DWCNT suspension and the entire leaf with the same suspension of DWCNTs using the 633 and the 1064 nm laser. (A) With the laser 633 nm: acquisition time 2 sec, 3 acquisitions; with the laser 1064 nm: acquisition time 20 sec, 3 acquisitions. For both no baseline correction. (B) For both spectra: acquisition time 2 sec, 3 acquisitions, and no baseline correction. (C) Acquisition time 60 sec, 3 acquisitions, baseline correction.

With the 1064 nm laser, the signal intensity was much lower (around 100 counts with the 1064 nm laser vs 20000 counts with the 633 nm) (Figure 40C). That is why we had to increase the acquisition time of the spectra (from 2 sec for the 633 nm laser to 60 sec for the 1064 nm laser), thus making possible the detection of the Raman signal of CNTs dropped on the surface of the leaf. Only the G band was detected, which is the band with the highest intensity. However, no signal could be detected when studying cucumber leaves grown in a 100 mg/L CNT suspension for 15 days.

3.1.2. Digested leaves

For this experiment, we only used the 633 nm laser, since there was no challenge about the fluorescence background. We first assessed the impact of the digestion protocol on the DWCNTs themselves. Indeed, earlier work from the group at CIRIMAT has evidenced that DWCNTs can be functionalized but also partly degraded during the interaction with HNO₃ in reflux conditions (130°C) (Bortolamiol *et al.*, 2014). The Raman analysis confirmed that the digestion, performed at 62°C during 12 hours significantly increased the D band relative intensity in comparison to the G band, from 0.23 for raw DWCNTs to 0.85 after digestion (Figure 41A). This ratio being a classical indicator of the sample structural quality, it is obvious that the digestion step significantly increased the amount of structural defects.



Figure 41 Effects of the digestion process on the suspension of DWCNTs (A) and detection of DWCNTs on control digested leaves and CNT-spiked digested leaves (B) both using 633 nm laser. For all the spectra: 60 sec of acquisition time, 3 acquisitions, baseline correction.

We reproduced the same conditions as in the protocol of Das et al. 2018: we spiked the digested leaves at a concentration of 25 mg of DWCNTs/kg of dry plant weight. Results of the control digested leaves and of the leaves spiked with CNTs are shown in Figure 41B. In the control digested leaf, no Raman signal was detected, and no fluorescence background was present. On the digested leaves spiked with CNTs, we were able to detect the signal of the CNTs with the two typical bands (D and G).


Figure 42 (A) Raman mapping (selected range between 1200 and 1700 cm⁻¹ corresponding to the two more intense Raman bands of DWCNTs) and (B) the corresponding picture with optical microscope (dark spots are the CNTs).

A mapping was performed on the spiked sample in order to increase the statistic of the analysis (Figure 42). The red spots on the map reveal the presence of DWCNTs in the digested sample.

However, when switching to the analysis of cucumber leaves grown in hydroponic conditions in the presence of DWCNTs (100 mg/L), we were not able to detect their presence. This was in contradiction with the study of Das et al. 2018 where they were able to detect MWCNTs on roots, stem and leaves of lettuce grown hydroponically with a maximum MWCNT concentration of 20 mg/L. In their study, they used different experimental conditions: different plant species, different CNTs and a longer exposure duration (18 days) which might explain this difference.

3.1.3. Discussion

Using Raman spectroscopy, it was possible to detect the presence of CNTs in plant leaf tissues, either using the 1064 nm laser on the fresh leaves to avoid chlorophyll fluorescence background or with the 633 nm laser on digested leaves. However, in the case of exposed plants (not spiked plants), it was not possible to evidence the presence of the DWCNTs, highlighting a lack of sensitivity for this technique when used under more "environmentally" relevant conditions.

Using the whole fresh leaf was convenient since it required no sample preparation. Nevertheless, a very high fluorescence background was detected and decreased further the sensitivity of the technique. In the literature, Raman spectroscopy was mostly used on whole fresh roots or seeds to detect CNTs. In these parts of the plant, less fluorescence background is present since there is no pigment such as chlorophylls. However, in few studies, the authors were able to detect CNTs in leaves using Raman spectroscopy (Begum and Fugetsu, 2012b; Larue *et al.*, 2012; Das, You, *et al.*, 2018). They did not report any problem related to fluorescence background. Begum and Fugetsu 2012 dried the fresh leaves before the analysis and used an acquisition time of 500 seconds (much higher than in our experiment) in order to detect CNTs with a 785 nm laser. However, increasing too much the acquisition time may add noise to the Raman spectrum. Larue

et al. (2012) analyzed leaf cross sections of 1 mm² fixed with glutaraldehyde and paraformaldehyde and dehydrated (TEM sample preparation) using a 532 nm laser. Looking to cross sections was not tested in this study but it could be done. The thin thickness of the sample should decrease the fluorescence intensity. And Das *et al.* (2018) analyzed the digested leaves.

3.2. Transmission electron microscopy

Transmission Electron Microscopy (TEM) is widely used to detect CNTs in environmental samples such as a wide variety of plant species (*e.g.* maize, canola, spinach, tomato) and different organs (leaves, stems, roots, seeds and cells). The main advantage of this technique is its very good lateral resolution, however TEM observation requires the use of thin cross sections (about 80 nm thick) which can induce some bias. During cutting, CNTs which are on the surface of the organ of interest (such as roots for example) may be transported inside the section by the knife. It is thus difficult to make a clear distinction between internalized and non-internalized CNTs. Another drawback is the lack of formal CNT identification. Indeed, in the literature, the detection of black elongated structures leads to the conclusion that they are CNTs. Some studies associated another techniques (*e.g.* Raman spectroscopy) in order to make sure that they were CNTs (Khodakovskaya *et al.*, 2009; Larue *et al.*, 2012). Also in some cases, especially in the case of MWCNT, it could be possible to use HRTEM and measure the spacing between the walls (a distance of 0.34 nm between wall fringes certifies that it is graphitic carbon). The combination of TEM and EDS analysis, when possible in the same microscope, can also be used to co-localize CNTs and residual catalytic nanoparticles. However, in some of the published studies, there is no other technique used to prove that the dark structures are indeed CNTs.

Finally, another disadvantage of this technique is the small field of analysis (less than one cell) which makes the analysis highly time consuming since we are looking for a rare event (the presence of CNTs). It is thus very complicated to obtain reliable statistics since a high cell number would need to be examined.

In most cases, TEM has been used for the detection of large-diameter MWCNTs, and not so much for CNTs with a low number of walls (1 to 3). Only Giraldo et al. 2014 were able to detect SWCNTs in chloroplast cross sections after chloroplasts incubation in SWCNT suspension. When looking at the different pictures in the literature where CNTs were detected inside plants, most of the time short CNTs (less than 500 nm) were observed (Figure 43). Serag et al. (2011) studied the length distribution of CNTs through various subcellular organs of the plant cells after exposure to a suspension of CNTs with lengths between 0.5 and 2 μ m. They showed that MWCNTs with a length between 50 and 100 nm were localized in vacuoles, plastids but also in the nucleus. Longer nanotubes (but still rather short, mostly between 100 and 300 nm) were observed in most of the other organelles. Two hypotheses can be made to explain why only short CNTs were imaged inside plant tissues: (i) since there is a length distribution for the CNTs in the suspension,

only the shorter CNTs were internalized in the plants, (ii) a transformation occurred inside the plants or inside the medium (soil or hydroponics) and CNTs were "cut" into smaller CNTs.



Figure 43 A. High magnification of root cells of *Lotus japonicus* treated with MWCNTs (diameter 8-15 nm and length 5-50 μm) (Yuan et al., 2017). B. High magnification of *Triticum aestivum* leaves exposed to MWCNTs (mean diameter 41.2 nm and length 1-10 μm) (Larue et al., 2012). C. High magnification of *Catharanthus.roseus* protoplasts incubated with MWCNTs (diameter 20-30 nm and length 0.5-2 μm) (Serag et al., 2011). Red arrows indicate CNTs, green arrows indicate cell wall.

In this work, we analyzed both roots and leaves of cucumber grown in DWCNT suspension. Roots were used as positive control to examine DWCNT aspect inside tissues since they were highly adsorbed at the root surface (can be seen with naked eye) (Figure 44).



Figure 44 High magnification of root cross sections of cucumber grown in DWCNT suspension (100 mg/L for 15 days). A. focused picture. B. Defocused picture showing the CNTs.

The Figure 44A showed an accumulation of dark agglomerates. We also confirmed that slightly defocusing the TEM can help to detect the CNTs by increasing their contrast (Bourdiol *et al.*, 2013) (Figure 44B). It is possible to identify CNT walls. Those CNTs were detected away from the root itself suggesting that they were displaced by the knife during the cutting step; TEM picture interpretation has thus to be performed very carefully.

In the root cross sections of exposed plants, little dark spots with size between 10 and 50 nm were identified (Figure 45A and B). No such similar spots were visible in the control roots, so we hypothesize that it corresponds to CNTs.



Figure 45 High magnification of root sections of cucumber grown in DWCNT suspension (100 mg/L) for 15 days. Red arrows are pointing to potential CNT agglomerates.

During the internalization process, CNTs may be modified and do not keep a straight structure since they are not completely rigid. They may form balls of CNTs. Some authors also reported the presence of such spots (of around 50 nm) which they associated to CNTs internalized in plants (Figure 46).



Figure 46 A. High magnification TEM image of tobacco cells exposed to MWCNTs (Khodakovskaya et al., 2012).B. High magnification TEM image of tomato seeds exposed to MWCNTs (Lahiani et al., 2017). C and D. High magnification TEM images of soybean seeds exposed to MWCNTs (Lahiani et al., 2013). Black arrows are showing potential CNTs.

It is possible to see in Figure 45 that these spots seem to have been displaced during the sample preparation. Indeed, close to the dark spots, we can see holes with a similar size. As CNTs are very hard material in contrast to plant matrix, we can suppose that the knife was not able to cut them, and shifted then slightly away from their original location.

High resolution TEM was also used in order to confirm the presence of CNTs in the root cross sections. Indeed, using this kind of equipment it is possible to distinguish the walls of the CNTs when looking at a CNT suspension (Figure 47).



Figure 47 Typical high resolution picture of DWCNTs.

It is also possible to use an EDS detector to identify if the spots contain Co. However, our analyses using both HR-TEM and EDS were inconclusive.

Cross sections of cucumber leaves exposed to CNTs were also analyzed by TEM, but unfortunately nothing was identified as being potentially CNTs.

Using TEM, we were thus able to detect potential CNTs inside plant tissues. However, this is just a hypothesis as we have no clear evidence that these dark spots are indeed CNTs.

3.3. Broadband microwave biosensor

Usually when microwave techniques are used to detect CNTs, authors measure the temperature rise of a sample at a certain microwave wavelength (Irin *et al.*, 2012; Cano *et al.*, 2016). Indeed, CNTs have the capacity to heat at high temperature when they are submitted to microwave irradiation (Irin *et al.*, 2012). However, this technique was mostly applied to roots of plants exposed to CNTs in hydroponic conditions or to CNT-spiked materials, which is not representative of an environmental sample. The established detection limit of this method is around 0.1 μ g/g (Cano *et al.*, 2016).

In this work, we used the microwave irradiation in a different way. We took advantage of the intrinsic high conductivity of CNTs in the range of microwave frequencies. It was indeed reported several times that CNTs have a high microwave permittivity and behave like a dielectric material at high frequency (HF) waves (Wu and Kong, 2004; Dragoman *et al.*, 2006). We used this property to detect CNTs in plant tissues using

a HF biosensor. It was already successfully applied earlier in our laboratories to detect the presence of the same DWCNTs in amphibian larvae (Bourdiol *et al.*, 2015). With this technique, only liquid samples can be analyzed. Here, we tested digested leaf tissues of control and contaminated plants at several frequencies (from 0.2 to 20 GHz). Because the suspension of DWCNTs was not fully stable at the scale of the analysis (partial sedimentation), measurements were acquired at different times (0, 2, 4 and 6 min).

First, we analyzed the nutritive medium in which the plants were grown, as well as the same medium spiked with DWCNTs *vs* time. Results are shown only at 0.2 GHz (Figure 48) but the same response was found up to 20 GHz.



Figure 48 Permittivity (real and imaginary parts) at 0.2 GHz of the plant growth medium and the medium spiked with DWCNTs (100 mg/L) at different measurement times (0, 2, 4 and 6 min).

The medium alone can be considered as stable since there was no modification of the dielectric response over the time. However, when DWCNTs were added to the medium, the dielectric response changed with time. This is probably due to the agglomeration and settling down of the CNTs in the medium. Even if the medium spiked with the DWCNTs contained CMC (dispersant) and was sonicated immediately before analysis, the agglomeration was still noticeable at such a focused scale. This can be explained by the high DWCNT concentration spiked in this medium (100 mg/L). To be able to compare all the results, we decided to take the dielectric response of all the studied samples at 6 min to decrease the influence of the sedimentation occurring mostly over the first minutes. The following results were normalized by the response of the control medium.



We then assessed the shape of the dielectric response of the DWCNTs as a function of the electromagnetic wave frequency between 0.2 and 20 GHz (Figure 49).

Figure 49 Contrast of the real part \mathcal{E}' (A) and imaginary part \mathcal{E}'' (B) of the permittivity of the DWCNTs in the medium as a function of the EM wave frequency between 0.2 and 20 GHz.

The behavior of the real part and the imaginary part of the permittivity over frequencies was characteristic of a liquid-based dielectric relaxation, regardless of the presence of the DWCNTs in the medium. The maximum real permittivity was obtained at the lowest frequency (0.2 GHz) which was followed by a continuous decrease until the highest studied frequency (20 GHz). The imaginary permittivity decreased rapidly at the lowest frequencies, followed by a rapid rise until 20 GHz. 0.2 GHz seemed to be the best frequency to study the DWCNTs since it showed the highest permittivity. However, at low frequencies there is a high influence of the different ions present in the studied media. We thus examined the results over the whole frequency range (from 0.2 GHz to 20 GHz).

As a second step, we analyzed the different digested samples of the control plants and the plants grown in the DWCNT suspension. Only results at 0.2 GHz are shown (Figure 50). 5 replicates per condition were measured. The dielectric response of the different samples from the same condition was highly different. This high variability made it impossible to identify a difference between the two conditions. Even if suspensions looked well dispersed, we observed a small accumulation of remaining digested leaves on the biosensor. It may explain why we observed so much variability between our samples.



digested control plants and plants grown in the DWCNT suspension at 100 mg/L during 15 days. Measurement time = 6 min.

The permittivity recorded for the digested leaves of the control plants was different from the medium. However, the digested leaves from the plants grown with the DWCNTs were located close to those of the control plants and far from the DWCNT suspension permittivity, suggesting that CNT concentration was probably not high enough to be detected. This method does not seem to be sensitive enough for the detection of CNTs in plant leaves. The experiment of Bourdiol *et al.*, (2015) was realized with Xenopus larvae which had higher CNT concentration. They achieved a detection limit of 0.15 mg/mL (the amount of detectable CNTs in the sensing area was 0.02 μ g). Since this experiment, the broadband microwave sensor was optimized. However, it is still not enough sensitive for our plant samples.

3.4. Two-photon excitation microscopy

Two-photon excitation (TPE) microscopy is a technique that provides many advantages for threedimensional and deep tissue imaging. It is nowadays widely used in cell biology and especially in plant cell biology (Feijo and Moreno, 2004). Among other advantages, TPE microscopy does not require specific sample preparation and whole plant tissues can be observed almost directly. Since it can provide deep tissue imaging, it is possible to make the distinction between internalized NMs and surface adsorbed NMs. TPE microscopy was for example used to assess the penetration depth into cells of different NMs such as gold NPs (Rane and Armani, 2016). Wild and Jones 2009 also used TPE microscopy for the detection and imaging of different NMs including MWCNTs in living wheat roots. Likewise, Verneuil (2015) were able to detect internalized MWCNTs in the cytoplasm of the diatom *Nitzschia linearis*, and specifically in the area surrounding the nucleus. In this work, we used TPE microscopy to evaluate DWCNT internalization inside plant tissues (roots and leaves) of cucumber. We compared control samples (non-exposed roots and leaves, digested non-exposed leaves) with plant samples spiked with DWCNTs and finally digested exposed leaves.

Figure 51 shows representative images of the DWCNT suspension alone. With an excitation wavelength at 880 nm, DWCNTs are emitting in a broad range of the spectrum.



Figure 51 (A) Optical image of the DWCNTs. (B) Corresponding 2D tomographic image with the two-photon excitation microscopy of the DWCNTs. Excitation at 880 nm and emission range between 500 and 600 nm. DWCNTs are in blue (false color).

For plant tissues we chose a different excitation wavelength (720 nm) because higher signal was detected at this wavelength in comparison to 880 nm (Figure 52).



Figure 52 Tomographic 3D images of (A) a control root and (B) a control leaf with the two-photon excitation microscopy. Excitation at 720 nm. Green color is representing the plant cell wall and cytoplasm. Orange color is attributed to chloroplasts.

In the roots, there was no strong emission. It was possible to identify the plant cell walls, but no cell organelle could be distinguished. In order to identify cell organelles using this microscopy, dyes could be be used (Feijo and Moreno, 2004). However, in the leaves a strong autofluorescence signal was observed from the plant cell walls and from the chloroplasts.

After spiking the plant root and leaf surfaces with a DWCNT suspension, we were able to distinguish the CNT signal at the root surface (Figure 53) but not in the leaves because the signal from the plant tissues and from the DWCNTs were overlapping in the entire emission range..



Figure 53 Two photon excitation microscopy observation of cucumber roots spiked with the DWCNTs. Excitation at 880 nm and emission range between 500 and 600 nm. DWCNTs are in blue (false color). Optical image in the background.

In order to cope with this issue of chlorophyll autofluorescence, digested leaves were prepared and analyzed. By using this type of sample preparation, the lateral resolution information is lost but we should be able to identify CNT translocation in leaves.



Figure 54 Two photon excitation microscopy observation of control digested cucumber leaves (A) and control digested leaves spiked with DWCNTs (25 mg of DWCNTs/kg of dry plant weight (B). Excitation at 880 nm and emission range between 500 and 600 nm. DWCNTs are in blue (false color). Optical image in the background.

At both excitation wavelengths (720 and 880 nm), no emission signal was detected in the digested control leaves (Figure 54A). There were still pieces of not well digested leaves. When the digested leaf sample was spiked with DWCNTs (25 mg of DWCNTs/kg of dry plant weight), it was possible to clearly identify their

presence (Figure 54B). But, again no CNT signal could be detected in the digested leaves of plants grown in the presence of DWCNTs.

As a conclusion, TPE microscopy was used here to detect the internalization of CNTs inside root and leaf tissues. We demonstrated that it is possible to detect DWCNTs in whole roots without any sample preparation. However, due to the high emission range of photosynthetic tissues, it was not possible to detect CNTs in whole leaves. More importantly, we were not able to detect the presence of CNTs in digested leaves grown in the presence of DWCNTs, which suggests that we were below the detection limit of this technique.

3.5. Hyperspectral imaging

Hyperspectral imaging (HSI) is an analytical tool that was developed to address current analytical challenges for nanoscale materials (Roth *et al.*, 2015). For instance, some authors successfully identified AgNPs in human macrophage cell lines (Lim *et al.*, 2012). In another work, HSI was used to localize CNTs in suspension in a solvent in order to study the potential of synergistic toxic effects between CNTs and herbicides on photosynthetic algae (Schwab *et al.*, 2013). HSI can also be applied to *in vivo* samples. It permitted to localize AgNPs in nematodes after ingestion (Meyer *et al.*, 2010). A study in mouse investigated CNT degradation in lung tissues by HSI (Kotchey *et al.*, 2012). Thus HSI seems to be a promising tool for the detection of NMs in complex matrices. However, it is not without limitations arising from its light-based mode of operation. Its spectral resolution (2.0 nm) is not precise enough to make the distinction between individual particles and their agglomerates. Additionally, this technique is highly dependent on the material used. Indeed, reflectance spectra of some materials are more easily detected and characterized than others (Roth *et al.*, 2015). In most cases, the use of HSI relies on the comparison with controls because there is no absolute signature of a NM as it strongly depends on its interactions with the surrounding environment.

There are several types of HSI imaging machines. A relatively new one (2012) is the CytoViva[®] nanoscale microscope and HSI system. This technique employs a novel dark-field-based illuminator. As a result, images have better contrast and higher signal-to-noise ratio compared to classical HSI images (Roth *et al.*, 2015).

In this study, we used the CytoViva[®] nanoscale microscope and HSI system on leaves of cucumber exposed by roots to DWCNTs (Figure 55).



Figure 55 (A) Hyperspectral imaging of cucumber leaves exposed for 15 days to 100 mg/L DWCNT with (B) the spectral profile corresponding to the red square in A and (C) the zoom with in red the pixel identified as CNT based on the spectral region between 550 and 650 nm.

After a quick scan of the different cross sections (leaves of the control and exposed plants), a CNT spectral profile was identified in the exposed plants (Figure 55B) which was not present in the control samples. The Figure 55C showed that CNTs were located at the periphery of the cell (at the plant cell wall). Those cells were identified as cells of the conductive vessels. CNTs may enter the plants through the roots and be translocated to the upper part of the plants by traveling with nutrients in the conductive vessels as it has been reported in the literature (Liné, Larue and Flahaut, 2017).

The CytoViva[®] nanoscale microscope and HSI system was able to detect the presence of CNTs in cucumber leaf exposed to 100 mg/L DWCNTs in hydroponics. However, it was just a quick feasibility test and further investigations have to be done before drawing definitive conclusions.

3.6. Synchrotron micro X-Ray fluorescence

In the CNTs, even after a purification process, there is still the presence of some remaining metal catalysts (Pumera 2007; Ge et al. 2011) that could be used as a proxy to detect CNTs. It was measured that metal impurities contribute in CNTs between 0.44 and 3 wt. % (Ge *et al.*, 2011), other authors even reported

until 30 wt. % depending of the type of synthesis used (Remy *et al.*, 2015). The DWCNTs used in this study had 3.99 wt. % of Co and 0.96 wt. % of Mo. Thus, the CNT concentration expected in the above-ground part of the plants is very low. It was demonstrated for some plants than less than 0.005‰ of the applied dose of MWCNTs was actually translocated from the roots to the leaves of the plants (Larue *et al.*, 2012). In such a case, the metal impurity concentration would be around 19.9 ng/kg dry weight for Co and 4.8 ng/kg dry weight for Mo. There is thus a need of extremely sensitive techniques to be able to detect such a low concentration of residual catalyst.

ICP-MS could be used (Hanna, Miller and Lenihan, 2014; Schierz *et al.*, 2014) which allows an accurate and selective determination of many elements (*e.g.* Cr, Co, Cu, Fe, Mo, Ni, Y, Zn) with a high sensitivity in the range of ng/L or even sub ng/L levels (Doudrick *et al.*, 2013; Petersen *et al.*, 2016). However, a complete dissolution of the matrices is required prior to analysis. Indeed, an incomplete sample digestion would lead to the partial release of metal ions from the CNTs and thus to a biased quantification (underestimation), or an external contamination could lead to an important bias in the determination of the bulk metal content (over-estimation). Moreover, classical ICP-MS machine do not give access to the localization of CNTs (Petersen *et al.*, 2016).

Alternatively, synchrotron radiation based X-Ray fluorescence (μ XRF) is a very sensitive technique that allows the mapping of elementary elements present in a sample with a high spatial resolution. It is more and more used to investigate the interactions between plants and engineered NMs (Castillo-Michel *et al.*, 2017). In this study, we used μ XRF at ESRF on beamline ID21. The detection limit was around 0.1 μ g/g and the spatial resolution was 0.2 x 0.8 μ m² (Tiwari *et al.*, 2013). However, these limits are established for an imaging technique, which cannot be compared to limits of a bulk technique such as ICP-MS.

Co is an element that can be detected using μ XRF on ID21 beamline and was the most concentrated catalyst in DWCNTs. The emission lines for Co are around 6.93 keV for K α and 0.77 keV for L α . Only Fe has emission energies close to Co (6.41 keV for K α and 0.71 keV for L α , but the sensitivity of the beamline is good enough to distinguish the two elements. Furthermore, several studies have successfully used μ XRF to localize Co inside plants (Punshon, Guerinot, and Lanzirotti 2009; Leonardo et al. 2014; van der Ent et al. 2018).

The first step of the experiment was to confirm the presence of Co in the DWCNT suspension. We then analyzed roots of cucumber plants grown in hydroponics in the presence of DWCNTs, and also further confirmed that Co detection in the roots was possible.

Finally, we analyzed Co distribution in the leaves of cucumber plants (Figure 56A, B). In exposed plants, it was mainly located at the basis of the plant trichomes. This distribution was further confirmed by analyzing the fitting of the XRF spectrum of each area. Indeed, we can see that in the control sample, forcing Co in the fitting procedure does not change anything to the quality of the fit (Figure 56C, E). On the opposite, if Co is excluded from the fitting procedure of the exposed plants, the peak at 6.9 keV corresponding to the Co emission energy is not properly fitted (underestimation) and the fit overall quality is clearly decreased.

CHAPTER 4 CNT DETECTION



Figure 56 μX-Ray maps of leaf trichomes of control plant (A) and plant exposed to 100 mg/L of DWCNTs during 15 days (B) with the corresponding XRF spectra and fits without Co (C and D) or with Co (E and F). Co emission energy is at 6.9 keV.

It has already been observed that some NMs are accumulated in plant trichomes. For example, Servin *et al.* (2012) observed that Ti from TIO_2 -NPs was transported from the roots to the trichomes suggesting that trichomes are possible sinks or excretory system for Ti.

Using synchrotron radiation based μ XRF it was possible to detect Co as a proxy for CNTs in the leaves of exposed plants. This technique is accurate for very low concentrations of CNTs. However, we can wonder if the Co is still co-localized with the CNTs once in the plant leaves. As a reminder, residual Co nanoparticles in the raw DWCNT samples are usually encapsulated within concentric graphitic shells, attached to CNT surface. Since the behavior of these carbon-encapsulated nanoparticles is not well known, dissolution in

the soil or in the plant cannot be excluded, or these nanoparticles may be separated from the CNTs during their journey in the plant. In this case, the co-localization hypothesis would not be true anymore, and Co could be found independently from the CNTs. However, literature suggests that CNTs are expected to remain in the environment without degradation or modification (Chen, Qin and Zeng, 2017; Phan *et al.*, 2018). We can thus hypothesize that Co is still attached to the CNTs. To validate this hypothesis, other techniques should be combined with this one (*e.g.* TEM imaging, Raman spectroscopy).

However, the main disadvantage of this technique is the limited access to synchrotron facility. There are not so many synchrotrons worldwide and beam time is restricted. Also as the acquisition time can be long (about 3h for a 200 x 200 μ m map), few samples can be analyzed during a shift (8h). For those reasons, this technique does not seem to be suitable for routine measurements of CNTs.

3.7. Techniques that require enriched CNTs

It is possible to isotopically enrich CNTs. This can be done during the synthesis, or later by functionalization of the outer wall. The two common isotopes of carbon used for isotopic labelling of CNTs are ¹⁴C and ¹³C. In this study we used MW¹³CNTs and DW¹⁴CNTs.

3.7.1. Isotopic-Ratio mass spectrometry

The use of IRMS to determine the ¹³C/¹²C ratio is common in many fields such as food safety and quality (Jamin, Martin and Martin, 2004; Calderone, 2005). Indeed, the analysis of stable isotopic ratios of bio elements allows the control of frauds and forgeries in the food and drink industry. Since the technique is highly sensitive, it is possible to use it to determine the geographical origin of products (*e.g.* plants) implying only minor changes in their isotopic ratio (Longobardi *et al.*, 2015). This technique is thus mainly used to analyze natural enrichment of stable isotope such as ¹³C. It is also possible to use IRMS in order to detect changes in the isotopic signature of a sample after extra addition of a specific isotope in the medium. Bourdiol et al. (2013) used this technique to quantify CNTs in the blood and liver of Xenopus larvae exposed to ¹³C-enriched DWCNTs and MWCNTs confirming that IRMS was efficient to detect the presence of enriched ¹³CNTs in biological matrices. They highly recommended not to use tissues which were directly in contact with the CNTs since it makes it impossible to make the difference between the internalized CNTs and those only deposited at the surface. They also recommended paying great attention during the sample preparation process to avoid cross-contamination of the samples.



Figure 57 Graphical representation of the mean (± standard deviation) δ^{13} C obtained after analysis of cotyledons and leaves of control plants and plants grown in 2 wt. % DW¹³CNT suspension (100 mg/L) for 15 days.

In this work, we used DW¹³CNTs enriched at 2 at. %. A low enrichment was used in order to preserve the equipment from a contamination that may occur when a product with a high relative amount of ¹³C is analyzed. The isotopic dosage of the MW¹³CNTs in suspension gave a result of δ^{13} C = 3300 ‰. It confirmed that DW¹³CNTs were enriched since the natural abundance is around -28 ‰. According to this result, in case of translocation of CNTs from the medium to the leaves of the plants, the latter should have values of δ^{13} C higher than the natural abundance. Here, the average of δ^{13} C for control plants was around -28.19 ‰ (Figure 57).

A Kruskal-Wallis test was applied to all the data and no significant difference could be highlighted. It was thus not possible to attest the presence of MWCNTs in any of the leaves of the plants.

Those results do not allow to confirm that IRMS can be used for the detection of ¹³CNTs using these specific exposure conditions. However, a recommendation for further work would be to use CNTs with a higher $^{13}C/^{12}C$ isotopic ratio. Indeed, Bourdiol et al. (2013) used $^{13}CNTs$ enriched at 20 at. %. However, the sample preparation protocol is valid and can be used further, since no contamination was identified in the control samples.

3.7.2. Micro ion beam analysis

Ion-beam Analysis (IBA) can be used for a wide range of applications (from chemical analysis at the subcellular scale to reaction analysis on materials) in many different domains (*e.g.* biomedicine, environment, microelectronics or material sciences). Life sciences constitute an important part of the application of IBA due to Rutherford Backscattering Spectroscopy (RBS), Particle Induced X-ray Emission (PIXE) and Scanning Transmission Ion Microscopy (STIM). For example, ion beam analyses were used to localize Ni in the halophyte *Sesuvium portulacastrum* (Fourati *et al.*, 2019). NMs, and especially TiO₂-NPs. Some authors evidenced the internalization of these NPs and their preferential localization in the cytoplasm of keratinocytes after exposure (Simon *et al.*, 2011). Larue et al. (2014) used µPIXE coupled to RBS to determine concentration of Ti in lettuce leaves exposed to TiO₂-NPs. Besides, NRA (Nuclear reaction analysis) is used for the detection of light elements (from He to F) and their isotopes. For instance, Siegwolf *et al.* (2001) analyzed the stable isotope ¹⁴N (indicator in the metabolic pathway of the uptake of gaseous compounds of nitrogen), ¹³C and ¹⁸O (to reveal the inherent coupling between the plant carbon, water and nitrogen relation) in poplar leaves in order to highlight differential effects of soil nitrogen and nitrogen dioxide on the water use efficiency.

Using this technique, we analyzed cross sections of plants grown in hydroponic conditions with MW¹³CNTs at 100 mg/L during 15 days. We first analyzed the MW¹³CNT suspension, then the whole leaf of control plant and plant exposed to the MW¹³CNTs. We also observed a whole control leaf spiked with CNT suspension. Finally we analyzed cross sections of the control and the exposed plants. Leaf cross sections were placed between two layers of polycarbonate film to maintain the section structure.

We used NRA analysis in order to be able to distinguish the ¹³C from the ¹²C in the sample. We were able to identified ¹²C and ¹³C signal among the contribution of other light elements (Figure 58).



Figure 58 NRA spectrum of the leaf cross section of the plant exposed to MW¹³CNTs at 100 mg/L during 15 days. ¹²C and ¹³C from the leaf section and from the polycarbonate film were identified as well as other light elements.

Two distinct peaks were identified for the ¹³C, one can be associated with the plant cross-section and the other with the polycarbonate film covering it. Based on that, we were able to make counting of the different signals by integrating the signal under the ¹³C peak (Table 15).

Nature	A ¹³ C Polycarbonate	A ¹³ C leaf sample	Ratio ¹³ C sample/ ¹³ C Polycarbonate
MW ¹³ CNT suspension	73	2042	27.97
Polycarbonate film 2 μm	41	None	-
Leaf of control plant	993	1415	1.42
Leaf of control plant + MW ¹³ CNT spiked	536	1184	2.21
Leaf of exposed plant	980	1310	1.34
Cross section of leaf control plant	393	987	2.51
Cross section of leaf exposed plant	268	1426	5.32
Cross section of leaf exposed plant	358	630	1.76

Table 15 Counting based on the NRA analysis of ¹³C corresponding to the polycarbonate film and to the different analyzed samples. Control plant are highlighted in green.

A higher ¹³C signal was identified for the MW¹³CNT suspension. A¹³C signal was also detected in control leaves which is normal since plants naturally contain naturally ¹³C (Mariotti, 1991) as already characterized by IRMS. An increase of 55% in the ratio ¹³C sample/¹³C polycarbonate in the section was observed for the entire leaf spiked with the suspension. In the leaf of the exposed plant, we did not identify a relative ¹³C enrichment. However for leaf cross sections, we observed a higher signal for two of the cross section of the exposed plants in comparison to the one of the control plant which suggests the presence of MW¹³CNTs. However, the ¹³C signal from the polycarbonate film made the result interpretation more difficult.

To try to increase the quality of the ¹³C signal, we tested another sample preparation excluding the polycarbonate film. The leaf cross sections were placed between two gold rings in order to avoid having a layer of polycarbonate film between the sample and the detector. Unfortunately, we were not able to

analyze these samples: the high vacuum in the chamber together with beam induced damage lead to the destruction of the "unprotected" samples during the first minutes of acquisition.

A series of test samples (control, leaf spiked with the ¹³CNT suspension) were analyzed in order to highlight the possibility of detecting ¹³C using RBS/NRA. We were able to observe the ¹³C variation in the studied samples but it was difficult to go further due to the sample preparation using polycarbonate which add supplementary signal. Plants exposed to ¹³CNT were analyzed. However, more tests should be done in order to confirm the CNT presence in the exposed plants. An adequate preparation of sample and a higher ¹³C enrichment may be used in order to make the detection easier.

3.7.3. Autoradiography

Autoradiography is a measurement of beta emissions from ¹⁴C. It can provide a spatial distribution of radioactivity with a good accuracy. So far, it is the technique that provides the best quantification of CNTs in complex matrices (Petersen *et al.*, 2016). However, it includes many restrictions: (i) a high cost to synthetize radioactively labeled CNTs, (ii) safety concerns since it is a radioactive product, (iii) dedicated installations to manipulate ¹⁴C, which are usually not "plant-friendly".

This technique has been used several times to assess the accumulation and the behavior of CNTs in the aquatic ecosystem: *e.g.* Zhang et al. 2012 observed the interactions of MW¹⁴CNTs with soil minerals in water, while other authors studied the accumulation and distribution of MW¹⁴CNTs in zebrafish (Maes *et al.*, 2014). Parks et al. (2013) worked the bioaccumulation of MW¹⁴CNTs in benthic organisms at the base of the marine food chain. This technique was also applied to assess the accumulation and behavior of CNTs in earthworms (Petersen *et al.*, 2008; Petersen, Huang and Weber, 2008). In plants, this technique has been used only once. Authors demonstrated that in wheat and rapeseed, less than 0.005 ‰ of the applied MW¹⁴CNTs dose was taken up by plant roots (hydroponic conditions) and finally translocated to the leaves (Larue *et al.*, 2012).

In this study, we used this technique to evaluate the translocation of DW¹⁴CNTs in cucumber grown in 100 mg.L⁻¹ and in 50 mg.L⁻¹ during 15 days. The DW¹⁴CNTs used were labelled at 4 μ Ci/mg. We were able to detect ¹⁴C in leaves of the plants exposed to the CNTs. For the plant grown in 100 mg.L⁻¹, DW¹⁴CNTs were found in the three leaves of the plant (Figure 59). In total, 0.263 µg of DWCNTs were found inside leaves (0.173 µg in the oldest leaf (leaf 1), 0.069 µg in the second (leaf 2) and 0.021 µg in the youngest). For the plant grown in 50 mg.L⁻¹ of DW¹⁴CNTs, CNTs were also detected in all leaves with in total 0.115 µg of CNTs (0.058 µg in the oldest (leaf 1) and 0.056 µg in the youngest (leaf 2)).

With this technique, we were able to show that DWCNTs are translocated from the roots to the upper part of the plant. The concentration of DWCNTs used showed that less CNTs are translocated when the concentration is lower. When half of the concentration was used, less than 56% of CNTs were found inside leaves. The translocation of CNTs in this experiment seems to be dose-dependent.



Figure 59 Pictures (A and C) and autoradiography pictures corresponding (B and D) of two cucumber plants grown with DW¹⁴CNTs at 100 mg.L⁻¹ (A and B) and 50 mg.L⁻¹ (C and D) during 15 days.

4. CONCLUSION

We chose to analyze leaves to avoid getting confounding signal possibly coming from CNTs adsorbed on the root surface after a root exposure. Analysis of leaves is also more challenging since CNT concentration is probably lower and chlorophyll signal lead to a high autofluorescence background.

Among the different techniques used to detect CNTs in the upper part of plants exposed to CNTs, autoradiography with ¹⁴CNTs was the most efficient. Indeed, it allowed the detection and quantification of CNTs in cucumber leaves after 15 days of exposure at 100 mg/L. Sample preparation and analysis are simple data treatment quite straightforward. However, the use of radiolabeled CNTs is expensive and requires dedicated installation to handle radioactivity.

To overcome this issue, we tested the other isotope of C: ¹³C. Using µNRA, we were able to detect a ¹³C enrichment in one of the exposed leaf cross-sections. However, the experiment needs to be repeated better understand from where comes from the high variability among samples seen in our first experiment and thus ensure about the repeatability of the technique. Higher enrichment should also be used to be able to properly implement the 2D mapping mode (here the statistic at the pixel level was too low to draw maps). IRMS was also tested, but results were not conclusive. A test using a higher enrichment in ¹³CNTs should be done. Another technique that could be investigated for the detection of ¹³CNTs at the subcellular scale is the nanoscale secondary ion mass spectrometry (nanoSIMS).

Using non-enriched CNTs, several techniques were tested. If TEM is one of the most used techniques, CNT identification is not unequivocal. Raman spectroscopy as well as TPE were not enough sensitive to evidence the presence of CNTs in cucumber leaves (either fresh whole leaf or digested leaf). We also investigated the broadband microwave sensor which has never been used before for the detection of CNTs in plants, but again the detection limits were too high to detect CNTs in plant leaves. Hyperspectral imaging can be a promising technique but more tests are still needed to confirm our preliminary results.

Several other techniques were not tested in this work by lack of time such as thermogravimetric analysis (TGA) or photoacoustic techniques. TGA consists on the quantification of mass percentage of phases with distinct thermal stabilities under a variety of reactive atmosphere. It is a fast technique theoretically allowing quantification in complex samples such as plants (Petersen *et al.*, 2016). Photoacoustic techniques measure the acoustic response to a rapid volume change. It is suitable in liquids but also in complex media such as plants. It has already been shown to detect CNTs in plants (Petersen *et al.*, 2016).

One of the most important challenges in the detection of CNTs in plants is the sample preparation. Sample preparation is often poorly reported in research articles which makes the replication of approaches complicated or even impossible in some cases. Also, in the literature, the reproducibility and the robustness of a given technique remain unclear. It would be helpful to report in an open database the techniques used even if they failed to help the community to make faster progress on this major technical bottleneck.

In the context of food safety, CNT concentration in the edible parts of the plants will be a major concern and so far only few techniques can give access to absolute quantification.

CHAPTER 5: ASSESSING PLANT RESPONSE TO 2 NMS USING FTIR SPECTROSCOPY

CNTs and TiO₂-NPs are among the most used nanomaterials thanks to their excellent optical, mechanical, electrical and thermal properties. During their lifecycle, CNTs and TiO₂-NPs will be spread in the environment during production, use, destruction, reuse or potential accidents in production units or during transportation. For this reason, it is essential to evaluate their behavior and potential impacts on ecosystems and particularly on plants. Indeed, plants are found at the interface between the three ecosystems (air, soil and water) and are the base of the food chain.

To date effects of NMs on plants are not well understood (see chapter 1). One of the main reasons is that there is a multitude of methods to evaluate impacts of NMs on plants. Many biomarkers can be used from morphological scale to genetic scale and most of the time only few are investigated. There is a need for a routine, standardized and widely informative method to evaluate NM toxicity. Fourier Transform InfraRed spectroscopy (FTIR) is a powerful and rapid technique based on the vibrational state of the molecules. This technique permits to obtain the biomacromolecular composition of a sample. Data acquisition is easy and fast. However, data treatment is tedious due to the complexity of obtained spectra. We worked on the development of the FTIR approach to evaluate impacts of the two NMs (TiO₂-NPs and CNTs).

Three-week-old tomato plants (*Solanum lycopersicum*, var. red robin) were grown in soil contaminated with CNTs or TiO₂-NPs at different concentrations (0, 100 and 500 mg/kg) during different exposure durations (5, 10, 15 and 20 days). The aim was to assess and compare the effects of the two NMs according to exposure duration and to evaluate if the effects were linked with the physicochemical characteristics of the NMs (in particular form and chemical nature). Phytotoxicity was assessed through morphological markers, physiological markers and impact on biomacromolecules studied by FTIR. Figure 60 summarizes the experimental methodology used.

CHAPTER 5 IMPACTS OF TWO DIFFERENT NMS



Figure 60 Summary of the experimental design to disentangle impacts of TiO₂-NPs and CNTs on tomato plants using FTIR

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ARTICLE: FOURIER TRANSFORM INFRARED SPECTROSCOPY CONTRIBUTION TO DISENTANGLE NANOMATERIAL (DWCNT, TIO₂) IMPACTS ON A CROP PLANT Clarisse Liné^{*a,b}, Juan Reyes-Herrera^c, Valentin Costa^a, Mansi Bakshi^{a,d}, Hiram Castillo-Michel^c, Emmanuel Flahaut^b and Camille Larue^a

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1. INTRODUCTION

Over the last two decades, nanotechnologies became more and more important. Indeed, nanomaterials (NMs) present unique properties such as high specific surface area (Christian *et al.*, 2008) which can be useful in many domains such as electronics, materials or food industry (Jeevanandam *et al.*, 2018). In 2019, the Dutch Nanodatabase revealed that a total of 3036 consumer products officially contained NMs (Danish Consumer Council, The Ecological council, 2019). Investigations about their possible use in medicine (Murthy, 2007) or in agriculture (Duhan *et al.*, 2017) are also in progress.

Carbon nanotubes (CNTs) and titanium dioxide NPs (TiO₂-NPs) are among the most used NMs (Vance *et al.*, 2015). CNTs are part of the carbon-based NMs family. They have remarkable properties (optical, electrical, thermal, mechanical and chemical) (Dresselhaus, Dresselhaus and Avouris, 2003). They are mainly used in batteries, plastic additives or sporting goods (Terrones, 2004). TiO₂-NPs are well known for photocatalytic applications. Some of their main applications are semiconductors (Gupta and Tripathi, 2011), food additives (Weir *et al.*, 2012) or cosmetics (Lu *et al.*, 2015). Since applications of both of these NMs are still increasing, their release into the environment, intentionally or not, is of great concern.

Assessing their concentrations in the environment is a major issue in ecotoxicology. Modelling studies were carried out on some nanomaterials in order to evaluate their concentrations in the different environmental compartments. TiO₂-NPs have been identified as one of the most concerning nanomaterials due to the high forecast concentrations. In sludge treated soils, TiO₂-NPs environmental concentrations were predicted to be around 61 mg.kg⁻¹ whereas for CNTs it was 12 μ g.kg⁻¹ (Sun *et al.*, 2016a).

NM impacts on terrestrial ecosystem are still controversial (Liné, Larue and Flahaut, 2017). Indeed, some authors reported higher germination rate and better yield after exposure to CNTs while other studies highlighted decreased root length or oxidative stress (Liné, Larue and Flahaut, 2017). The same conclusions were reached for TiO₂-NP impacts on plants (Cox *et al.*, 2016). While some beneficial effects were reported such as a higher germination rate or longer root and shoot length (Zheng *et al.*, 2005; Clément, Hurel and Marmier, 2013; Hatami and Ghorbanpour, 2014), other works described that TiO₂-NPs decreased the germination rate or plant growth and caused genotoxic effects (Ghosh, Bandyopadhyay and Mukherjee, 2010; Castiglione *et al.*, 2011). Until now, the specific action mechanisms of NMs are still to be identified and required further research (Reddy *et al.*, 2016).

One possible explanation to these contrasted phytotoxicity results is the method used to evaluate NM impact on plants. A lot of biomarkers can be evaluated from the morphological to the metabolism scale showing diverse sensibility. Their use to evaluate plant health is accurate, especially when many of them are combined. But in the literature, most of the studies use only 5 biomarkers leading to a potentially partial image of the toxicity effects. The availability of routine, standardized and widely informative analytical methods to evaluate NM toxicity is a key to fill this current gap of knowledge (Ray, Yu and Fu, 2009).

Fourier Transform Infrared (FTIR) spectroscopy is a powerful, easy to access and fast technique based on the vibrational state of molecules. This analytical technique gives access to the metabolic composition of a sample (Kumar, Manoj and Giridhar, 2015), it allows the acquisition of a signal combining information on a multitude of compounds unlike chemical dosages (*e.g.* specific enzymes or secondary metabolites) which give access only to one compound at a time. With FTIR, the bulk of a sample is measured and sample preparation is very simple (grinding of dry materials) thus reducing artefacts. There are two types of acquisition mode: either bulk analysis of a sample or imaging mode (the acquisition can take few hours) (Baker *et al.*, 2014).

In plant biology, FTIR has been mainly used to characterize plant cell wall components (Largo-Gosens *et al.*, 2014; Chylińska, Szymańska-Chargot and Zdunek, 2016; Gierlinger, 2018). It allows a highly sensitive characterization of all cell wall components using a fast and easy technique compared to classical methods that require isolation, extraction and fractionation of the different cell wall components (Szymanska-Chargot and Zdunek, 2013; Largo-Gosens *et al.*, 2014). Recently, FTIR was used in ecotoxicological studies to analyze changes on biological materials (Baker *et al.*, 2014). Dao *et al.*, (2017) demonstrated that the level of carbohydrates and lipids increased while proteins and phosphorylated molecules decreased in microalgae exposed to lead. FTIR was also used to evaluate the leaf biochemical composition of Cassava in response to *Bacillus subtilis* which was modified mainly in the epidermis and mesophyll tissues (Thumanu *et al.*, 2015).

FTIR data processing is however tedious due to the complexity of spectra. Indeed, since FTIR is widely informative, spectrum contains signals coming from many molecular bonds which can be convoluted together. The difference in the spectrum from one sample to another may be very small, making it difficult to observe it in the raw spectra with bare eyes. Sometimes, minor differences may contain critical information. The direct comparison between samples can also be tricky since the baseline may vary a lot. For those reasons, it is important to process and analyze the data in a more systematic way using statistical approaches (*i.e.* supervised classification, clustering method (Gautam *et al.*, 2015)) in order to obtain meaningful information.

Aims of this study were to develop the FTIR approach in order to evaluate impacts of NMs on plants, and to use it to disentangle impacts of two different NMs taking into account: (i) different exposure durations and (ii) different NM concentrations.

Seedlings of tomato were grown in soil contaminated with CNTs or TiO₂-NPs at two different concentrations (100 and 500 mg.kg⁻¹of soil) during different exposure 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 and metabolism biomarkers were also used (height, biomass, number of leaves, photosynthetic pigment, flavonoid and total phenolic compound contents and lipid peroxidation).

2. MATERIALS AND METHODS

2.1. Nanomaterials

 TiO_2 -NPs (Sigma-Aldrich, ref 718467, Aeroxide P25) were composed of 80% anatase and 20% rutile with a nominal diameter of 25.0 ± 5.7 nm (Figure 61A). They had a specific surface area of 46 ± 1 m².g⁻¹. A fresh TiO_2 -NP suspension at 1 g.L⁻¹ was prepared with deionized water, just before use.



Figure 61 TEM pictures of TiO2-NPs (A) (Vijayaraj et al., 2018) and DWCNTs (B)

DWCNTs (Figure 61B) were synthetized at 1000°C by catalytic chemical vapor deposition (CCVD) of a mixture of CH₄ (18 mol.%) and H₂ using a Co:Mo/MgO-based catalyst composed of Mg_{0.99}Co_{0.0075}MgO_{0.0025} (Flahaut *et al.*, 2003a). After CCVD, the composite powder was processed overnight with an aqueous HCl solution to dissolve oxides and non-protected residual catalyst NPs without degrading CNTs. Then, the sample was filtered through cellulose nitrate membrane (pore 0.45 μ m) and washed few times with deionized water until neutrality. Transmission Electronic Microscopy (TEM) observations were performed to determine material shape and diameter (JEOL TEM 1400). The outer diameter ranged from 1 to 3 nm (Figure 61B). The length varied from 1 to 100 microns (Flahaut *et al.*, 2003a). The specific surface area was 980 m²/g (Brunauer, Emmett and Teller (BET) method; Micrometrics Flow Sorb II 2300; 2h degassing at 100°C in N₂ and adsorption of nitrogen gas at liquid nitrogen temperature; measurement accuracy ± 3%). A stable suspension at 1 g.L⁻¹ was prepared from a wet powder of CNTs in deionized water. It was dispersed using a BRANDSON digital sonifier S-250D with a 1/8 inch tapered microtip (200W; amplitude: 35% 1s/1s on/off) for 30 min. Just before use, suspension was re-dispersed using a sonication bath for 10 min (Elmasonic S30H, 280 W).

2.2. Soil characteristics and contamination

A silty sand soil (according to USDA criteria) was used for this experiment (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, $0.06 \pm$

0.01 % of nitrogen, had a pH of 4.9 \pm 0.3 and a cation exchange capacity of 4.3 \pm 0.6 meq/100 g. The soil water capacity was 60 mL for 100 g of soil.

Briefly, CNT or TiO_2 -NP suspensions were added to the dry soil in order to reach 100 or 500 mg of NMs per kg of dry soil (ratio liquid:soil = 1:1 in weight). After 2 hours on a shaker table, the soil mixture was filtered in order to remove the water in excess. After homogenization, pots of 170 g of soil were prepared. This soil preparation protocol ensured a soil contamination as homogenous as possible.

2.3. Plant material and cultivation

Organic seeds of tomato *Solanum lycopersicum* (var. Red Robin) were obtained from the French seed company Germinance and surface-sterilized using Ca(ClO)₂ (1%). Seeds were placed in compost soil to germinate for 7 days. At cotyledon appearance, seedlings were transferred in hydroponic conditions during 3 weeks in order to obtain plants with 5 leaves. Plants were placed into control or contaminated soil until harvest (after 5, 10, 15 or 20 days of exposure). The experiment was performed in an environmental chamber with controlled parameters: 10 hours of light per day, 22°C during the night, 24°C during the day and a hygrometry rate of 85%.

Five different exposure conditions were used: control (only soil without NM contamination), 100 mg of CNTs per kg of dry soil (CNT 100), 500 mg of CNT per kg of dry soil (CNT 500), 100 mg of TiO₂-NPs per kg of dry soil (TiO₂ 100) and 500 mg of TiO₂-NPs per kg of dry soil (TiO₂ 500), with 5 biological replicates each.

Morphological parameters were monitored every day (plant height and number of leaves). Upon harvest, other physiological parameters were measured (total fresh biomass and foliar surface area using a camera and ImageJ software). Part of the leaves was frozen at -80°C for biochemical analyses and the other part was dried at 50°C during 24h for FTIR analysis.

2.4. FTIR analysis

About 20 mg of dry leaves were ground using a FastPrep grinding machine (2 x 15 sec at maximum speed). Each powdered sample was analyzed in attenuated total reflectance (ATR) mode using a diamond crystal (Thermo Nicolet, Nexus, Smart Orbit). Infrared spectra were collected in the range 4000 - 400 cm⁻¹. All the samples were analyzed in (technical) triplicates and each spectrum was the sum of 64 scans. OMNIC software was used to export experimental spectra.

2.5. Chemometric analysis for FTIR data

A chemometric analysis of FTIR spectra was developed using Orange software (BioLab) (Demšar *et al.*, 2013). The first step of spectrum analysis was the pre-processing of data. This step is required in order to eliminate effects of unwanted signals (detector noise, atmospheric background, *etc.*), to improve the robustness and the accuracy of subsequent analyses and to increase the interpretability of the data by correcting issues associated with spectral data acquisition (Baker *et al.*, 2014). During this step, a Savitzky-Golay filter was applied (window of 21, polynomial order of 2 and derivative order of 2). This filter is based

on simplified least square procedures and allows to remove various instrumental and scattering effects (Trevisan *et al.*, 2012). A vector normalization was then applied in order 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 (Baker *et al.*, 2014; Türker-Kaya and Huck, 2017). 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 mostly for proteins. The region between 1800 and 2700 cm⁻¹ was removed because it mainly corresponds to background interferences.

A multivariate analysis was performed on pre-processed spectra with first: a principal component analysis (PCA) followed by a linear discriminant analysis (LDA) (Gautam *et al.*, 2015). The PCA is an unsupervised method which looks for projections to maximize the variance meanwhile the LDA is a supervised method looking 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 is lower than the number of variables such as in this work. PCA allows to reduce the number of variables/wavenumbers (from 1246 variables per samples to 10 components), the reduced dataset being then analyzed by the LDA to identify groups.

In order to identify the wavenumbers contributing the most to differences among groups, a logistic regression was run on the pre-process 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 extracting the features of the logistic regression. In order to test the robustness of the statistical model used, area under a receiver operating characteristics (ROC) curve (AUC) and cross validation were used. In order to compare the different spectra among them, areas under the different peaks were calculated by integrating the area starting from 0 on the pre-processed spectra.

2.6. 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. Then, a PCA was performed with all the data. All statistical analyses were carried out using the RStudio statistical software (Team, 2015) (version 1.1.453) with car (Fox, 2002), multcompView (Graves, Piepho and Selzer, 2015), Ismeans (Lenth, 2016), pgirmess (Giraudoux *et al.*, 2018), ggplot2 (Wickham, 2009), FactoMineR (Lê *et al.*, 2008) and factoextra (Kassambara and Mundt, 2017) packages.

3. RESULTS

3.1. Morphological responses

No significant difference was identified for plant height after 5, 10 and 20 days of exposure (Figure 62A-D). However, at 15 days, plants of the condition TiO_2 100 were significantly smaller than the control (- 57%, p-value < 0.05) (Figure 62A). For the TiO_2 500 condition, the difference was not significant (p-value = 0.27) but there was a decrease in size of 28% compared to the control. Although plants exposed to both CNT concentrations were not significantly different in growth from the control plants, there was an increase of 126% in CNT 100 (p-value = 0.32) and 28% in CNT 500 conditions (p-value = 0.27 with plants growing of 2.28 cm, 2.88 cm and 2.92 cm, respectively.



Figure 62 Results of the morphological parameters (plant height (A), leaf number (B), leaf surface area (C) and total fresh biomass (D)) after tomato exposure (for 5, 10, 15 or 20 days) to TiO₂-NPs and CNTs at 100 or 500 mg/kg. For each graph, statistical analyses were done separately for each exposure durations. Different letters imply statistical differences (p<0.05).

The number of leaves during exposure was not significantly different among conditions for the 4 exposure durations (Figure 62B). At 5 days, plants grew up on average of 0.44 leaf, 1.72 after 10 days, 1.84 after 15 days and 3.00 after 20 days.

The foliar surface area was not significantly different for the different exposure durations except at 15 days (Figure 62C). Indeed, at 15 days, it was significantly lower for the two TiO_2 -NP exposed groups: 7.20 cm² for TiO_2 100 (p-value = 0.004) and 8.95 cm² for TiO_2 500 (p-value = 0.033) with the control at 18.96 cm². On the other hand, no significant difference was found for plants exposed to CNT (p-value = 0.99 for CNT 100 and 0.92 for CNT 500).

The total biomass of plants was not significantly different after the different exposure durations among treatments (Figure 62D). However, after 15 days, plants of the condition CNT 500 exhibited a trend for higher biomass than the control (1.31 times, p-value = 0.60) while plants of the condition TiO_2 100 tended to be lighter than the control (2.77 times, p-value = 0.05). The difference in-between those two conditions was significant (p-value = 0.002).



Figure 63 PCA on the morphological parameters of tomato plants (plant height, number of leaves, leaf area and total biomass) for the four exposure durations (5, 10, 15 and 20 days) and the different exposure conditions (Control, CNT 100, CNT 500, TiO₂ 100 and TiO₂ 500). Bigger symbols are the barycenters of ellipses.

The PCA analysis of different morphological parameters according to the 4 exposure durations did not permit to identify significant differences among the different exposure conditions (Figure 63A-D).

The 15-day exposure led to more differences than the other conditions. Groups of TiO_2 100 and TiO_2 500 were distant from the control group when comparing barycenter of the ellipses.

We tried to assess the biochemical response of the plant using different biomarkers such as lipid peroxidation, pigments and secondary metabolites. However, the plant biomass was not enough to realize an efficient analysis.

3.2. FTIR analysis

Figure 64 shows the PC-LDA of the FTIR data for the 4 different exposure durations. After 5 days of exposure, the PC-LDA showed that exposed plants were well separated from the control plants mostly along PCA1. The CNT 100 group overlapped with the CNT 500 group and the TiO_2 500. The TiO_2 100 group was segregated from the others along PC2. Looking at the distance of the barycenter of the ellipses, all the exposed conditions were almost at the same distance from the control condition.



Figure 64 PC-LDA of the FTIR spectra (between 800-1800 and 2700-2900 cm⁻¹) acquired on tomato leaves for the four exposure durations (5, 10, 15 and 20 days) with the different exposure conditions (Control, CNT 100, CNT 500, TiO₂ 100 and TiO₂ 500). PC-LDA were run with Orange software and drawn with RStudio (ggplot2).

After 10 days of exposure, the PC-LDA highlighted that CNT 100 and TiO_2 100 plants had a leaf composition similar to the control plants, while both conditions at 500 mg.kg⁻¹ were impacted.

15 days of exposure was again the most discriminant exposure duration with all conditions being significantly different from the control and among each other according to the PC-LDA. Looking at the distance of the barycenter of the ellipses, CNT 500 had the highest differences in plant composition and CNT 100 the lowest in comparison to the control. Both TiO₂-NPs were almost at the same distance from the control, but in the opposite direction of CNT groups along the component 1 axis.

After 20 days of exposure, both conditions of TiO₂-NPs were overlapping with the control conditions in the negative part of the component 1 axis while both CNT conditions were segregating in the positive part (similarly as after 15 days of exposure).

By investigating the logistic regression results, wavenumbers contributing the most to the observed differences were almost the same for the four exposure times. As the 15-day exposure was the condition evidencing more differences in leaf composition, the rest of the analysis will be focused on this condition (Figure 65, Table 16 and Table 17).



Figure 65 Normalized FTIR spectra for tomato plants exposed for 15 days to TiO₂ or CNT at 100 or 500 mg.kg⁻¹. Peaks contributing the most to differences among are highlighted in yellow. Peak A = 2848-2852 cm⁻¹, lipid region. Peak B = 1537-1550 cm⁻¹, amide II region. Peak C = 1312-1320 cm⁻¹, carboxyl region. Peak D = 1155-1160 cm⁻¹, polysaccharide region (cellulosic compounds). Peak E = 1070-1082 cm⁻¹, polysaccharide region (hemicellulose compounds). Peak F = 990-1052 cm⁻¹, pectin and various polysaccharide region.
Wavenumbers (cm ⁻¹)	Band	Assignment	Main compounds	References
2848-2852	A	CH ₂ symmetric stretch	Lipids	Schulz et al., 2007 Türker-Kaya et al., 2017
1537-1550	В	N-H and C=N Amide II		Sene et al., 1994 Regvar et al., 2013
1312-1320	с	C-H bend	Carboxyl groups from ligands, proteins, various polysaccharides (cellulosic compounds)	Bonetta et al., 2002 Schulz et al., 2007 Alonso-Simon et al., 2011 Regvar et al., 2013 Türker-Kaya et al., 2017
1155-1160	D	OH or C-O stretch	Various polysaccharides (mainly cellulosic compounds)	Schulz et al., 2007 Regvar et al., 2013 Alonso-Simon et al., 2011 Türker-Kaya et al., 2017
1070-1082	E	C-O ring stretch	Various polysaccharides (hemicelluloses in particular)	Alonso-Simon et al., 2011 Regvar et al., 2013 Türker-Kaya et al., 2017
990-1052	F	O-H and C-OH stretch	Pectin, various polysaccharides	Schulz et al., 2007 Regvar et al., 2013 Türker-Kaya et al., 2017

Table 16 Most relevant peaks extracted from the logistic regression for 15 days of exposure with the band letter corresponding to the figure 4, the band assignment and the main compounds that can be found with the cited references.

All the interpretations are based on the normalized FTIR spectra, studied biomacromolecules are thus expressed as relative amount. A difference was highlighted in the lipid region (Figure 65, peak A) with higher relative amounts for the four different treatments in comparison to the control with the highest amount for plants grown on soil contaminated by CNT at 500 mg.kg⁻¹ (areas under the peak of control = 0.0408, and CNT 500 = 0.0539; Table 17). In the amide II peak (Figure 65, peak B), plants grown on contaminated soils exhibited a decrease in comparison to the control except for CNT 500 (12% decrease for CNT 100, 6,5% for Ti 100 and 6,1% for Ti 500). In the peaks C (1312-1320 cm⁻¹) and D (1155-1160 cm⁻¹) (Figure 65) both corresponding mainly to various polysaccharides, exposed plants had higher relative amount than the plants from the control. Between 1070 and 1080 cm⁻¹ (Figure 65, peak E), a decrease in hemicellulose relative content was detected for the plants from all contaminated soils. Finally, concerning the peak corresponding to pectin and various polysaccharides (Figure 65, peak F), a decrease was noticed for the plants grown in soil contaminated with CNTs and an increase for the plants grown with TiO₂-NPs. Most of the differences identified in the FTIR spectra correspond to cell wall components (cellulosic compounds or hemicelluloses).

Wavenumbers (cm ⁻¹)	Band	Control	CNT 100	CNT 500	TiO ₂ 100	TiO ₂ 500
2848-2852	А	0.0408	0.0516	0.0539	0.0461	0.0435
1537-1550	В	0.2851	0.2511	0.2885	0.2668	0.2676
1312-1320	С	0.1774	0.1866	0.2041	0.1834	0.1836
1155-1160	D	0.1479	0.1466	0.1404	0.1468	0.1471
1070-1082	E	0.5971	0.5674	0.5598	0.5784	0.5895
990-1052	F	4.0931	4.0388	3.8417	4.1885	4.4404

Table 17 Area under the peak of the 6 peaks contributing the most to differences among plants exposed to the 5 conditions: control, CNT 100, CNT 500, TiO₂ 100 and TiO₂ 500 after 15 days of exposure. Area under the peak was calculated using Orange software by integrating the different peak from O.

4. DISCUSSION

In this study, FTIR spectroscopy appears to be a more sensitive technique than both morphological and physiological biomarkers traditionally used. Indeed, FTIR analysis evidenced a plant response to NM contamination even at the shortest time of exposure (5 days). Looking at the morphological parameters, few differences were highlighted at 15 days (decrease in plant height for NTC 100 and decrease in leaf area for Ti 100 and Ti 500) but not earlier. And no impact was detected for both NMs using biomarkers for lipid peroxidation, photosynthetic pigments or secondary metabolites whatever the exposure duration. FTIR spectroscopy is thus a good technique to identify NMs early impacts on plants. It allowed assessing the impacts on several biomacromolecules (*i.e.* lipids, various polysaccharides, cellulosic compounds) in one single fast analysis. The developed chemometric analysis was very useful to highlight differences among conditions, which might not have been possible to identify by looking with the naked eye.

CNTs and TiO_2 -NPs are two very different NMs; in particular they are varying in shape (tubular for CNTs and spherical for TiO_2 -NPs), in surface chemistry (metal oxide and carbon), in size (1-3 nm diameter for CNTs and 25 nm for TiO_2 -NPs). Their behavior and their impacts are expected to be different (Rico *et al.*, 2011; Hatami *et al.*, 2016). However, in this study they had quite similar impacts, especially on cell wall components which might suggest a common response of plants to exposure to CNTs and TiO_2 -NPs.

The impacts of both NMs tended to increase with time until 15 days of exposure. At 15 days, impacts were the most important for both NMs, which were less marked after 20 days. This decrease in impacts after 15 days of exposure might suggest than there was some sort of plant recovery. Over 20 days, plants were possibly able to withstand NM contamination since there was no detectable difference in the morphological and physiological markers at this exposure duration and lower impact on biomacromolecules. It would thus be interesting to look at the impacts on biomacromolecules after a longer exposure time to confirm that hypothesis. In the literature, for instance, it was reported that TiO₂-NPs had no major impact on tomato plants upon harvest but some markers indicated that plants might have gone through oxidative stress earlier in their life cycle (Bakshi *et al.*, 2019).

The plant response to NMs did not seem to be dose-dependent. Most of the time, impacts were not larger at the highest concentration. One hypothesis possibly explaining this result is that NMs can have different behaviors depending on the concentration used. Indeed, when the concentration is increased, it also lead to more chances CNT interactions and homoagglomeration phenomena which would results in decreasing NM mobility and bioavailability in soils.

Differences in the FTIR spectra occurred in the protein region. Many studies reported that NMs can impact proteins (increased or decreased content), depending on the exposure dose and the type of plant species (Hatami *et al.*, 2016). TiO₂-NPs have been observed to increase the level of proteins in different plants such as *Linum usitatissimum* (Aghdam *et al.*, 2016) and *Spinacia oleracea* (Yang *et al.*, 2006). Using FTIR analysis, it has also been reported that TiO₂-NP contamination decreased the relative amount of amide of cucumber fruits (Servin, *et al.*, 2013).

FTIR spectra also showed that relative amount of lipids were increased by both NMs. Lipid content modification is one of the plant response to various stresses such as high temperature, drought or heavy metal (HM) (Niu and Xiang, 2018). Changes in the lipid composition and/or interactions between lipids and specific membrane proteins can occur in order to reinforce the cell to resist to the stress. This result is also consistent with the literature: in a study on spinach, TiO₂-NPs also increased the level of lipids after a foliar contamination (Gao *et al.*, 2013). Using FTIR analysis, several studies reported that metal NMs impacted the lipid region of the spectra. CeO₂-NPs provoked changes in the lipid region of *Coriandrum sativum*, *Oryza sativa* and *Cucumis sativus* (Morales *et al.*, 2013; Rico *et al.*, 2013; Zhao *et al.*, 2014). Ag-NPs also impacted the lipid region of radish sprouts (*Raphanus sativus*) (Zuverza-Mena *et al.*, 2016).

Plant cell wall components were the most impacted after exposure to both NMs, in particular cellulosic compounds and hemicellulose. Plant cell walls an important barrier against NM intrusion (Houston et al., 2016) thanks to its complex structure composed mainly of cellulose microfibrils and non-cellulosic neutral polysaccharides. And 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 (Tenhaken, 2014; Gall et al., 2015; Houston et al., 2016; Zhu, 2016). Both components are located inside the primary cell walls and are responsible of the cell thickness (Gall et al., 2015). Cellulose provides mechanical strength for load bearing thanks to the cross-linking by hemicelluloses (Tenhaken, 2014). During heavy metal contamination, plant cell wall also plays a crucial role as a physical barrier against the entry of heavy metals into the symplastic compartment and can act as a buffer by sequestrating them (Sattelmacher and Horst, 2007) through binding with hemicellulose and pectin. Cell wall thickenings also represents a plant response to mechanical intrusion of pathogens (Voigt, 2014; Schwab et al., 2015). For example, the cell wall can be actively reinforced through the deposition of cell wall appositions (papillae) at site of interaction with pathogens (Voigt, 2014). It has been found that cellulose deficient mutant plants are more sensitive to abiotic stress than wild type plants (Kesten et al., 2017). For example water deficit has been reported to increase the level of cellulose content; this could be in order to maintain cell wall integrity and cell turgor pressure which allows continuous cell growth under low water potential (Gall et al., 2015). It has already been reported earlier that NPs can affect region of the FTIR spectra corresponding to cell wall components. Ag-NPs affected cellulose and hemicellulose region of FTIR spectra in radish sprouts (*Raphanus sativus*) (Zuverza-Mena *et al.*, 2016). TiO₂-NPs were also able to increase lignin band area of the FTIR spectra of cucumber fruit (Servin *et al.*, 2013). It has finally been reported that metal lignin complexes may be formed and could be responsible of changes in the chemical environment of the plants (Morales *et al.*, 2013; Servin *et al.*, 2013).

Another phenomenon that can be responsible of the 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) (Hatami *et al.*, 2016; Liné *et al.*, 2017). For instance, CNTs increased ROS content in epidermis cells after 15 days of exposure in hydroponic conditions (Begum and Fugetsu, 2012b). CNTs have also been observed to increase the content of antioxidant enzymes such as peroxidases (POX) (Smirnova *et al.*, 2012). TiO₂-NPs also increased the level of catalase (CAT) and ascorbate peroxidase (APX) content in leaves of cucumber exposed for 150 days in sandy loam soil contaminated with TiO₂-NPs (Servin *et al.*, 2013). Those ROS can be associated with cell wall modification since a sudden burst of ROS can lead to catalytic oxidation of various substrates of the cell wall which results in cell wall cross-linking and growth arrest (Passardi *et al.*, 2004). Peroxidases, also implied in the regulation of oxidative stress, can promote cell wall loosening via the hydroxylic cycle (Schweikert *et al.*, 2002; Berni *et al.*, 2018). The modification identified in the cellulosic and hemicellulosic compounds may thus also be explained by the oxidative stress caused by the NMs tested which could be independent from NM internalization.

5. CONCLUSION

The use of FTIR spectroscopy in this study has allowed identifying a similar impacts of CNTs and TiO₂-NPs on tomato cell walls mainly hemicellulose and cellulose. FTIR is an easy-access, fast and powerful technique. Although data processing is not straightforward, we have proposed a strategy based on simple statistical analysis of the data which proved to be able to highlight very slight modifications induced by NM exposure. While no toxicity could be detected using more classical morphological and biochemical parameters. We observed a plant response even at the shortest exposure durations (5 days). The plant response tended to increase until 15 days of exposure for both NMs and effects were less marked at 20 days of exposure. The plant response did not seem to be dose-dependent for both NMs.

CHAPTER 6: ASSESSING PLANT RESPONSE OF 4 TYPES OF PLANTS TO DWCNT EXPOSURE

CNT impacts on plants are controversial. Some authors reviewed positive impacts and this reinforces the idea of the use of CNTs as growth regulators in agriculture. Other authors reported negative effects of CNTs. Finally in other studies, no effects were identified (see chapter 1 1.7).

It has been reported several times that the impacts of NMs may be dependent on plant species. Some studies have shown that some species are more sensitive to CNTs than others. For instance, Begum, Ikhtiari and Fugetsu (2014) concluded that spinach and lettuce are more sensitive to MWCNTs than rice and cucumber. Some authors even identified opposite effects of CNTs according to the plant species. Canas *et al.*, (2008) reported an inhibition of root growth in tomato but an enhancement in onion after MWCNT contamination. To date, no correlation between the parameters that defined the plant species (type of photosynthesis, monocots *vs* dicots, *etc.*) and the CNT impacts was identified.

In this chapter, we aimed to assess the effects of DWCNTs on 4 plant species (canola, cucumber, maize and tomato). These four species are plants of agronomic interests and present several differences (monocots *vs* dicots, C3 photosynthesis *vs* C4 photosynthesis, different size, *etc.*). The FTIR analyze developed in chapter 4 has also been used as well as plant morphology and metabolism analyses. Summary of the experimental set-up is presented in Figure 66.



Figure 66 Summary of the experimental design used to assess the impacts of DWCNTs on four different plant species.

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ARTICLE: COMPARATIVE RESPONSES OF FOUR CROP SPECIES (CANOLA, CUCUMBER, MAIZE AND TOMATO) TO A DOUBLE WALLED CARBON NANOTUBE CONTAMINATION IN SOIL

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1. INTRODUCTION

Research about nanotechnologies, and especially carbon nanomaterials (NMs) has intensively increased over the last few decades. Among the carbon NM family, carbon nanotubes (CNTs) are one of the most promising (Yang *et al.*, 2007). 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 length of few microns (Dresselhaus *et al.*, 2003). Thanks to their extraordinary thermal, electrical and mechanical properties, CNTs are used in many applications (*i.e.* batteries, plastic additives, sporting goods) and are promising for many other applications (Ajayan and Tour, 2007; De Volder *et al.*, 2013). Agriculture is one of the potential sectors for the use of CNTs and nanotechnology in general. Indeed, NMs may be used as fertilizers to enhance plant growth, pesticides for pest and disease management and finally sensors in order to monitor plant health and soil quality (Gogos *et al.*, 2012; Servin *et al.*, 2015; Mukherjee *et al.*, 2016).

Analytical data on CNT concentrations in the environment is not yet available due to the technological issue of detecting CNTs in complex carbonaceous matrices (Sun *et al.*, 2016a). Gottschalk et al. (2015) used models to predict the 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, it could reach 11.7 μ g/kg (data for Denmark). Gogos *et al.* (2012) calculated that the application dose of CNTs as plant protection products or fertilizers could be 3 to 12 g/ha which would correspond to a flux of 1.1 to 4.3 μ g/kg per year. Therefore, it is crucial to understand the toxicity of this emerging contaminant in agroenvironment and evaluate related health risk for humans.

However, despite this increasing concern, CNT effects on plant morphological, physiological, biochemical 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 time that exposure to CNTs can lead to enhancement of plant productivity (Khodakovskaya et al., 2013; Lahiani et al., 2016; McGehee et al., 2017). But other studies have shown that CNTs can lead to phytotoxic effects: decreasing plant growth, increasing generation of reactive oxygen species (ROS) or decreasing cell dry weight (Lin et al., 2009; Begum and Fugetsu, 2012a). Finally, other authors identified that CNTs exhibited no effect on different plant species (Lin and Xing, 2007; Larue et al., 2012; Hamdi et al., 2015). Such controversial effects could be explained by the type of CNT used, the experimental set-up as well as by the type of plants. Few studies investigating potentially different impacts of CNTs according to plant species have been published. For example, Canas et al. (2008) concluded that MWCNTs inhibited root elongation in tomato but enhanced it in onion and cucumber. Begum et al. (2014) identified that red spinach and lettuce were more sensitive to MWCNTs than rice and cucumber, with a diminution of root and shoot lengths. They also observed no toxic effects on chili, lady's finger and soybean. It is also interesting to note that almost all these studies were realized in hydroponic conditions and focused most of the time only on the impacts on seeds (germination, root and shoot length) (Canas et al., 2008; Begum et al., 2014; Lahiani et al., 2015).

Vithanage et al. 2017 concluded that CNTs tended to stimulate plant growth of many plants but their exact physiological functions depend on the genetic traits of a particular plant species and this is largely unknown. Several hypotheses can be stated: (i) interactions between plant seed and NMs will be higher with larger seed sand could lead to a higher CNT sensitivity; (ii) the plant response may change according to plant family: monocots *vs* dicots. Indeed, several mechanisms as well as plant architecture are different between the mono and the dicots (*e.g.* conductive vessel or root architecture). (iii) Not to mention plant types, every species is different from one to another (*e.g.* height, number of leaves, foliar area, *etc.*). This diversity may be an explanation to the differences in plant responses to NMs. For example, the higher leaf surface area can enhance the water exchange between the soil and the atmosphere (Wang *et al.*, 2019) and thus leading to a higher CNT accumulation in the leaves and more detectable effects.

The aim of this study was to try to identify relevant biological parameters influencing plant response to a DWCNT contamination using exposure conditions as realistic as possible: soil exposure and duration (5 weeks). We selected crop plants divided in three dicot species: cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*), canola (*Brassica napus*) () and one monocot: maize (*Zea mays*). CNT effects of s were evaluated at different biological levels: (i) plant morphology: germination, plant height, number of leaves, fresh and dry biomass as well as leaf area; (ii) plant metabolism: chlorophyll, flavonoid and phenolic compound concentrations and finally (iii) plant biomacromolecules using Fourier-transformed infrared spectroscopy (FTIR).

2. MATERIAL AND METHODS

2.1. DWCNT preparation and characterization

DWCNTs were synthetized at 1000°C by catalytic chemical vapor deposition (CCVD) of a mixture of CH₄ (18 mol.%) and H₂ using a Co:Mo MgO-based catalyst composed of Mg_{0.99}Co_{0.0075}MgO_{0.0025} (Flahaut *et al.*, 2003a). 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 μ m) 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 DWCNT suspension just before use since the different steps of the preparation protocol may modify the CNT 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 N₂ and adsorption of nitrogen gas at the temperature of liquid nitrogen; measurement accuracy \pm 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, $\lambda = 633$ 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 DWCNTs (XPS Kalpha ThermoScientific). Finally, zeta potential was determined in ultrapure water (Zetameter ZETACAD, CIRIMAT, Toulouse).

2.2. Soil characteristics and contamination

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

The amount of DWCNT suspension used to contaminate the medium was calculated to add half of the water capacity to the soil (here 30 mL for 100 g of soil) to avoid deconstructing the soil and reach a final concentration of 100 mg/kg of dry soil. To obtain a DWCNT distribution in the soil as homogeneous as possible, the suspension was spread on a tray of soil with a maximum soil thickness of 2 centimetres. The soil was then mixed and distributed in pots each containing the equivalent of 150 g of dry soil.

2.3. Plant material and cultivation

Organic seeds of tomato *Solanum lycopersicum* (var. Red Robin), cucumber *Cucumis sativus* (var. Le Genereux), canola *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 hours of light per day, 22°C during the night, 24°C during the day and a hygrometry rate of 85%). The exposure duration was 5 weeks. Two conditions were used: control and plants exposed to 100 mg/kg dry weight of DWCNTs with 5 replicates per condition. Four seeds were introduced per pot. After the appearance of the cotyledons, only one plant per pot was kept.

Morphological parameters were monitored all along the experiment: germination, plant height and number of leaves. The plant height was measured only from day 14 of the experiment, before they were too small for an accurate measurement. Upon harvest, the different parts of the plants were weighted in order to obtain the fresh biomass. Roots were dried at 50°C during 24h and weighted. 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. The other part was dried at 50°C during 24h for FTIR analysis.

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 glass bead of 4 mm and a bead-mill. In total, 5 biomarkers were assessed: photosynthetic pigment concentration (chlorophylls a, b and carotenoids) and secondary metabolites (phenolic compounds and flavonoids). Briefly, around 20 mg of ground fresh leaves were introduced into a 96 well microplate of 2 mL (3 technical replicates per plant). 1.5 mL of methanol 95% was added in each well. Plates were mixed for 2 min and covered with aluminum foil in order to avoid light degradation. Incubation time was 24h in the dark for photosynthetic pigments and 48h for secondary metabolites. After incubation, plates were centrifuged at 4500 rpm for 5 min. For pigments, 100 μ L of supernatant was transferred into microplates and absorbance was measured at 470, 652 and 666 nm (Lichtenthaler, 1987). The concentration was expressed as milligram per gram of fresh weight (mg/g f. wt.) using standard curves.

For total phenolic compounds, concentrations were calculated based on Folin Ciocalteu assay (Ainsworth and Gillespie, 2007). Briefly, 20 μ L of supernatant were mixed with 40 μ L of Folin reagent (10% v/v) and 0.10 mmol of sodium hydrogen carbonate (NaHCO₃). The mixture was incubated during 2h at room temperature until colour development. Absorbance was then measured at 760 nm. The concentrations were calculated using a standard curve of gallic acid and expressed as milligram of gallic acid equivalent (GAE) per gram of fresh weigh (mg GAE/g f. wt.).

Finally, flavonoid concentrations were determined based on aluminum chloride method (Settharaksa *et al.*, 2014). The reaction mixture contained 25 μ L of supernatant, 7.25 μ M of sodium nitrite (NaNO₂), 0.11 μ M of aluminium chloride (AlCl₃) and 0.02 mM of sodium hydroxide (NaOH). The mixture was homogenized during 1 min and absorbance was read at 595 nm. Concentrations were calculated using a standard curve of catechine and expressed as milligram of catechine equivalent (CE) 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 FastPrep machine (2 x 15 sec at maximum speed). FTIR analyses were performed at the European Synchrotron Radiation Facility (ESRF) in Grenoble (France) 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 the samples were analyzed in (technical) triplicates and one spectrum was the sum of 64 scans. OMNIC software was used to export experimental spectra.

Spectra were analysed with Orange software (BioLab) (Demšar *et al.*, 2013). First, they were pre-processed by restricting the areas of interest (between 1800 and 800cm⁻¹ and between 2900 and 2700 cm⁻¹). These two regions correspond to the protein and lipid regions. The region between 2700 and 1800 cm⁻¹ was removed because it mainly corresponds to background interferences. Data were then normalized using vector normalization and a Savitzky-Golay filter was applied with a window of 21, a polynomial order of 2

and a derivative order of 2. After pre-processing, a principal component analysis (PCA) was applied. This analysis permitted to check if different groups could be identified between conditions. When different groups were found, a logistic regression (LASSO) was performed on the pre-processed data, allowing the identification of the areas (wavenumbers) contributing the most to differences.

2.6. Statistical analysis

Data were checked for homoscedasticity and normality. When assumptions were met for parametric analyses, a one-way ANOVA was used. Otherwise, a Kruskal-Wallis test was applied. Then, a PCA was realized with all the data. All statistical analyses were performed using the RStudio statistical software (version 1.1.453) with car (Fox, 2002), multcompView (Graves, Piepho and Selzer, 2015), Ismeans (Lenth, 2016), pgirmess (Giraudoux *et al.*, 2018), ggplot2 (Wickham, 2009), FactoMineR (Lê *et al.*, 2008) and Factoextra (Kassambara and Mundt, 2017) packages.

3. RESULTS

3.1. DWCNT characterization

According to TEM observations, the mean outer diameter of the DWCNTs was 2.05 ± 0.7 nm (Figure 67A). The median inner diameter was 1.35 nm and the length between 1 and 100 μ m (Flahaut *et al.*, 2003a). The sample was composed of 15% of CNT triple-walled, 80% double-walled and 15% single-walled (Flahaut *et al.*, 2003a).



Figure 67 DWCNT characterization (A) TEM image of the purified DWCNTs. (B) 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).

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 67B). 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 ± 0.00 suggesting very little structural defects in the sample.

The first derivative of the TGA curve demonstrates that the DWCNTs were thermally stable up to *ca.* 310°C and the maximum rate of decomposition of the nanotubes was at 421°C (Figure 67C).

The specific surface area was 985 m²/g (Figure 67D).

The elemental analysis evidenced the composition of the DWCNTs: 89.75% carbon and 2.13% oxygen (Figure 67D). The catalyst amount remaining in the sample was 3.99% for cobalt and 0.96% for molybdenum. These metals were tightly encapsulated within graphitised layers of carbon and fully protected from their environment (no possible leak) (Flahaut *et al.*, 2002).

The DWCNT 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 67D). The soil solution was obtained by mixing miliQ water with water during 3 hours and then filtrating through filter paper.

3.2. Impacts of DWCNTs on plant morphological response

The leaf number was rapidly higher for cucumber plants exposed to DWCNTs in comparison to the control with a significant difference (p-value = 0.0161) at 16 days of exposure (Figure 68A). For canola, no difference was evidenced with in average 4.2 leaves at the end of the experiment (Figure 68B). For maize, the leaf number was the same for both conditions during the whole experiment with 3 leaves at 10 days of exposure, 4 at 10 and 5 at 33 (Figure 68C). For tomato, a higher leaf number was found at 19 and 20 days of exposure. After 35 days however, plants growing in both conditions had on average 3 leaves per plant (Figure 68D).



Figure 68 Leaf number during the experiment from day 1 to day 35 for the four plant species (cucumber, canola, maize and tomato) grown in a silty sand soil without (control) or with DWCNTs (CNT) at 100 mg/kg of dry soil. Significant differences are labeled with a star * (student test, p-value < 0.05).

Height of exposed cucumber plants was on average higher of 0.5 cm than the control plants all along the experiment (Figure 69A). However, differences were not significant (p-value > 0.5 for every day). For canola, from day 18 of exposure, exposed plant were on average 1.10 cm longer than the control plants, but this was also not significant (p-value > 0.5 for all the days) (Figure 69B). Exposed maize plants were smaller than the control plants, especially at the end of the exposure with significant differences at days 32 (0.84 cm smaller, p-value = 0.0254), 33 (0.90 cm smaller, p-value = 0.0249) and 35 (1.2 cm smaller, p-value = 0.0159) (Figure 69C). Finally, exposed tomato plants were on average 0.9 cm longer than the control plants during the whole exposure period, but this was again not significant (p-value > 0.05).



Figure 69 Plant height from day 14 to day 35 of the experiment for the four plant species (A cucumber, B canola, C maize and D tomato) grown in a silty sand soil without (control) or with DWCNTs (CNT) at 100 mg/kg of dry soil. Significant differences are labeled with a star * (student test, p-value < 0.05).

Germination started for all the plants 3 days after the beginning of exposure. The germination rates were not impacted by DWCNT exposure (Figure 70A). On average, the germination rate was 88% for cucumber, 70% for canola, 78% for maize and 75% for tomato.

At the end of exposure, the number of leaves was not significantly impacted by DWCNT exposure for none of the species (Figure 70B). Canola had on average more (4.2 on average) in comparison to the others (3.5 for cucumber, 5 for maize and 3 for tomato). Maize height at the end of exposure was the highest among the 4 species and a significantly decreased from 10.2 cm to 8.8 cm (- 13.7%) was noticed for plants exposed to DWCNTs in comparison to the control, going (p-value = 0.011) (Figure 70C). No significant difference was identified for the other plant species. On average plants measured 4.3 cm for canola, 3.5 cm for cucumber, 9.5 cm for maize and 3.7 cm for tomato. The leaf fresh biomass was significantly increased by 55.1% for canola and 70.8% for cucumber (p-value = 0.021 and 0.041 respectively) but unchanged for the other two species (Figure 70D). Maize had the highest leaf fresh biomass (1225 mg on average, 5.6 times more than canola, 6 times more than cucumber and 7.8 times more than tomato). The root fresh and dry biomasses as well as the total fresh biomass were not impacted by the DWCNTs (Figure 70(E-G)). Maize had the highest fresh and dry root biomass (688 mg for the fresh biomass and 45 mg for the dry one). The total leaf area at the end of the experiment was increased by 58.4% for canola and 64% for cucumber (pvalue = 0.033 and 0.040, respectively) (Figure 70H). When the mean leaf area per leaf was considered, canola and maize were significantly increased by 62.9% for canola and 58.4% for maize (p-value = 0.040 and 0.033 respectively) (Figure 70I). Maize had the highest mean area per leaf in comparison to the other plants (8.9 for maize, 2.1 for canola, 3.75 for cucumber and 2.1 for tomato).



CHAPTER 6 PLANT SCREENING

Figure 70 Results of the morphological analyses for the four studied plants (canola, cucumber, maize and tomato) grown in a silty sand soil without (Control) and with DWCNTs (CNTs) at 100 mg/kg of dry soil. A germination rate, B number of leaf, C height of the plant, D leaf fresh biomass, E root fresh biomass, F total fresh biomass, G root dry biomass, H leaf area and I leaf area per leaf. Significant differences are labeled with a star * (Kruskal-Wallis test for non-parametric data and one-way ANOVA for parametric data, p-value < 0.05).

3.3. Impacts of DWCNTs on plant biochemical responses

Chlorophylls of cucumber were significantly impacted (Figure 71) with an increase in chlorophylls a + b of 29.15% (p-value = 0.033) (Figure 71A). Canola was the plant with the highest chlorophyll concentration in comparison to the other plants: 837 mg/g f. wt for the total chlorophylls of canola and 476 mg/g f. wt for maize for example.

The concentrations of phenolic compounds and flavonoids were not impacted by DWCNT exposure (Figure 71D and E). The flavonoid concentration was on average 2 times lower for cucumber in comparison to the other plant species (1182 mg CE/g f. wt. for cucumber and 2613, 3286 and 1493 mg CE/g f. wt. for canola, maize and tomato). Likewise, lower phenolic compound concentrations were found for cucumber and tomato (respectively 20 mg GAE/g f. wt. and 13 mg GAE/g f. wt.) in comparison to the other plants (90 mg GAE/g f. wt. for canola and 112 mg GAE/g f. wt. for maize).





Figure 71 Results of the biochemical analyses for the four studied plants (canola, cucumber, maize and tomato) grown in a silty sand soil without (Control) and with DWCNTs (CNTs) at 100 mg/kg of dry soil: total chlorophyll concentration (A), flavonoid concentration (B), and total phenolic compound concentration (C). Significant differences (p<0.05) are labeled with a star * (Kruskal-Wallis test for non-parametric data and one-way ANOVA for parametric data, p-value < 0.05).

PCA were performed with all the biomarkers (physiological and biochemical) for the four plants (Figure 72). The factor mainly driving the PCA along axis 1 was the plant species, indeed maize which performed best on most assessed parameters segregated on the right side of the PCA while all other species were overlapping in the left side. Taking plant species into account, differences arising from DWCNT contamination played a minor role in the PCA.



Figure 72 PCA of morphological (germination rate, number of leaves, height of the plant, fresh and dry biomasses, leaf area) and biochemical parameters (total chlorophyll, flavonoid and phenolic compound concentration) for the four types of plants (canola, cucumber, maize and tomato) grown in a silty sand soil without (Control) and with DWCNTs (CNTs) at 100 mg/kg of dry soil. PCA were run and drawn with RStudio (FactoMineR and ggplot2). Bigger points are the barycenter of ellipses.

3.4. Impacts of DWCNTs on the plant biomacromolecules

FTIR spectra obtained on the 4 crops were analysed using a PCA approach highlighting mainly differences among plant species macromolecules with tomato and maize groups individualized and canola and cucumber grouped together (Figure 73). PCA for cucumber, maize and tomato did not show two groups well separated. DWCNT exposure did not lead to noticeable differences when plant species was considered. However, this parameter set apart, DWCNT and Control treatments for each species segregated (data not shown).



Figure 73 PCA of the FTIR spectra (between 800-1800 and 2700-2900 cm⁻¹) for the four types of plants (canola, cucumber, maize and tomato) with the different conditions (Control and CNTs). PCA were run with Orange software and draw with RStudio (ggplot2).

There were more differences between the control plants and the exposed plants in the FTIR spectra for canola compared to the other plants. However, there are still few differences for the other plants. Biomacromolecule compositions were different for maize and tomato in comparison to the other plants.

The identified differences for each plant are presented in Figure 74. Table 16 summarizes the most relevant peaks identified for the four plants. Differences in the region around 990 cm⁻¹ were identified for canola and maize (peak d Figure 74A and C). This area represents the peaks of pectin, various polysaccharides and mainly cellulose. The area under the peak at this wavenumber was lower for plants grown in soil contaminated with CNTs compared to control plants which indicates that the relative amount of cellulose related compounds decreased with CNT exposure. Differences were also identified in the peak between 1610 and 1660 cm⁻¹ for cucumber and tomato (peak b Figure 74B and D). This peak corresponds to the amine I region according to the literature. For cucumber, another significant difference was spotted at 1078 cm⁻¹ (peak c Figure 74B). This area is attributed to hemicellulose and in particular xyloglucan. For

maize, another difference was identified at *ca.* 2849 cm⁻¹ (peak a Figure 74C). This peak is in the lipid region, and corresponds to the CH_2 symmetric stretching.



Figure 74 Normalized FTIR spectra for the four types of plants (canola, cucumber, maize and tomato) with the two conditions (Control and CNTs). Peaks contributing the most among the different groups are highlighted in vellow.

Table 18 Most relevant peaks extracted from the logistic regression for the four types of plants with the band letter corresponding to the figure 6, the area under the peak, the band assignment and the main compounds that can be identified with the cited references.

Wavenumbers (cm ⁻¹)	Band	Plants	Area under the peak	Assignment	Main compounds	References
2848-2852	а	Maize	0.168 Control 0.174 CNTs	CH ₂ symmetric stretch		Schulz et al., 2007 Türker-Kaya et al., 2017
1610-1640	b	Cucumber	0.554 Control 0.551 CNTs	C=O strotch	Amide I: protein, pectin, water associated with cellulose or lignin	Türker-Kaya et al., 2017 Regvar et al., 2013
		Tomato	0.587 Control 0.586 CNTs	C=O stretch		
1070-1080	с	Cucumber	0.489 Control 0.493 CNTs	C-O ring stretch	Various polysaccharides (hemicelluloses in particular xyloglucan)	Alonso-Simon et al., 2011 Regvar et al., 2013 Türker-Kaya et al., 2017
990	d	Canola	0.630 Control 0.609 CNTs		Pectin, various polysaccharides, cellulose	Schulz et al., 2007 Regvar et al., 2013 Türker-Kaya et al., 2017
		Maize	0.51 Control 0.52 CNTs			

4. DISCUSSION

In the literature, most of the studies evaluating impacts of CNTs on plants focused on one single plant species. It is tricky to compare effects of CNTs on different plant species using different articles since many parameters usually vary from one study to another (*e.g.* exposure time, growth media, type of CNTs). In the studies comparing effects of CNTs among different plant species, similar effects were most of the time described. Lahiani *et al.* (2013) established that seed germination was activated for barley, soybean and maize after MWCNT deposition on seed surfaces. Using the same 3 species, they also had an enhanced development in hydroponics up to 100 µg.mL⁻¹ of MWCNTs. They recorded several other positive phenotypical changes as well as the enhancement of photosynthesis in exposed plants (Lahiani *et al.*, 2017). Srivastava and Rao (2014) also reported enhancement of plant growth and biomass for 4 wheat, maize, peanut and garlic exposed at 50 µg.mL⁻¹ of MWCNTs in hydroponics. Lahiani *et al.* 2013 compared the effects of functionalized MWCNTs on seeds of barley, soybean and maize and evidenced early germination and higher seedling growth for all the tested plants. In 2015, they also identified "positive" impacts of SW carbon nanohorns (at 50, 100 and 200 µg.mL⁻¹) on maize, tomato, rice and soybean but no impact was found for barley and switchgrass (Lahiani *et al.*, 2015b).

Here, our results highlighted a significant decreased in maize height while a significant increased biomass and leaf area was detected for canola and cucumber. Tomato plant appeared to be the less sensitive plant species with no significant impact of DWCNT upon harvest. Contrasting with literature studies, plants were exposed in soils to DWCNTs. NM behavior in soil is not yet fully understood (Shrivastava *et al.*, 2019). Nevertheless, we can expect NMs to behave differently in soil than in suspension or in jellified growth medium affecting their interactions and thus their impact on plants. Many parameters differ from maize to the other plants. Maize had the biggest seeds in comparison to the other plants which increase the surface contact between plant and CNTs in soil. However, the smallest seeds were canola seeds which was not the least sensitive species. Likewise, in the literature, no difference was reported according to the size of the seeds (Liné *et al.*, 2017; Chen *et al.*, 2018). Jain *et al.*, (2017) established no correlation between seed size and ZnO NP toxicity. However, they reported that seed surface anatomy played a crucial role in determining the phytotoxicity of ZnO NPs. Least 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 seeds which may ease the interactions between nano ZnO and seeds.

Maize was also the plant exhibiting the longest roots. Since plant exposure was made by the roots, a more developed root system means potentially more contact between plants and CNTs in the soil.

The leaf surface area was also larger in maize. Plants with higher surface area have a higher transpiration rate which leads to increase the exchange of water between the soil and the plant (Wang *et al.*, 2019). We can thus expect a higher CNT accumulation in maize plant. Indeed, several studies have shown that uptake of NMs is varying according to the plant species (Pérez-de-Luque, 2017). Zhu *et al.*, (2012) showed that radish and ryegrass accumulated larger amounts of AuNPs than rice and pumpkin. Larue *et al.*, (2012) established that CNT accumulated more in wheat than in rapeseed. Once inside the plant, it has been recorded several times than CNTs may cause negative effects when they are in contact with cells: *e.g.* increase level of ROS, decrease cell viability (Tan *et al.*, 2009; Liné *et al.*, 2017).

Both effects of CNTs on pigment concentrations were reported. As we also found for cucumber plant, Park and Ahn, (2016) recorded an increase in the chlorophyll content in carrots after an exposure to MWCNTs.

Biomacromolecules such as cellulose, hemicellulose, proteins and lipids were impacted by CNT contamination. The effects of NMs on proteins have been shown to be species and cultivars specific (Hatami *et al.*, 2016). For example AgNPs increased more the protein level in leaves in Foxi than in Flowerfairy (two cultivars of pelargonium), while in tomato AgNPs were shown to decrease the level of total soluble proteins (Mehrian *et al.*, 2015). TiO₂-NPs have been observed to increase the protein level in spinach (Yang *et al.*, 2006) while Servin *et al.*, (2013) reported a decrease in amide after exposure of cucumber to TiO₂-NPs. Plant cell wall components such as cellulose and hemicellulose have been shown to be impacted by CNTs. These two compounds were reported many times to be impacted by abiotic and biotic stresses (Tenhaken, 2014; Le Gall *et al.*, 2015; Houston *et al.*, 2016). For example, during exposure to heavy metals (HM), plant cell wall will act as a barrier against the entry of HM. It has already been reported that NPs can affect the region of the FTIR spectra corresponding to cell wall components: TiO₂-NPs increased lignin band area of cucumber fruits (Servin *et al.*, 2013).

5. CONCLUSION

In this study, four plant species (canola, cucumber, maize and tomato) were used to assess the impacts of DWCNT contamination. Different endpoints were used to evaluate the impacts (*i.e.* morphological parameters, biochemical analyses and biomacromolecule analyses using FTIR). We were able to demonstrate that the impacts of DWCNTs are species dependent Concerning the morphological parameters, maize was slightly inhibited (plant height) but canola and cucumber were boosted (leaf area and leaf biomass). Tomato was not impacted. No significant differences were found for the biochemical parameters for cucumber, canola and tomato. However, total chlorophylls were higher for maize with the CNT contamination. FTIR was used to evaluate the response on the biomacromolecules. A common response was found for the four plant species with impacts of the DWCNTs on the plant cell wall components. However, impacts were more or less important according to the plant species.

CHAPTER 7: EVALUATION OF THE INFLUENCE OF THE PHYSICOCHEMICAL PARAMETERS OF CNTS ON PLANT RESPONSE UNDER OPTIMAL CONDITIONS AND HEAT STRESS

There are a multitude of CNTs varying in several physicochemical parameters (*e.g.* diameter/number of walls, length, specific surface area, functionalization, *etc.*). It has been reported that the plant response may differ according to the type of CNTs used. For instance, Zhai *et al.* (2015) demonstrated that the surface charge and the length of CNTs caused relative differences in their uptake and translocation in maize and soybean. Likewise, Canas *et al.* (2008) evidenced that non-functionalized SWCNTs affected root length of several crop species (cucumber, lettuce, inion, tomato, cabbage and carrot) more than raw SWCNTs.

In another hand, some studies reported an increased plant response to NMs when exposure was combined with another stressful condition (*i.e.* abiotic and biotic stress). For example, TiO₂-NPs were shown to reduce the impacts of drought on dragonhead plants (*Dracocephalum moldavica*) (Mohammadi, Esmailpour and Gheranpaye, 2016). In another study, MWCNTs were able to increase the seed and seedling tolerance of Caucasian alder (*Alnus subcordata*) to drought stress (Rahimi *et al.*, 2016).

In this paper, we aimed to investigate canola (*Brassica napus*) response to five types of CNTs (DWCNTs, DWCNTs functionalized, MWCNTs, MWCNTs functionalized and short MWCNTs) in soil at three different concentrations (0.1, 10 and 100 mg/kg of soil) under optimal and heat stress conditions. Morphological (*i.e.* germination rate, plant height, number of leaves, leaf area and biomass) and biochemical (*i.e.* nutrient concentrations in roots and leaves, pigment concentrations and lipid peroxidation level) analyses were assessed. A graphical abstract of the experimental set-up is presented in Figure 75.

This experiment has been carried out at the Connecticut Agricultural Experiment Station (CAES) in New Haven, USA in the lab of Jason White as part of collaboration. This research stay was funded by two grants from Toulouse University and Toulouse INP





Figure 75 Summary of the experimental design used to assess the impacts of 5 different types of CNTs under optimal and heat stress conditions.

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ARTICLE: INFLUENCE OF THE PHYSICOCHEMICAL PARAMETERS OF CNTS ON THEIR IMPACTS ON CANOLA PLANT UNDER OPTIMAL AND ABIOTIC STRESS CONDITIONS

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1. INTRODUCTION

Carbon nanotubes (CNTs; graphene layer(s) rolled around themselves to form tubes) are among the most used nanomaterials (NMs). The worldwide market was estimated around USD 4.55 billion in 2018 and is expected to grow and reach more than USD 9 billion by 2023 (MarketsandMarketsTM, 2019). Their unique structure brings them remarkable attributes (*e.g.* optical, electrical, thermal, mechanical and chemical) (Terrones, 2004). Consequently, they are used in many applications such as field emission displays, energy storage, integrated circuits, nanocomposites, *etc.* (Monthioux *et al.*, 2014). It exists many types of CNTs: they can have different numbers of layers which make their diameter varies from a few nm for the single walled CNTs (SWCNTs) to several tens of nm for the multi walled CNTs (MWCNTs). The length of the tubes ranges most of the time between 1 and 100 μ m, but they can also be shorter in some cases, especially after some processing steps such as functionalization and/or sonication. CNTs can be surface functionalized (*i.e.* attachment of chemical groups at the external surface of the tubes) or not. These different physicochemical parameters can change their properties and behavior in the environment after release.

Indeed, all along their lifecycle, from production to destruction, CNTs may be spread into the environment (Gottschalk *et al.*, 2013b). For example, they can accumulate in soils due to unintentional release from the wear of CNT-containing tires on the roads (Nowack *et al.*, 2013), or intentionally as growth regulators in agriculture or contaminant removal in soil and water remediation (Upadhyayula *et al.*, 2009). Nowadays, there is no agreement concerning the effects and the behavior of CNTs on the terrestrial ecosystems and especially on plants (Liné *et al.*, 2017). One of the main reasons for this great disparity between results is the diversity of the CNT used. For example, Zhai *et al.* (2015) identified that CNT surface charge and length caused relative differences in their uptake and translocation in maize and soybean. In maize only the neutral MWCNTs moved to the xylem and phloem, positively charged MWCNTs were attracted to the surface of the xylem cells while the negatively charged CNTs were repulsively dispersed in the xylem cells. Likewise, Canas *et al.* (2008) concluded that overall non-functionalized SWCNTs affected root length of several crop species (cucumber, lettuce, onion, tomato, cabbage and carrot) more than raw SWCNTs. The non-functionalized CNTs inhibited root elongation in tomato and enhanced it in onion and cucumber.

Recently, some authors evidenced that NMs could help plants to cope with abiotic stress. Nano TiO₂ with a diameter between 10 and 15 nm at a concentration of 10 mg.L⁻¹ reduced the impacts of drought on dragonhead plants (*Dracocephalum moldavica*) (Mohammadi *et al.*, 2016). Cerium dioxide nanoparticles decreased oxidative stress in Sorghum (*Sorghum bicolor* L.) grown under drought conditions leading to a higher photosynthesis and grain yield (Djanaguiraman *et al.*, 2018). Likewise, nano zinc alleviated salinity stress on cotton plants (*Gossypium barbadense* L.) (Hussein and Abou-Baker, 2018).

In this study, we aimed to assess impacts of five different CNTs (DWCNTs, functionalized DWCNTs, MWCNTs, functionalized MWCNTs and short MWCNTs) on canola plants (*Brassica napus*) under two different conditions (optimal conditions and heat stress). First, CNTs were thoroughly characterized. Canola plants were grown for five weeks in an agricultural soil contaminated at three CNT concentrations

(0.1, 10 and 100 mg.kg⁻¹ of soil). After exposure, several parameters were assessed to evaluate CNT impact in the two exposure conditions: morphological parameters, metabolism biomarkers and nutrient concentration.

2. MATERIALS AND METHODS

2.1. Nanomaterials

Five different CNTs were used during this experiment. DWCNTs were synthetized by catalytic chemical vapor deposition (CCVD) using a Co:Mo/MgO-based catalyst (Flahaut *et al.*, 2003a). Purification process was made with an aqueous HCl solution (37%). MWCNTs were purchased from Cheaptubes[®]. They were synthetized also by CCVD but with Ni and Fe as catalysts. Functionalized DWCNTs and MWCNTs were prepared from these two pristine powders. In the case of DWCNTs, the functionalization was performed directly after catalyst removal, from a wet sample (deionized water). For the commercial MWCNTs, provided in the form of dry powders, they were used as such. CNTs were sonicated few minutes with HNO₃ 3M (1 mL of HNO₃ solution per mg of dry CNTs). The mixture was heated using a reflux heating system for 24h at 130°C. Then, the sample was cooled down to room temperature and filtered through polypropylene membrane (Merk Mollipore, 0.45 μm) and rinsed several times until neutrality. Short MWCNTs were purchased from NanoGrafi[®]. They were also synthesized by CCVD, with Fe and Co as catalysts.

For preparation of stock suspensions (1 g/L), an ultrasonic probe (Vibra Cell 75042, 20 kHz, 500 W, 12.5 mm diameter rod) was immerged in the suspension, placed into an ice bath. The sonication followed a square wave composed of 1s pulse and 1s relaxation with an amplitude of 35% for 30 min. Immediately before use, suspensions were re-sonicated for 10 min in an ultrasonic bath (Elmasonic S30H, 280 W) in order to homogenize the samples.

Since the preparation of CNT suspension may modify their physicochemical properties, characterization was performed immediately after this step. Indeed, it was proven that sonication could change their morphology: creation of structural defects, shortening by breaking especially for MWCNTs)) (Kaur *et al.*, 2017).Nanotube shape and diameter were observed by Transmission Electron Microscopy (TEM; JEOL TEM 1400). The specific surface area (SSA) was assessed using the Brunauer, Emmett and Teller (BET) method (Micrometrics Flow Sorb II 2300; 2h degassing at 100°C in N₂ and adsorption of nitrogen gas at liquid nitrogen temperature; measurement accuracy ± 3%). Chemical elemental analysis was performed using micro-analyzers for C, O, S and N measurements and Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) for catalyst metal content (Institut des Sciences Analytiques, UMR 5280, CNRS Lyon). Zeta potential was determined with a Zetasizer in deionized water (Malvern).

2.2. Soil characteristics and contamination

An agricultural soil from the Connecticut agricultural experiment station (CAES) experimental farm was used. The pH of the soil was at 6.20. The percentage of organic matter was 2.49%. The cation exchange capacity (CEC) was 11.52 mol/kg.

The amount of the CNT suspension to add to the soil was calculated in order to add half of the water holding capacity of the soil and reach a final concentration of 0.1, 10 or 100 mg/kg of dry soil. In order to achieve a homogeneous repartition of the CNTs, the suspension was spread on a tray of soil with a thickness of a few centimetres. The soil was then mixed manually and distributed in pots (equivalent of 150 g of dry soil per pot).

2.3. Plant material and cultivation

Seeds of canola *Brassica napus* were surface sterilized using $Ca(CIO)_2$ (1%) and introduced in the soil (4 seeds per pot). Pots were placed in a greenhouse. After cotyledon appearance, only one plant per pot was kept. Exposure duration was 5 weeks with 10 biological replicates per condition.

Morphological parameters were recorded daily: germination, plant height and number of leaves. Upon harvest, fresh biomass was recorded, and 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 biochemical analyses. The other part was dried at 50°C during 24h, weighted and used for ICP-AES (roots and leaves).

A first set of experiments were carried out under optimal growth conditions for canola plant. The outside average temperature was around 4°C with a maximum temperature at 19°C and a minimum at -16°C. It was then replicated during spring-summer. The average temperature was 14°C with a maximum temperature recorded at 31°C and a minimum at -3°C. Experiments were performed in the greenhouse.

2.4. Biochemical response analysis

Biochemical analyses were performed on liquid nitrogen frozen leaves after a grinding step using a mortar and pestle. Then photosynthetic pigments and lipid peroxidation were assessed.

2.5. Nutrient concentration analyses

Briefly, leaves and roots were rinsed several time with MilliQ water, then oven-dried at 100° C for 72h and digested for 25 min on a hot block with concentrated HNO₃ at 115° C. After 30 min, 1 mL of H₂O₂ was added to each digestion tube and samples were digested for an additional 30 min and diluted with 50 mL of MilliQ water. Samples were anayzed using ICP-AES.

2.6. Statistical analysis

Data (morphological and physiological 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 principal component analysis (PCA) was performed with all the data. Statistical analyses were carried out using the RStudio statistical software (version 1.1.453) with car (Fox, 2002), multcompView (Graves, Piepho and Selzer, 2015), Ismeans (Lenth, 2016), pgirmess (Giraudoux *et al.*, 2018), ggplot2 (Wickham, 2009), FactoMineR (Lê *et al.*, 2008) and factoextra (Kassambara and Mundt, 2017) packages.
3. RESULTS

3.1. CNT characterisation

Results of the CNT characterisation are presented in Table 19.

Table 19 Characteristics of the different CNTs used. *Information provided by Flahaut et al (2003) ** Information provided by Cheaptubes© *** Information provided by Nanografi© (letters) statistical analysis using Kruskal Wallis (p-value < 0.05) and ND = non-determined. Different techniques were used: TEM for diameters and length, BET method for SSA measurement, organic micro-analyzers for elemental analysis (CNOS), ICP-AES for metal analysis, the I_D/I_G ratio with Raman spectroscopy and the zeta potential with a zetasizer.

	DWCNTs	DWCNTs f	MWCNTs	MWCNTs f	Short MWCNTs	
Catalysts used	Co/Mo	o-MgO	Fe/I	Fe/Co***		
Mean inner diameter	1.4 ± ND*	1.4 ± ND*	9.4 ± 2.6	8.2 ± 2.9	5.6 ± 1.9	
(nm)	(median)	(median)	(a)	(a)	(b)	
Mean outer diameter	2.1 ± 0.7	2.0 ± 0.8	24.2 ± 8.6	23.4 ± 10.8	12.3 ± 4.0	
(nm)) (a) (a)		(b)	(b)	(c)	
Length (microns)	1 to 100*	1 to 100*	1 to 20**	1 to 20**	<0.5***	
Specific Surface Area (m²/g)	985	240	90	90	60	
Elemental analysis (CNOS)	C 89.75% O 2.13%	C 76.93% O 17.25% N 0.30%	C 94.88% O 0.56%	C 93.16% O 1.92% N 0.10%	C 87.65% O 2.18% N 0.22%	
Elemental analysis (Metals from catalysts)	Co 3.99% Mo 0.96%	Co 0.99% Mo 0.12%	Fe 0.21% Ni 1.90%	Fe 0.32% Ni 1.28%	Fe 0.18% Co 0.66%	
I_D/I_G intensity ratio	0.23 ± 0.00	0.22 ± 0.00	1.28 ± 0.00	1.18 ± 0.00	1.77 ± 0.02	
Zeta potential in DI water (mV)	-27.5 -40.4		-17.8	-41.5	-18.7	

The mean inner diameter of both DWCNTs (functionalized or not) was not possible to determine using a classical TEM. Flahaut *et al.* (2003) on the same material used a high resolution TEM and measured a median inner diameter of 1.35 nm. We consider that the inner diameter for the DWCNTs f was the same since functionalization does not change the outer diameter of CNTs in such experimental conditions. MWCNTs and MWCNTs f had an inner diameter of 9.4 and 8.2 nm and the short MWCNTs of 5.6 nm (p-values < 0.0001). DWCNTs had the smaller outer diameter (around 2 nm). The other CNTs had a

diameter much higher, 6 times higher for the short MWCNTs (p-value < 0.0001) and 12 times higher for the other MWCNTs (p-value < 0.0001). Functionalization did not impact the outer diameter.

The length of the tubes varied within the same sample (large distribution range). Furthermore, using TEM, the tubes appeared too entangled and overlapped to obtain a reliable length measurement. For DWCNTs, the length was estimated in a previous study using the very same material between 1 and 100 μ m (Flahaut *et al.*, 2003a). For MWCNTs, the supplier indicated a length between 1 and 20 μ m. Finally, the short MWCNTs were bought for their small length claimed by the manufacturer (< 500 nm). However, according to our TEM images, their length was rather shorter than 2000 nm. Over 150 tubes measured, most of the tubes (40%) had a length between 500 and 1000 nm. However, more than 16% reached more than 2000 nm. In conclusion, short MWCNTs were longer than what the producer announced but most of them were still smaller than the other CNTs.

The SSA varied a lot according to CNT diameter/number of walls and functionalization. Not surprisingly, DWCNTs reached the highest SSA (985 m²/g) due to their small diameter and limited number of walls. The functionalization decreased their SSA (240 m²/g). For the other tubes, the SSA were much lower due to their larger diameter (90 for both MWCNTs and 60 for short MWCNTs).

The elemental analysis revealed that samples were mostly composed of C, > 75 wt. % for all the samples. The O content increased with the functionalization (*e.g.* 2.13 wt. % for DWCNTs *vs.* 17.25 wt. % for DWCNTs f). N was only detected in DWCNTs f, MWCNTs f and the short MWCNTs, respectively at 0.20 wt. %, 0.10 wt. % and 0.22 wt. %. Remaining metal catalysts were detected in all samples: Co and Mo for DWCNTs, Fe and Ni for the MWCNTs from Cheaptubes and Fe and Co for the short MWCNTs. The functionalization decreased the content of metals (*e.g.* Co content of 3.99% for the DWCNTs *vs.* 0.99% for the DWCNTs f). The zeta potential was modified by the oxidizing functionalization. CNTs became more hydrophilic which makes them more stable in suspension (Ernst *et al.*, 2017). Suspensions of particles with a zeta potential of more than +30 mV or less than -30 mV are considered stable (Gupta and Trivedi, 2018).

3.2. Stress effects on plant growth

Table 20 gathers the different morphological parameters of the control plants for the experiment carried out under optimal growth condition and the one under heat stress condition.

	Experiment without stress (optimal conditions)	Experiment with stress (abiotic stresses)	Differences (%)	
Germination (%)	88.00	32.00	- 56.00	
Number of leaves	5.80 ± 0.75	12.50 ± 2.89	+ 46.40 *	
Height (cm)	9.27 ± 0.79	33.50 ± 7.42	+ 72.30 *	
Fresh leaf biomass (g)	0.74 ± 0.27	1.70 ± 0.20	+ 56.50 *	
Leaf dry matter content (%)	25.91 ± 4.28	10.47 ± 2.30	- 59.59 *	
Fresh root biomass (g)	0.76 ± 0.13	1.03 ± 0.28	+25.80 *	
Root dry matter content (%)	8.64 ± 0.73	9.18 ± 3.21	+ 6.25	
Total leaf area (cm ²)	23.40 ± 4.51	24.40 ± 7.42	+ 4.10	
Leaf area / leaf (cm²/leaf)	4.04 ± 0.67	2.02 ± 0.72	- 50.00 *	
SLA	170.95	138.63	+ 18.47	
Flowers	No	Yes		

Table 20 Morphological parameters of the control canola plant in the two experiments (with and without stress) with the differences in percent. * indicates statistical differences (p-value < 0.05). SLA = specific leaf area

Only 32% of the seeds germinated in the experiment with stress (56.00% less than in optimal growth conditions). Plants exposed to abiotic stress had a significantly higher number of leaves (+46.40%, p value = 0.0194). Plants were also significantly taller (+72.30%, p value = 0.0050). Consequently, they had more biomass (1.70 vs 0.74 g of fresh leaves in optimal growth conditions). Despite a higher number of leaves, the total leaf area was not significantly different between the two experiments. The leaf area per leaf was even two times lower for the stressed plants. During heat stress, plants reached the flowering stage but this was not observed in the other experiment. The development of plants was much faster in the second experiment.

3.3. CNT impacts in optimal growth conditions

Impacts on the different parameters (germination, number of leaves, height, leaf area, leaf area per leaf, dry and fresh biomass, SLA, total chlorophylls and lipid peroxidation) were studied. No significant difference was found between the control and the different CNT conditions for most of the parameters such as germination rate, number of leaves, height of the plant, total dry weight, chlorophylls and water contents. However, plants grown with MWCNTs had a lower leaf area (-11.6% in comparison with the control, p-value = 0.0021), a lower leaf area per leaf (-5.6%, p-value = 0.2890), a lower total fresh weight (-16%, p-value = 0.0043) and consequently a lower SLA (-4%, p-value = 0.0037). Plants grown with DWCNTs showed a trend to have a higher leaf area per leaf (+15% in comparison with the control, p-value = 0.2720), a higher SLA (+28%, p-value = 0.0222) but a lower ratio roots/leaves (-33%, p-value = 0.0001). The PCA (Figure 76) summarizes all these data and highlights the fact that the different CNTs had no major impact on plant development in optimal growth conditions.



Figure 76 PCA of the different morphological and biochemical parameters (germination, number of leaves, height, leaf area, leaf area per leaf, dry and fresh biomass, SLA and total chlorophylls) of the canola plants grew with or without the different CNTs (DWCNTs, DWCNTs f, MWCNTs, MWCNTs f, short MWCNTs) at three concentrations (0.1, 10 and 100 mg/kg⁻¹) in a fine sandy loam soil during 5 weeks.

3.4. CNT impacts combined with heat stress

Germination rate was differently impacted by the CNT contamination under abiotic stress (Figure 77). DWCNTs f increased significantly the germination especially at the highest concentration (two times higher at 100 mg/kg) (p-value = 0.0009). DWCNTs at the highest concentration decreased the germination but it was not significant due to the high standard deviation. The other CNTs tended to increase the germination rate in comparison to the control, but it was not significant.



Figure 77 Germination rate of the control plants and plants grown in contaminated soil during five weeks with the five CNTs at the three different concentrations. Kruskal Wallis tests were used for non-parametric data. Different lowercase letters indicate significant differences (p-value < 0.05).

The number of leaves was not impacted by the different CNT contamination (Figure 78A). A slight nonsignificant increase was detected for the DWCNTs f in comparison to the control: 12.50 leaves for the control and 17.32 leaves on average for the DWCNTs f (p-value = 0.07). There was also a trend for a decreased number of leaves for plants exposed to MWCNTs in comparison to the control. However, there was a significant difference between plants exposed to DWCNTs f and MWCNTs (p-value = 0.0046).

More differences were evidenced for plant heights (Figure 78B): plants exposed to DWCNTs were higher while the others tended to be smaller in comparison to the control. The control plants had an average height of 33.5 cm, while after exposure to DWCNTs the average height was 56.1 cm. The height of plants exposed to DWCNTs, MWCNTs, MWCNTs f and short MWCNTs were respectively 21.6 cm, 25.6 cm, 21.7 cm and 20.0 cm. A significant difference was also found between DWCNTs and DWCNTs f (p-value < 0.0001). There was no dose dependent effect.

The total leaf area was impacted by the different CNTs (Figure 78C). The plants grown with DWCNTs f had a surface area 2.3 times higher than the control (p-value = 0.0008). A small increase was found for the number of leaves of plants grown with DWCNTs f. It explains the higher total surface area found for this

condition. When this surface area is divided by the number of leaves, no significant difference was noticed between the different conditions (p-value = 0.1590) (Figure 78D).

Looking at the specific leaf area (SLA), we detected an increase for plants grown with DWCNTs in comparison to control (p-value = 0.0140) and plants grown with DWCNTs f (p-value < 0.0001) (Figure 78E). The SLA was 2.4 times higher than the control and 2.8 times higher than the DWCNTs f condition. At the smallest concentration (0.1 mg/kg), higher SLA were obtained but it was not significant in comparison to the control (p-value = 0.1143). Concerning the water content in plants, significant differences were found between the two DWCNTs (p-value = 0.0002) (Figure 78F with an average of. 92.87% for DWCNTs and 88.12% for DWCNTs f in comparison to the control (on average 89.53%). For leaves and roots, higher fresh and dry biomasses were highlighted after exposure to DWCNTs f (Figure 79). There was no clear effect related to CNT concentrations on these different parameters.

The biomass (leaves and roots, fresh and dry) was higher for the plants exposed to DWCNTs f in comparison to the control at all concentrations. For plants exposed to DWCNTs, the fresh leaf biomass was: 1.45 ± 0.62 g, 1.82 ± 0.47 g and 1.94 ± 1.39 g for 0.1, 10 and 100 mg/kg resp. and after exposure to DWCNTs f: 4.18 ± 0.42 g, 3.97 ± 0.38 g and 4.24 ± 0.77 g. Fresh root biomass was 43.47% higher for DWCNTs f exposed plants in comparison to those exposed to DWCNTs (p-value = 0.0002). Likewise, dry leaf biomass was 2.7 times higher for plants exposed to DWCNTs f in comparison to DWCNTs (p-value < 0.0001). Finally for the dry root biomass, there was an increase of 26.66% at 0.1 mg/kg, 53.33% at 10 mg/kg and 11.11% at 100 mg/kg for the plants exposed to DWCNTs f in comparison to the DWCNTs (p-value < 0.0001).

For the biochemical parameters, no significant difference was determined for pigments (chlorophyll a and b, and carotenoids) between plants exposed to the different CNTs and control plants (Figure 80A, B, C and D). On average, leaves had 42.75 \pm 14.2 mg of chlorophyll a/ g of FW, 9.63 \pm 3.33 mg of chlorophyll b / g of FW and 13.19 \pm 4.62 mg of carotenoids / g of FW. For chlorophyll b, a small non-significant decrease was evidenced for plants exposed to DWCNTs f (6.67 \pm 2.29 mg/g of FW on average in comparison to 9.93 \pm 3.93 mg/g of FW for the control) and a small increase for those exposed to MWCNTs f (11.21 \pm 2.61 mg/g of FW) and short MWCNTs (10.01 \pm 2.00 mg/g of FW). Lipid peroxidation was not impacted by the different exposure conditions (Figure 80E) with on average 1.16 \pm 0.31 nmol/g f.wt.



Figure 78 Morphological parameters (height, number of leaves, leaf area, leaf area per leaf, SLA and water content) of the control plants and the plants grown in soil contaminated with the different CNTs at three concentrations (0.1, 10 and 100 mg/kg). Letters indicate the differences.



Figure 79 Fresh (A) and dry biomass (B) for leaves and roots of the control plants and the plants grown in soil contaminated with the different CNTs at three concentrations (0.1, 10 and 100 mg/kg). Letters indicate the differences.



Figure 80 Biochemical parameters: A. Chlorophyll a, B. chlorophyll b, C. total chlorophylls, D. carotenoids and E. lipid peroxidation of control plants and plants grown in soil contaminated with the different CNTs at three concentrations (0.1, 10 and 100 mg/kg). Different letters indicate significant differences (p<0.05). Data are mean ± standard error, n = 5).

No significant difference was identified on nutrient concentrations (B, Ca, Cu, K, Cd, Mn, Mg, Na, P, Si, S, and Ti) in the different parts of the plants (leaves and roots) exposed to the different CNTs (Figure 81). However, looking at both PCA (leaves and roots), plants grown in soil with DWCNTs f can be distinguished from the other groups. In details, they tended to have lower concentrations of calcium, magnesium, sodium and sulfur in leaves, and lower concentrations of magnesium and sulfur in roots.



Figure 81 PCA of the nutrient contents (B, Ca, Cu, K, Cd, Mn, Mg, Na, P, Si, S and Ti) in leaves and roots of the canola plants grown with or without the different CNTs (DWCNTs, DWCNTs f, MWCNTs, MWCNTs f, short MWCNTs) at 3 concentrations (0.1, 10 and 100 mg.kg⁻¹) in a fine loamy soil during 5 weeks.

4. DISCUSSION

DWCNTs reduced the germination rate at the highest concentration under heat stress. The other CNTs did not impact the germination. It has been reported several times that CNTs can affect plant germination (C. Liné, Larue and Flahaut, 2017). For instance, MWCNTs have been shown to enhance the germination of wheat (*Triticum aestivum*) (Joshi *et al.*, 2018b), tomato (*Solanum lycopersicum*) (Khodakovskaya *et al.*, 2009; Ratnikova *et al.*, 2015) or maize (*Zea mays*) (Tiwari *et al.*, 2014). MWCNTs used in these different studies were most of the time functionalized or stabilized in gallic acid and with an intermediate diameter (15 nm) between DWCNTs and MWCNTs used in this study. This confirms that smaller CNTs seem to impact more germination rate. No effect of any CNTs was found on the other morphological parameters under optimal growth conditions of canola.

Under abiotic stress, some CNTs such as DWCNTs f were able to mitigate drought stress and permitted to reach the same germination rate than in optimal conditions. Also DWCNTs f exposure lead to a higher number of leaves, and more developed plants in comparison to stressed control plants. In the literature, improved development after exposure has already been reported several times in tomato and rice for instance but rather under optimal growth conditions (Khodakovskaya *et al.*, 2009)(Khodakovskaya *et al.*, 2013b). (Hao Zhang *et al.*, 2017). Impacts of CNT under stressful conditions has never been investigated before.

Sometimes, boost effect on plant growth was associated to higher water uptake (Khodakovskaya *et al.*, 2009; Khodakovskaya *et al.*, 2012; Martínez-Ballesta *et al.*, 2016). Thanks to this higher water uptake, plants were able to better cope with abiotic stress such as high salinity. In our study, no higher water content was found with plants grown with DWCNTs. However, water regulation is a dynamic phenomenon, modification can appear only during stress peaks and come back to a basal level after

In our study, only the DWCNTs, with the smallest diameter, significantly impacted plant germination and growth. One of the hypotheses that can be made is that only the DWCNTs were able to penetrate the seeds thanks to their small diameter and affect plant development. Indeed, CNTs were found to penetrate plant seed coat in several studies (Liné *et al.*, 2017). Khodakovskaya *et al.* (2009) demonstrated using Raman spectroscopy and TEM that MWCNTs (5 nm) were able to penetrate the thick seed coat of tomato. Ratnikova *et al.* (2015) also demonstrated that MWCNTs were seen inside tomato seed embryos after exposure. Unfortunately, no information about CNT diameter was given. In both studies, this internalization was coupled to germination promotion.

By looking at the different TEM images where CNTs were reported inside plant cells, most of the time only short CNTs (less than 500 nm) were found (Liné *et al.*, 2017). Length of CNTs can thus play a crucial role on their entry and their toxicity on plants. However, no difference was found between the MWCNTs (1 to 20 μ m) and the short MWCNTs (less than 500 nm) in our study. It might be related to the large length distribution in the two samples. Lahiani *et al.* (2016) tested the impacts of four types of carbon based NMs: helical MWCNTs (outer diameter between 100 and 200 nm, length between 1 to 10 μ m), long

functionalized MWCNTs (outer diameter between 13 and 18 nm, length between 1 and 12 μ m), short functionalized MWCNTs (outer diameter between 20 and 30 nm, length between 0.5 and 2 μ m) and graphene sheets. They concluded that the 4 NMs were able to impact positively callus cells and tomato plant growth, without any effect of the morphology. Here raw and functionalized DWCNTs had opposite effects on plants. The functionalization consists to graft carboxylic groups (-COOH) on the outer surface of the nanotubes. One of the main consequences of the functionalization is to make CNTs more hydrophilic (Ernst et al., 2017). We can expect the functionalization to change the behavior of CNTs in soil. Indeed, Villagarcia et al. (2012) evidenced that the surface chemistry of CNTs plays an important role on biological effects on plants. They used MWCNTs (outer diameter between 8 and 35 nm, length of several microns) with different types of functionalization: carboxylated (more negatively charged) or PEG coated (less negatively charged). They concluded that the more negatively charged MWCNTs induced higher germination and a more significant increase in fresh biomass. However, the fact that one of type of functionalization was covalent while the other one was not was not discussed (possible detachment of adsorbed PEG coating). Likewise, Canas et al. (2008) reported that the non-functionalized SWCNTs (diameter not reported) affected more the root elongation, but the effect was species dependent (inhibition for tomato and enhancement for onion and cucumber). Another study showed that the functionalization of SWCNTs with quantum dots significantly inhibited root growth and exhibited severe symptoms of leaf senescence while the same non-functionalized SWCNTs did not impact tomato plants (Alimohammadi et al., 2011a).

Recently, it has been demonstrated that mass concentration is not the only dose-metric to consider in nanotechnology studies, and that SSA could be more informative (Hull *et al.*, 2012; Mottier *et al.*, 2016). Indeed, NMs are known for their high SSA which makes them more reactive and gives them new properties (Auffan *et al.*, 2009). Some studies performed on amphibians demonstrated that SSA is the most accurate metric in ecotoxicological assessment and it better explained the toxicity of CNTs and other carbon based NMs (Mottier *et al.*, 2016; Lagier *et al.*, 2017). In our study, the highest surface concentration was found for the DWCNTs at 100 mg/kg (98.5 m²/kg of soil) (Table 21 however no dose response was found on canola plants according to surface concentrations.

Finally, the amount of remaining metal impurities can also impact the toxicity of CNTs since they can be released into the media and directly impact organisms (Jakubek *et al.*, 2009; Petersen, 2014). Even if the amount of residual metals is usually rather low, since high concentration of CNTs may be tested in toxicological studies, this metallic contamination needs to be considered. Several metal catalysts were used in the different CNTs of this study with the highest concentration found for Co. The average natural concentration of Co in soils is around 8 mg/kg (Singh *et al.*, 2010). Toxic effects of Co on plants were reported on tomato, French bean or mung bean (*e.g.* chlorosis of leaves) at concentration above 40 mg/kg of soil (Liu *et al.*, 2000; Gopal *et al.*, 2003; Chatterjee *et al.*, 2006). However, EC₅₀ were found for canola plants between 7 and 966 mg/kg of soil of Co (Singh *et al.*, 2010). In this study, Co was present at 3.99 mg/kg DWCNTs at the highest concentration. Even if all the Co would have been released into the soil from

CNTs (ionic form), which is unlikely, this should not have caused any toxic effects on canola plants. To reach a concentration of 40 mg/kg of Co in the soil, DWCNT concentration in soil should be around 1000 mg/kg. Mo was also found to impact plants at concentration around 40 mg/kg of soil (*e.g.* chlorosis of the leaves) (McGrath *et al.*, 2010; Singh *et al.*, 2010), which is much higher that the highest concentration potentially available in our study (*i.e.* less than 1 mg/kg). The heavy metal Ni is another essential trace nutrient and concentration below 20 mg/kg of soil were demonstrated to be beneficial for plants (Singh *et al.* 2010; Shahzad *et al.* 2018). However, at high concentration, Ni can lead to chlorosis and necrosis of leaves (Rahman *et al.*, 2005; Gajewska and Skłodowska, 2007; Llamas *et al.*, 2008). The toxicity threshold for Ni was found in ryegrass around 30 mg/kg (Singh *et al.*, 2010). The highest Ni concentration in CNTs is 1.90 mg/kg of soil (with 100 mg/kg of MWCNTs f) which is again much lower than the reported toxicity value. Likewise Fe at high concentrations can reduce photosynthesis and increase oxidative stress, resulting in damages to membranes, DNA and proteins in plants (Nagajyoti *et al.*, 2010). The highest concentration of Fe in this study was less than 2 mg/kg of soil. which is negligible in comparison with natural soil Fe concentration ranging from 20 to 550 mg/kg (USEPA, 2003).

Table 21 Conditions expressed in other metrics than mass concentrations (mg/kg): surface concentration (m²/kg), elemental concentrations (mg/kg) for C, O, N, Co, Mo, Fe and Ni. NM = element not measured.

	Mass	Surface	Elemental concentrations (mg/kg soil)						
CNTs	concentrations (mg/kg CNT)	concentrations (m ² /kg) CNT	С	0	N	Со	Мо	Fe	Ni
DWCNTs	0.1	0.0985	0.0897	0.0021	NM	0.0040	0,0010	NM	NM
	10	9.8500	8.9750	0.2130	NM	0.3990	0,0960	NM	NM
	100	98.5000	89.7500	2.1300	NM	3.9900	0,9600	NM	NM
DWCNTs f	0.1	0.0240	0.0769	0.0172	0.0003	0.0010	0,0001	NM	NM
	10	2.4000	7.6930	1.7250	0.0300	0.0990	0,0120	NM	NM
	100	24.0000	76.9300	17.2500	0.3000	0.9900	0,1200	NM	NM
MWCNTs	0.1	0.0090	0.0949	0.0006	NM	NM	NM	0.0002	0.0019
	10	0.9000	9.4880	0.0560	NM	NM	NM	0.0210	0.1900
	100	9.0000	94.8800	0.5600	NM	NM	NM	0.2100	1.9000
MWCNTs f	0.1	0.0090	0.0393	0.0019	0.0001	NM	NM	0.0003	0.0013
	10	0.9000	9.3160	0.1920	0.0100	NM	NM	0.0320	0.1280
	100	9.0000	93.1600	1.9200	0.1000	NM	NM	0.3200	1.2800
Short MWCNTs	0.1	0.0060	0.0876	0.0022	0.0002	0.0007	NM	0.0002	NM
	10	0.6000	8.7650	0.2180	0.0220	0.0660	NM	0.0180	NM
	100	6.0000	87.6500	2.1800	0.2200	0.6600	NM	0.1800	NM

5. CONCLUSION

In this study, we assessed the influence of 5 different CNT (DWCNTs, functionalized DWCNTs, MWCNTs, functionalized MWCNTs and short MWCNTs) physicochemical characteristics on their impact on canola crop plant under both optimal and abiotic stress (*i.e.* heat) conditions. Under optimal conditions, canola did not show any response to the different CNT contaminations in terms of plant development. However, under heat stress, this response was different according to the type of CNTs used. Functionalized DWCNTs were able to alleviate the effects of the abiotic stress on the plants while the non-functionalized DWCNTs increased the inhibition of plant development. Plants were more sensitive to CNTs when they were submitted to concomitant heat stress. Canola was more sensitive to CNTs with the smallest diameter and the highest specific surface area, but it was also observed that the functionalization greatly modulated the plant response. Those results highlight the need of a full characterization of NMs before ecotoxicological studies. A lack of characterization leads to hinder comparison among studies and will limit the ability to understand mechanistic insights and slow down risk assessment related to NM dissemination in the environment.

CHAPTER 8: CONCLUSION & PERSPECTIVES

1. SCIENTIFIC CONCLUSION

The increase in the number of nanotechnology applications and therefore in the use of CNTs will inevitably lead to an increase of their release into the environment. To date, data is scarce and often contradictory regarding CNT impacts and behavior in plants (see Introduction, Liné *et al.*, 2017). Indeed, many parameters can influence plant response to CNT exposure. One of the purpose of my PhD was to evaluate some of these parameters: (i) type of NMs, (ii) plant species, (iii) CNT physicochemical properties and finally (iv) combined stress, in order to assess plant response to CNTs in a more accurate way. My second goal was to investigate different spectroscopic techniques to investigate CNT internalization and impact on plants. The summary of the experiments done during my PhD is presented at the end of the scientific conclusion (Figure 82).

1.1. Influence of different parameters on plant response to CNT exposure

1.1.1. Influence of the plant species

There is a wide variety of plant species used in the literature when assessing NM phytotoxicity. For example, on the 46 publications studied for the review (Liné *et al.*, 2017), 30 different plant species were used.

As first hypothesized, we observed different plant response to DWCNT contamination according to the species. Maize displayed a decreased development while for the other species (canola, cucumber and tomato), we identified mostly an enhanced development. Many parameters differ from maize to the other plants for instance it has (i) bigger seeds, increasing the surface contact with CNTs, (ii) a more developed root system also possibly leading to an enhanced surface exchange with soil elements and NMs, (iii) a higher leaf surface area which could lead to an increased transpiration rate and thus increased exchanges of water between the soil and the plant and possibly higher NM flux going through the plant system. However, a common response was also identified for all the plant species: alteration of plant biomacromolecules especially cell wall components (cellulose and hemicellulose). As for the previous experiment, we can hypothesize that it is related to an internalization of the CNTs in all plant species.

The results obtained here could be generalized in the future by repeating the same experiment using different plants. For example, other monocots can be used *i.e.* rice (*Oryza sativa*), wheat (*Triticum aestivum*) to verify if they have the same response as maize. Other plants with high leaf surface area as fava bean (*Vivia faba*) can be used in comparison to plants with small surface area like Thale cress (*Arabidopsis thaliana*).

1.1.2. Plant response to different types of NMs

The two NMs used in this study were chosen to be different: they are varying in shape (tubular for CNTs and spherical for TiO₂-NPs), in surface chemistry (carbon and metal oxide), in size (1-3 nm diameter for CNTs and 25 nm for TiO₂-NPs). Their similarity lies in the fact that they are both weakly soluble (if soluble at all) in our exposure conditions. Results showed that even after a 20-day exposure to each NM, very little effects were observed on the morphological and biochemical plant response. However, using FTIR spectroscopy, modification in biomacromolecules were identified in plants exposed to both NMs in particular in cell wall components. Mainly hemicellulose and cellulose were impacted in a similar way by both NMs. Contrary to our expectations, in this study, tomato plant tended to display a similar response to both TiO₂-NPs and CNTs despite their morphological differences. This common plant response may be explained by the internalization of both NMs inside the plant triggering the same cell wall damages. Oxidative stress is also known to be a common mechanism of actions for NMs and can also be responsible for plant cell wall modification (Cox *et al.*, 2016; Hatami *et al.*, 2016; Liné *et al.*, 2017).

As a perspective to better understand plant response to different NMs, it would be interesting to reproduce this experiment with a NM prone to dissolution (*e.g.* Ag NPs) and to evaluate if there is the same response in terms of impact on plant cell wall. If the plant response is different, it may suggest that Ag NPs, unlike CNT and TiO_2 NPs, are entering in plants in dissolved form. Another experiment that can be suggested in order to confirm that the plant cell wall modifications are related to the internalization of NMs, would be to dose them in the aerial parts of the plants. ICP-MS can be used to assess the Ti concentration while for CNTs, the best detection technique would the radiolabeling with ¹⁴C.

1.1.3. Influence of CNT physico-chemical properties

We observed that canola plants exposed in soils were more sensitive to DWCNTs than MWCNTs (mean outer diameter of 2.0 nm and 23.8 nm respectively). Plant response seemed to be more important when CNTs have a **small diameter**. However, in the experiment conducted with cucumber in hydroponic conditions, we observed limited effects of the $MW^{13}CNTs$ (leaf depigmentation, diameter 16.8 nm) while no effect was reported for the DWCNTs. This experiment was conducted in hydroponic conditions, thus the effects might be more ruled by the behavior of the CNTs in suspension in comparison to the dispersion and mobility of CNTs in a soil experiment. It has already been reported that CNTs with smaller diameter induced stronger plant response: Larue (2011) showed that DWCNTs (diameter of 2 nm) induced a root elongation in canola and wheat (*i.e.*) while MWCNTs (diameter of 54 nm) did not induce plant response. The author also conducted the same work with TiO₂-NPs and concluded that NMs with a diameter of less than 20 nm were more reactive towards plants.

In relation with CNT diameter, the **SSA** also differs greatly: 985 m²/g for the DWCNTs, 293 m²/g for the MW¹³CNTs and 90 m²/g for the MWCNTs and could also explain the previous results. Indeed, Mottier *et al.* (2016) demonstrated that the surface area of carbon based NMs was a more realistic dose-metric for ecotoxicological assessment. Regardless of the plant species and the exposure conditions, in our studies

we observed a more important plant response after exposure to DWCNT, followed by MW¹³CNTs and finally MWCNTs. CNTs with the highest SSA induced the most marked plant response.

We noticed that the **functionalization** also modulated plant response. Indeed, the functionalized DWCNTs triggered a higher plant growth compared to the non-functionalized DWCNTs, which even led to an inhibition of plant development. This is in agreement with the literature (Canas *et al.*, 2008; Alimohammadi *et al.*, 2011b; Villagarcia *et al.*, 2012). Functionalized CNTs appeared thus to be more reactive than their non-functionalized counterpart. Functionalization modifies greatly the CNT behavior since it makes them more hydrophilic (Ernst *et al.*, 2017) possibly leading to a different behavior of functionalized CNTs in soil and maybe more internalization in the plants or oxidative stress.

In this study, we did not report any influence of the **length** of the tubes (DWCNTs between 1 and 100 μ m, MWCNTs between 1 and 20 μ m, and the short MWCNTs about 2 μ m). Results were thus in contradiction with our hypothesis that smaller CNTs may induced a greater response of the plants. However, we used only one type of short CNTs (MWCNTs, diameter 12.3 nm). Since we observed more impacts after exposure to DWCNTs, we can hypothesize that short DWCNTs would have had even higher impacts on the plants. The synthesis of such a sample is however a long-term challenge.

CNTs contain remaining **metal impurities** coming from catalysts with different elements and different concentrations. Even if Hull *et al.* (2009) demonstrated that the release of metal impurities from carbon based NMs influenced greatly the aquatic toxicity, in our studies conducted in soil, the metal impurities did not seem to influence plant response. Nevertheless, they were present in low quantity in CNT (the highest concentration was for DWCNTs at 100 mg/kg containing Co: 3.99 mg/kg of soil) and we suspect that they do not dissolve since they are entrapped in a carbon solid shell. In DWCNTs in particular, residual catalytic nanoparticles are tightly encapsulated within concentric graphene shells and no dissolution may occur in the exposure conditions.

According to the results of our experiments, we can conclude that CNT diameter and functionalization are the two main parameters governing plant response to CNT contamination. Actually some authors reported that it may be inappropriate to classify all the CNTs into one single category in terms of their environmental regulation since their effects vary a lot according to their physicochemical characteristics (Kennedy *et al.*, 2008). Salieri *et al.* (2017), based on a statistical model, also concluded that the physicochemical properties of carbon based NMs (*i.e.* length, dispersion and diameter) play an important role to define the toxicity in algae, crustaceans and fish. However, due to the scarcity of information reported in the literature, they were not able to build a significant correlation between one specific CNT physicochemical characteristic and their toxicity. Likewise, in our study, several dose-metric expressions were tested (*i.e.* surface area, metal impurities concentrations (Co, Mo, Fe and Ni)); however, we did not have enough data to run an accurate model and failed to identify a formal correlation between these characteristics and plant response. Until now, it has not been possible to obtain a wide range of CNTs varying only in one parameter (for instance same diameter but different length) so we always assess the effects of a set of physico-chemical variations, thus reaching a conclusion is still challenging. A meta-analysis including many different types of well-characterized CNTs could be a key to fill this knowledge gap.

It can be interesting to compare plant response to CNTs and to other carbon based NMs such as graphene and graphene oxide. Graphene oxide is the functionalized version of graphene. It will allow knowing the influence of the functionalization on a 2D material. Another NM that can be used for comparison is boron nitride nanotube. It has exactly the same morphology as CNT but the surface chemistry is different.

1.1.4. Influence of CNT dispersion in the exposure medium

There is currently a lack of internationally-recognized standard dispersion protocols for CNTs. The use of these treatments affects the properties of the CNTs and therefore their interactions with living organisms (Petersen and Henry, 2012; Cerrillo *et al.*, 2015). When experiments are realized in soil, another question arises: how should the soil be contaminated? Most of the time in the literature, the protocol used to contaminate the soil is not well documented. In our studies, we first used a method that has been applied previously in the laboratory to contaminate soil with metals but also NMs such as TiO₂ NPs. This technique consisted in the preparation of a suspension of the contaminant and to mix this suspension with the soil with a ratio solid:liquid 1:10 during a few hours before removing the excess of water by filtration. However, this technique seems to be more appropriate for contaminants that are able to dissolve in water than for insoluble CNTs. For the other experiments, CNT suspension was deposited on a soil layer of a few cm (increased solid:liquid ratio) which we believe leads to a more accurate contamination (no potential loss of CNTs in the leachate and better soil structure preservation). The contamination method used has thus to be clearly defined in the publication.

It would be interesting to look at CNT aspect at the end of a long-term experiment conducted in soil. This would allow to inspect CNT after weathering in soil. However, the observation of CNTs in complex matrices such as soil is not easy. Several separation procedures of CNTs from complex media can be used (*e.g.* asymmetric flow field flow fractionation (AF4), matrix digestion, and sonication with surfactants). However, there is still a lot of work to do in order to obtain efficient yield. Another experiment that could have been done would have been to use ¹³CNTs in a soil column study with different types of soil (sandy, loamy silty and clayey soils). The soil column could then be cut in small fractions and the ¹³C concentration in each of them assessed using IRMS. It would give information about CNT mobility in soil and their risk of leaching towards aquifers. Furthermore, to investigate if CNT metal impurities are dissolving in the soil and possibly bioavailable and toxic to organisms, we could incubate CNTs in different types of soil and use sequential extractions to dose metal concentrations in soil solution by ICP-MS

1.1.5. Influence of an environmental stress

In our study, we observed that under optimal growth conditions, canola did not exhibit any response in terms of plant development and physiology to the different CNT contamination (DWCNTs, functionalized

DWCNTs, MWCNTs, functionalized MWCNTs and short MWCNTs). However, under heat stress, this response was different according to the type of CNTs: functionalized DWCNTs were able to alleviate the effects of the abiotic stress on plants while the non-functionalized DWCNTs increased the inhibition of plant development. As stated in the research question n°4, plants were thus more sensitive to CNTs when they were submitted to a concomitant heat stress. In the research for agricultural applications, NMs are being studied in order to assess their potential in protecting plants from abiotic stresses (*e.g.* drought, flood, nutrient deficiency, heat, light, metal stress, etc.) (Khan and Upadhyaya, 2019). For example, TiO₂ NPs have been shown to reduce the impacts of drought on dragonhead plants (Mohammadi *et al.*, 2016). Cerium oxide NPs have been identified to decrease the oxidative stress in sorghum under drought conditions (Djanaguiraman *et al.*, 2018). However, in some cases, multiple stresses can increase negative effects. For example, drought and heat stresses have synergistic effects on plant physiology, resulting in increasing negative impacts such as drastic yield reductions (Mittler, 2006).

However, stressful growth conditions certainly better reflect the reality that can be faced in the environment especially in the context of climate change (*i.e.* heat stress).

1.2. Techniques used for CNT detection, characterization and plant response evaluation

The detection of CNTs in plants is a challenge not yet faced. As we exposed in chapter 4, there are many methods which can be used, however, the technique giving the best results so far is autoradiography using ¹⁴CNTs. It is however expensive and impossible to set up in a "conventional" laboratory. Most of the other techniques presented a limit of detection higher than the actual CNT concentration in the studied organisms. Moreover, most of the times several techniques have to be combined together to prove the presence of CNTs. Sample preparation is also a huge challenge. For each detection technique, plant sample preparation should be defined and standardized as much as possible in order to be able to compare results among studies. However, standardized sample preparation may be tricky since there are many different plant species with different morphologies which can lead to different sample preparation requirements

Another option to test will be to fill the tube with an element that are not naturally occurring (or at very low concentrations such as Pb or Cd) in plants and that it is detectable with simple techniques such as ICP-MS.

As we demonstrated, the physicochemical characteristics of CNTs influence the plant response. This is why CNT characterization has to be done carefully in ecotoxicological studies. Many analytical methods can be used for their characterization (Herrero-Latorre *et al.*, 2015). Here we recommend a set of analytical techniques to be used for ecotoxicological studies. Some parameters (presence of amorphous carbon, diameter/number of walls, length) can be easily addressed using TEM. Nowadays, access to this technology in research laboratories is becoming more and more common. Furthermore, it does not require a complex sample preparation. Another important parameter to assess is the specific surface area (SSA) using BET method since it has been proven several times that it can condition CNT ecotoxicological effects.

This method is fast, easy and not expensive. However, the machine is not widespread in research laboratories which can make this analysis difficult. The metal impurities have also been reported as an important parameters influencing ecotoxicological responses. Metal concentrations can be assessed using techniques such as ICP-MS or ICP-AES which are becoming common in most research laboratories. It is also important to determine the functionalization degree and nature. If all ecotoxicological studies showed these basic parameters (*i.e.* diameters/number of walls, length, SSA, metal impurity concentration), the comparison among studies would be easier.

Another reason why CNT impacts on plants are so controversial is the lack of standardized methods to evaluate their effects (Ray *et al.*, 2009). In our study, we used morphological parameters (*i.e.* germination rate, plant height, number of leaves, surface area, root length, fresh and dry biomasses), some biochemical markers (chlorophyll pigment, flavonoid and phenolic compound concentrations, lipid peroxidation evaluation), and we developed the analysis of biomacromolecules using FTIR spectroscopy. In the different experiments, FTIR appeared to be the most sensitive in comparison with the other methods, evidencing some effects only after 4 hours of CNT exposure (data not shown in the manuscript) when no effect was detected using morphological and biochemical biomarkers even after 20 days of exposure. We determined using a chemometric analysis that the regions corresponding to plant cell walls were impacted. FTIR spectroscopy appeared as a powerful technique to evaluate CNT toxicity in plant leaves since it allows assessing the impacts on several biomacromolecules on samples easy to prepare (drying and grinding) with an analysis taking less than 2 min. The chemometric analysis developed in this work improved greatly the data processing usually performed by visual comparison of two spectra, and permitted to highlight differences between the spectra of the control and the exposed plants.

1.3. General perspectives

Almost all the experiments in this study were realized in soil. One important parameter that was not studied during this work is the influence of the bacteria that are present in the soil on the plant response to CNT contamination. Very few studies looked at CNT impacts on bacteria and effects found are controversial but mainly decreased soil microbial activity was reported for both MWCNTs and SWCNTs (Chung *et al.*, 2011b; Jin *et al.*, 2013, Liné *et al.*, 2017). In contrast, few other studies showed that MWCNTs stimulated mineralization of an agricultural soil by bacteria (Shan *et al.*, 2015a). There is no enough studies to conclude about the impacts of CNTs on bacteria. It is nevertheless important to study the bacteria response especially since CNT are introduced into the subsurface of the soil (as growth fertilizers).

The rhizosphere is defined as the thin layer of attached soil surrounding plant root surfaces. This region is highly biologically-active. Interactions between bacteria and root surface occurring in the rhizosphere are essential for plant growth (Huang, 2008). NM effects on plant growth may be linked to the impairment of the rhizosphere area. There is a lack of studies dedicating to this complex interface facing NMs. Some studies reported that TiO₂ and polystyrene NPs reduced rhizosphere bacteria and this was correlated with inhibited root and stem length of lettuce. We can suspected that our results in soil experiment may be

linked to soil bacteria and rhizosphere functioning. Further experiments are thus needed to identify interactions between CNTs and bacteria in the rhizosphere and more generally in soil.



Figure 82 Summary of the different experiments realized during this PhD word with the CNT characterization, the CNT detection and a summary of the different tested parameters than can influence the plant response to CNT contamination.

2. SIDE ACTIVITIES DURING THE PHD THESIS

During this PhD work, I kept developing a high throughput biomarker set that has been initiated during my M2 internship at the LGCgE laboratory. Thanks to that, I am co-author of a publication realized in my former laboratory:

Can Zn pollution of soil promote adaptive evolution in plants? Insights from a one-generation selection experiment. J. Nowack, H. Frérot, N. Faure, C. Glorieux, C. Liné, B. Pourrut & M. Pauwels. Journal of Experimental Botany, September 2018.

I used these different biomarkers to evaluate the plant response to CNT contamination but I also helped in different other projects conducted at EcoLab which gave me the opportunity to be also co-author of publications:

- Transfer and Ecotoxicity of Titanium Dioxide Nanoparticles in the Terrestrial and Aquatic Ecosystems: A Microcosm Study. V. Vijayaraj, C. Liné, S. Cadarsi, C. Salvagnac, D. Baqué, A. Elger, M. Barret, F. Mouchet & C. Larue. Experimental Science and Technology, October 2018.
- Assessing the impacts of sewage sludge amendment containing nano-TiO 2 on tomato plants: A life cycle study. M. Bakshi, C. Liné, D. E. Bedolla, R. J. Stein, R. Kaegi, G. Sarret, A. Pradas Del Real, H. Castillo-Michel, P.C. Abhilash & C. Larue. Journal of Hazardous Materials, February 2019.

The development of FTIR spectroscopy and associated chemometric analysis that I carried out was also shared in the laboratory and has led to the redaction of one paper currently submitted in Chemosphere:

Genotypes of the aquatic plant *Myriophyllum spicatum* with different growth strategies show contrasting sensitivities to copper contamination. E. Roubeau Dumont, C. Larue, H. Castillo Michel, H. Gryta, E. Maria Gross, C. Liné, D. Baqué & A. Elger.

During my PhD, I also had the opportunity to write for scientific popularization two publications with ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail) aimed at different governmental stakeholders:

- Utilisation des nanoparticules en remédiation et implications écotoxicologiques. C.Liné & C.Larue
- La problématique émergente des nanoplastiques. C.Liné & C.Larue

Also for scientific popularization, I participated in the writing of a paper for Techniques de l'ingénieur:

Toxicité des nanotubes de carbone envers l'homme et l'environnement. E. Flahaut, L. Evariste, L.
Gauthier, C. Larue, C. Liné, E. Meunier & F. Mouchet. Techniques de l'ingénieur, Octobre 2018.

During my 2nd and 3rd years of PhD, I choose to give 64 hours per year of teaching. My teaching hours were divided in two. 32 hours were given to the 1st year of the preparatory class of Institut National Polytechnique de Toulouse with tutorial classes and practical works in biochemistry. The second part of my teaching hours were done with pupils (élèves de 4^{ème} et 3^{ème}) of two schools of Toulouse (Collège Raymond Badiou and Collège Stendhal). This teaching load consists of the development and implementation of scientific workshops on environment and sustainable development in collaboration with teachers and engineer students. We organized different field trips (visit of a waste sorting center, scientific visit of Pic du Midi).

The scientific popularization is a subject that I care a lot about. I participated to different scientific popularization workshops: "Ma these en 180 secondes" and "La nuit européenne des chercheurs". I also organized visit of the EcoLab laboratory for different school classes.

CONCLUSION & PERSPECTIVES (FRANÇAIS)

L'augmentation du nombre d'applications des nanotechnologies et par conséquent de l'utilisation des NTC va mener inévitablement à une augmentation de leur rejet dans l'environnement. A ce jour, il y a peu de données sur l'impact et le comportement des NTC dans les plantes. Ces données sont en plus contradictoires. En effet, de nombreux paramètres peuvent influencer la réponse des plantes suite à une exposition aux NTC. Le but de ce travail de thèse était d'évaluer l'importance de certains de ces paramètres sur la réponse des plantes aux NTC : (i) le type de NM, (ii) les espèces de plantes, (iii) les paramètres physicochimiques des NTC et (iv) le stress combiné.

1. L'INFLUENCE DE LA REPONSE DES PLANTES A L'EXPOSITION AUX NTC

1.1. La réponse des plantes à différents types de NM

Les deux types de NM utilisés dans cette étude présentent des caractéristiques bien différentes. Ils diffèrent dans leur forme (tubulaire pour les NTC et sphériques pour les NPs de TiO₂), dans leur chimie de surface (carbone et oxyde de métal), dans leur taille (1 à 3 nm pour les NTC et 25 nm pour les NPs de TiO₂). Leur seule similarité réside dans le fait que ce sont tous les deux des NM difficile à dissoudre dans l'eau. Même après 20 jours d'exposition, très peu d'effets ont été observées au niveau de la réponse morphologique et biochimique des plants de tomate. Cependant, avec la spectroscopie FTIR, des modifications au niveau des biomacromolecules ont été identifiés pour les deux NM, en particulier dans la région des composés des parois cellulaires. La cellulose et l'hémicellulose ont été les composés les plus impactés. Dans cette étude, la tomate semble avoir une réponse plutôt commune aux 2 NM malgré leurs différences morphologiques. Les parois cellulaires sont les premières barrières contre les stress biotiques ou abiotiques (par exemple: une contamination aux métaux lourds contamination (Sattelmacher and Horst, 2007), une intrusion de pathogènes (Schwab *et al.*, 2015), *etc.*). Cette réponse commune peut être expliquée par l'internalisation des deux NM dans les plantes déclenchant les mêmes dommages aux parois cellulaires. Le stress oxydatif est aussi un mécanisme connu pour être déclenché par les NM. Il peut être à l'origine de modification des parois cellulaires des plantes.

Afin de mieux comprendre la réponse des plantes aux différents NM, il pourrait être intéressant de reproduire cette même expérience mais en utilisant cette fois des NM qui se dissolvent facilement (comme les NPs d'Ag). Si la réponse des plantes est différente, cela peut suggérer que ces NPs entrent dans la plante sous forme dissoute. Une autre expérience qui peut être effectué et de doser les parties aériennes des plantes afin d'évaluer si il y a eu une réelle internalisation des NM.

1.2. Influence des espèces de plantes

De nombreuses variétés de plantes différentes ont été utilisées dans la littérature pour évaluer la toxicité des NM. Par exemple, dans les 46 articles étudiés pour la revue scientifique (Liné *et al.*, 2017), 30 espèces de plantes ont été utilisés.

Comme nous l'avions d'abord supposé, la réponse des plantes à une exposition aux CNT double parois était différente selon l'espèce. Le maïs a montré une diminution de son développement alors que les

autres plantes (colza, concombre et tomate) ont plutôt montré des effets bénéfiques. Le maïs est très différent des autres plantes étudiées. Il possède (i) des graines plus grandes (ce qui peut augmenter la surface de contact avec les NTC), (ii) un système racinaire plus développer (augmentant la surface d'échange avec les éléments du sol et donc les NTC), (iii) une plus grande surface foliaire (augmentant le taux de transpiration et par conséquent l'augmentation des échanges d'eau entre le sol et la plante ce qui pourrait engendrer un flux de NTC plus important). Cependant, une réponse commune a également été identifiée. Les composés des parois cellulaires ont été impactés ce qui pourrait laisser à une internalisation des NTC dans toutes les plantes étudiées.

Afin de comprendre l'influence de l'espèce sur la réponse des plantes à la contamination aux NTC, la même expérience pourrait être effectué mais en utilisant d'autres plantes par exemple d'autres monocotylédones (le riz ou encore le blé ou d'autres plantes présentant une grande surface foliaire comme la fève ou encore au contraire des plantes présentant de faibles surface foliaire comme l'arabette.

1.3. Influence des propriétés physicochimiques

Les plants de colza exposés en sol ont été plus sensibles aux NTC double parois qu'aux NTC multi parois (diamètre des doubles parois 2.0 nm et des multi parois 23.8 nm). La réponse des plantes semblent donc être plus important lorsque le diamètre des NTC est faible. En relation avec le diamètre, la surface spécifique des NTC diffèrent grandement (985 m²/g pour les doubles parois et 293 m²/g pour les multi parois) et cela pourrait également expliquer la différence de réponses des plantes. Nous avons également observé que la fonctionnalisation des NTC modulait la réponse des plantes. En effet, les NTC double parois fonctionnalisés ont induit augmenter le développement des plantes tandis que les non fonctionnalisés ont plutôt inhibé les plantes. Dans cette étude, nous n'avons pas remarqué d'influence de la longueur des tubes (les doubles parois ont une longueur entre 1 et 100 µm, les multi parois entre 1 et 20 µm et enfin les multi parois courts ont une longueur d'environ 2 µm). Ces résultats sont en contradiction avec l'hypothèse que les NTC courts induisent une plus grande réponse des plantes. Cependant, nous avons regardé l'influence de la longueur sur un seul type de NTC (les multi parois). Il serait intéressant de comparer la réponse des plantes à des NTC double parois de longueurs différentes. Les NTC présentent des impuretés métalliques liées aux restes de catalyseurs. Ces impuretés métalliques ne semblent pas avoir modifié la réponse des plantes dans notre étude. Cependant, les concentrations de ces métaux dans notre étude étaient très faibles et nous n'avons pas d'indice en ce qui concerne leur dissolution (et donc leur biodisponibilité).

En se basant sur nos résultats, nous pouvons conclure que le diamètre et la fonctionnalisation des tubes sont les deux paramètres influant le plus la réponse des plantes suite à une exposition aux NTC. Jusqu'à présent, il n'a pas été possible d'obtenir un large éventail de NTC ne variant que d'un seul paramètre (par exemple, même diamètre mais longueur différente), de sorte que nous évaluons toujours les effets d'un ensemble de variations, ce qui rend la conclusion encore difficile ; une méta-analyse incluant différents types de NTC bien caractérisés pourrait être une clé pour combler cet écart dans nos connaissances.

1.4. Influence d'un stress environnemental

Dans notre étude, nous avons observé que dans des conditions de croissance optimales, le colza ne montrait aucune réaction en termes de développement aux différentes contaminations de NTC (NTC double parois, NTC double parois fonctionnalisés, NTC multi parois, NTC multi parois fonctionnalisés et NTC multi parois courts). Cependant, sous l'effet du stress thermique, cette réponse était différente selon le type de NTC : les NTCN fonctionnalisés ont pu atténuer les effets du stress abiotique sur les plantes tandis que les NTC non fonctionnalisés ont augmenté l'inhibition du développement des plantes. Les plantes étaient plus sensibles aux NTC lorsqu'elles étaient soumises à un stress thermique concomitant. Des conditions de croissance stressantes pourraient mieux refléter la réalité à laquelle la plante peut être confrontée dans l'environnement dans le contexte du changement climatique (comme le stress thermique).

1.5. Influence de la suspension de NTC

Il y a actuellement un manque de protocoles de dispersion normalisés reconnus à l'échelle internationale pour les NTC. L'utilisation de ces traitement peut impacter les propriétés des NTC et par conséquent leur interactions avec les organismes vivants (Petersen and Henry, 2012; Cerrillo *et al.*, 2015). Lorsque les expériences sont réalisées en sol, une autre question se pose : comment contaminer le sol ? La plupart du temps, dans la littérature, le protocole utilisé pour contaminer le sol n'est pas bien détaillé. Dans notre étude, nous avons d'abord utilisé une méthode utilisé en laboratoire pour contaminer le sol avec des métaux mais aussi des nanoparticules comme les NPs de TiO₂. Cette technique consistait à préparer une suspension et à mélanger cette suspension avec le sol pendant quelques heures, puis à éliminer l'excès d'eau par filtration. Toutefois, cette technique semble plus appropriée pour les contaminants qui sont capables de se dissoudre dans l'eau que pour les NTC qui sont eux insolubles. Pour les autres expériences, la suspension de NTC a été déposée sur une couche de sol de quelques centimètres (augmentation du rapport sol/eau) qui, selon nous, conduit à une contamination plus précise (aucune perte potentielle de NTC dans le lixiviat et une structure du sol mieux préservée). La méthode de contamination utilisée doit donc être clairement définie dans la publication.

Il pourrait être intéressant d'observer l'aspect des NTC à la fin d'une expérience menée dans le sol. Cela permettra de voir si l'aspect des NTC est altéré dans le sol. Cependant, l'observation des NTC dans des matrices complexes comme le sol n'est pas facile. De nombreuses procédures de séparation des NTC dans les milieux complexes ont été utilisées. Cependant, il reste encore beaucoup de travail à faire pour obtenir un rendement efficace. Une autre expérience qui aurait pu être faite est d'utiliser les MW¹³CNT dans une étude de colonne de sol. Différents types de sols peuvent être utilisés (sols sablonneux, limoneux ou argileux). La colonne de sol pourrait alors être coupée en petite fraction et la concentration de ¹³C dans chaque colonne dosée à l'aide de l'IRMS. Cela permettrait d'avoir des informations sur la mobilité des NTC dans le sol et leur risque de lessivage vers les aquifères. Les NTC peuvent aussi avoir été incubés avec différents types de sol peut être dosée à l'aide d'ICP-MS afin d'évaluer les concentrations

de métaux. Cela aidera à savoir si les impuretés métalliques présentes à l'intérieur des NTC se dissolvent dans le sol et sont donc plus disponibles pour les plantes et les autres organismes.

2. TECHNIQUES UTILISEES POUR LA DETECTION, LA CARACTERISATION DES NTC ET L'EVALUATION DE LA REPONSE DES PLANTES

La détection des NTC dans les plantes est un défi qui n'a pas encore été relevé. Comme nous l'avons exposé dans le chapitre 4, il existe de nombreuses méthodes utilisées pour détecter les NTC dans les plantes. Cependant, la technique qui donne les meilleurs résultats est l'autoradiographie avec des ¹⁴CNT mais elle est coûteuse et impossible à mettre en place dans un laboratoire "conventionnel". La plupart des autres techniques présentent une limite de détection supérieure à la concentration de NTC dans les organismes étudiés. Il y a un manque de robustesse et de précision des techniques actuelles utilisées puisque la plupart du temps, plusieurs techniques doivent être combinées pour prouver la présence de NTC. La préparation des échantillons est également un défi de taille. Pour chaque technique de détection, la préparation des échantillons de plantes doit être définie et normalisée afin de pouvoir comparer les résultats des différentes études. Cependant, la préparation d'échantillons standardisés peut s'avérer délicate car il existe de nombreuses espèces de plantes différentes avec une morphologie différente, ce qui peut conduire à une préparation différente de l'échantillon. La préparation de l'échantillon doit être définie en fonction de l'espèce végétale.

Comme nous l'avons démontré, les caractéristiques physicochimiques des NTC influencent la réponse des plantes. C'est pourquoi la caractérisation des NTC doit être faite avec soin dans les études écotoxicologiques. De nombreuses méthodes analytiques peuvent être utilisées pour la caractérisation des NTC (Herrero-Latorre et al., 2015). Nous recommandons ici un ensemble de techniques analytiques qui peuvent être utilisées pour les études écotoxicologiques. Certains paramètres peuvent être facilement évalués à l'aide du MET. De nos jours, l'accès à cette technologie dans les laboratoires de recherche est de plus en plus courant. De plus, il ne nécessite pas une préparation complexe de l'échantillon. L'analyse des NTC avec le MET permet d'avoir une idée de l'aspect des NTC (présence de carbone amorphe, diamètre/nombre de parois, longueur et état d'agglomération). Un autre élément très important qui doit être évalué puisqu'il a été prouvé à plusieurs reprises qu'il peut conditionner les effets écotoxicologiques des NTC est la surface spécifique. Cette surface est évaluée selon la méthode BET. Cette méthode est rapide, facile et peu coûteuse. Cependant, cette technique n'est pas très disponible dans les laboratoires de recherche, ce qui peut rendre cette analyse difficile. Les impuretés métalliques ont également été signalées comme des paramètres importants influençant les réponses écotoxicologiques. Les concentrations de métaux peuvent être évaluées à l'aide de techniques telles que l'ICP-MS ou l'ICP-AES. Ces techniques deviennent de plus en plus courantes dans les laboratoires de recherche. Il est également important de préciser si les NTC utilisés sont fonctionnalisés ou non, et si oui avec quel type de fonctionnalisation). Si toutes les études écotoxicologiques montrent ces paramètres (diamètres/nombre

de parois, longueur, état d'agglomération, SSA, concentration en impuretés métalliques), la comparaison entre les études sera plus facile.

L'une des raisons pour laquelle les impacts des NTC sont si controversés est le manque de méthodes normalisées pour évaluer leurs effets sur les plantes (Ray, Yu et Fu, 2009). Dans notre étude, nous avons utilisé des paramètres morphologiques (taux de germination, hauteur de la plante, nombre de feuilles, superficie, longueur des racines, biomasse fraîche et sèche), certains marqueurs biochimiques (concentration en pigments chlorophylliens, concentrations en flavonoïdes et en composés phénoliques, évaluation de la peroxydation lipidique) et nous avons développé l'analyse des biomacromolecules avec la spectroscopie FTIR. Dans les différentes expériences, la spectroscopie FTIR semble être la plus sensible par rapport aux autres techniques. Nous avons observé des effets visibles sur les spectres FTIR seulement après 4 heures d'exposition aux NTC (données non montrées dans le manuscrit) lorsqu'aucun effet n'a été détecté en utilisant des biomarqueurs morphologiques et biochimiques après 20 jours d'exposition. Nous avons observé que les régions correspondant aux parois cellulaires végétales des spectres FTIR étaient touchées. La spectroscopie FTIR est apparue comme une technique puissante pour évaluer la toxicité des NTC dans les feuilles des plantes puisqu'elle permettait d'évaluer les impacts sur plusieurs biomacromolécules en même temps sur des échantillons faciles à préparer (séchage et broyage) avec une analyse prenant moins de 2 minutes. L'analyse chémométrique développée dans ce travail a grandement amélioré le traitement habituel des données par comparaison visuelle de deux spectres et a permis de mettre en évidence les différences entre les spectres du contrôle et des plantes exposées.

3. PERSPECTIVES GENERALES

Presque toutes les expériences de cette étude ont été réalisées en sol. Un des paramètres importants qui n'a pas été étudié au cours de ces travaux est l'influence des bactéries et donc l'influence de la rhizosphère sur la réponse des plantes à la contamination par les NTC. En effet, il a été montré que les bactéries du sol peuvent être impactées par les NTC. Très peu d'études ont porté sur les impacts des NTC sur les bactéries et le peu d'effets observés sont controversés (Liné, Larue et Flahaut, 2017). Cependant, de nombreuses études ont rapporté que les NTC diminuaient l'activité microbienne du sol. Certains auteurs ont observé que les MWCNT et les SWCNT diminuaient l'activité enzymatique des bactéries du sol (Chung *et al.*, 2011b; Jin *et al.*, 2013). En revanche, d'autres études ont montré que les MWCNT stimulaient la minéralisation d'un sol agricole par des bactéries (Shan *et al.*, 2015a). Il n'y a pas assez d'études pour conclure sur les effets des NTC sur les bactéries. Il est néanmoins important d'étudier la réponse bactérienne d'autant plus que les NTC pourraient être introduits directement à la surface du sol (comme engrais de croissance).

La rhizosphère est définie comme la mince couche de sol qui entoure la surface des racines des plantes. Cette région est très active sur le plan biologique. Les interactions entre les bactéries et les surfaces racinaires présentes dans la rhizosphère sont essentielles à la croissance des plantes (Huang, 2008). Les effets des NM sur la croissance des plantes peuvent être liés à la rhizosphère. Cependant, la faible teneur en eau interstitielle et la présence de multiples interfaces rendent les propriétés physico-chimiques de la rhizosphère plus complexes que dans un sol (Kibbey et Strevett, 2019). Par conséquent, il y a un manque d'études sur les interactions nanomatériaux-bactéries dans la rhizosphère. Certaines études ont rapporté que les NPs de TiO₂ et de polystyrène réduisaient les bactéries de la rhizosphère, ce qui était corrélé avec la longueur inhibée des racines et des tiges de la laitue. Nous pouvons soupçonner que les résultats de nos expériences sur le sol peuvent être liés aux bactéries du sol et à la rhizosphère. D'autres expériences sont donc nécessaires pour identifier les interactions entre les NTC et les bactéries dans la rhizosphère et plus généralement dans le sol.
References

A

Adams, F. (2003) 'Synchrotron radiation micro-X-ray fluorescence analysis: A tool to increase accuracy in microscopic analysis', *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*. North-Holland, 199, pp. 375–381. doi: 10.1016/S0168-583X(02)01563-X.

Adams, F. (2010) 'Synchrotron X-ray fluorescence analysis in environmental and earth sciences', *EPJ Web of Conferences*. EDP Sciences, 9, pp. 165–180. doi: 10.1051/epjconf/201009013.

Aghdam, M. T. B., Mohammadi, H. and Ghorbanpour, M. (2016) 'Effects of nanoparticulate anatase titanium dioxide on physiological and biochemical performance of Linum usitatissimum (Linaceae) under well-watered and drought stress conditions', *Brazilian Journal of Botany*. Springer International Publishing, 39(1), pp. 139–146. doi: 10.1007/s40415-015-0227-x.

Agreste (2019) *Ministère de l'agriculture et de l'alimentation - agreste - La statistique, l'évaluation et la prospective agricole - Chiffres et données.* Available at: http://agreste.agriculture.gouv.fr/publications/chiffres-et-donnees/article/statistique-agricole-annuelle-2016 (Accessed: 20 May 2019).

Ainsworth, E. A. and Gillespie, K. M. (2007) 'Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent', *Nature Protocols*, 2(4), pp. 875–877. doi: 10.1038/nprot.2007.102.

Ajayan, P. M. and Tour, J. M. (2007) 'Materials Science: Nanotube composites', *Nature*, 447(7148), pp. 1066–1068. doi: 10.1038/4471066a.

Alimohammadi, M. *et al.* (2011a) 'Physiological responses induced in tomato plants by a two-component nanostructural system composed of carbon nanotubes conjugated with quantum dots and its in vivo multimodal detection', *Nanotechnology*, 22(29), p. 295101. doi: 10.1088/0957-4484/22/29/295101.

Alimohammadi, M. *et al.* (2011b) 'Physiological responses induced in tomato plants by a two-component nanostructural system composed of carbon nanotubes conjugated with quantum dots and its invivo multimodal detection', *Nanotechnology*, 22(29). doi: 10.1088/0957-4484/22/29/295101.

Álvarez-Torrellas, S. *et al.* (2017) 'Effective adsorption of non-biodegradable pharmaceuticals from hospital wastewater with different carbon materials', *Chemical Engineering Journal*. Elsevier, 320, pp. 319–329. doi: 10.1016/J.CEJ.2017.03.077.

Auffan, M. *et al.* (2009) 'Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective', *Nature Nanotechnology*, 4(10), pp. 634–641. doi: 10.1038/nnano.2009.242.

Ayouni-Derouiche, L. *et al.* (2014) 'Development of efficient digestion procedures for quantitative determination of cobalt and molybdenum catalyst residues in carbon nanotubes', *Carbon*. Pergamon, 80, pp. 59–67. doi: 10.1016/J.CARBON.2014.08.025.

Β

Baker, M. J. *et al.* (2014) 'Using Fourier transform IR spectroscopy to analyze biological materials', *Nature Protocols*, 9(8), pp. 1771–1791. doi: 10.1038/nprot.2014.110.

Bakshi, M. *et al.* (2019) 'Assessing the impacts of sewage sludge amendment containing nano-TiO2 on tomato plants: A life cycle study', *Journal of Hazardous Materials*. Elsevier, 369, pp. 191–198. doi: 10.1016/J.JHAZMAT.2019.02.036.

Barbillat, J. et al. (1999) 'Spectrométrie Raman', Techniques de l'ingénieur, 2, p. 2865.

Barth, A. (2007) 'Infrared spectroscopy of proteins', *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. Elsevier, 1767(9), pp. 1073–1101. doi: 10.1016/J.BBABIO.2007.06.004.

Basiuk, V. A. *et al.* (2019) 'Phytotoxicity of carbon nanotubes and nanodiamond in long-term assays with Cactaceae plant seedlings', *Fullerenes, Nanotubes and Carbon Nanostructures*. Taylor & Francis, 27(2), pp. 141–149. doi: 10.1080/1536383X.2018.1531393.

Begum, P. *et al.* (2012a) 'Phytotoxicity of multi-walled carbon nanotubes assessed by selected plant species in the seedling stage', *Applied Surface Science*. (Surface and Interface of Biomaterials - Structure and effect from macro to nano), 262, pp. 120–124. doi: 10.1016/j.apsusc.2012.03.028.

Begum, P., Ikhtiari, R. and Fugetsu, B. (2014) 'Potential Impact of Multi-Walled Carbon Nanotubes Exposure to the Seedling Stage of Selected Plant Species.', *Nanomaterials (Basel, Switzerland)*. Multidisciplinary Digital Publishing Institute (MDPI), 4(2), pp. 203–221. doi: 10.3390/nano4020203.

Berber, S., Kwon, Y.-K. and Tománek, D. (2000) 'Unusually High Thermal Conductivity of Carbon Nanotubes', *Physical Review Letters*, 84(20), pp. 4613–4616. doi: 10.1103/PhysRevLett.84.4613.

Berni, R. *et al.* (2018) 'Reactive oxygen species and heavy metal stress in plants: Impact on the cell wall and secondary metabolism', *Environmental and Experimental Botany*. Elsevier. Available at: https://www.sciencedirect.com/science/article/pii/S0098847218312784 (Accessed: 19 February 2019).

Bertrand, C. (2016) Nanomatériaux à travers un gradient de salinité : exposition et effets écotoxicologiques au cours de leur cycle de vie.

Bhattacharjee, S. (2016) 'DLS and zeta potential – What they are and what they are not?', *Journal of Controlled Release*. Elsevier, 235, pp. 337–351. doi: 10.1016/J.JCONREL.2016.06.017.

Bianco, A. *et al.* (2005) 'Biomedical applications of functionalised carbon nanotubes', *Chemical Communications (Cambridge, England)*, (5), pp. 571–577. doi: 10.1039/b410943k.

Birch, M. E. *et al.* (2013) 'Properties that influence the specific surface areas of carbon nanotubes and nanofibers.', *The Annals of occupational hygiene*. NIH Public Access, 57(9), pp. 1148–66. doi: 10.1093/annhyg/met042.

Bjorkl, R., Tobias, D. A. and Petersen, E. J. (2017) 'Increasing evidence indicates low bioaccumulation of carbon nanotubes', *Environmental Science: Nano*. Royal Society of Chemistry, 4(4), pp. 747–766. doi: 10.1039/c6en00389c.

Bonard, J.-M. *et al.* (2002) 'Carbon nanotube films as electron field emitters', *Carbon*. (Carbon Nanotubes:The Present State), 40(10), pp. 1715–1728. doi: 10.1016/S0008-6223(02)00011-8.

Bortolamiol, T. et al. (2014) 'Double-walled carbon nanotubes: Quantitative purification assessment,

balance between purification and degradation and solution filling as an evidence of opening', *Carbon*. Elsevier Ltd, 78, pp. 79–90. doi: 10.1016/j.carbon.2014.06.051.

Bouchard, D., Chang, X. and Chowdhury, I. (2015) 'Heteroaggregation of multiwalled carbon nanotubes with sediments', *Environmental Nanotechnology, Monitoring & Management*, 4, pp. 42–50. doi: 10.1016/j.enmm.2015.07.001.

Bourdiol, F. *et al.* (2013) 'Biocompatible polymer-assisted dispersion of multi walled carbon nanotubes in water, application to the investigation of their ecotoxicity using Xenopus laevis amphibian larvae', *Carbon*, 54, pp. 175–191. doi: 10.1016/j.carbon.2012.11.024.

Bourdiol, F. (2013) Evaluation de l'écotoxicité de nanotubes de carbone en milieu aquatique à l'aide du modèle amphibien Xenopus laevis : synthèse, protocoles d'exposition, détection et dosage.

Bourdiol, F. *et al.* (2015) 'Quantitative detection of carbon nanotubes in biological samples by an original method based on microwave permittivity measurements', *Carbon*, 81, pp. 535–545. doi: 10.1016/j.carbon.2014.09.086.

Brunauer, S., Emmett, P. H. and Teller, E. (1938) 'Adsorption of Gases in Multimolecular Layers', *Journal of the American Chemical Society*. American Chemical Society, 60(2), pp. 309–319. doi: 10.1021/ja01269a023.

Bureau, J.-M. (2016) 'Propriétés diélectriques des polymères', *Ref : TIP350WEB - 'Électronique'*. Editions T.I. | Techniques de l'Ingénieur. Available at: https://www.techniques-ingenieur.fr/basedocumentaire/electronique-photonique-th13/materiaux-pour-l-electronique-et-dispositifs-associes-42271210/proprietes-dielectriques-des-polymeres-e1850/ (Accessed: 26 February 2019).

Van Der Burg, W. J. *et al.* (1994) *Predicting tomato seedling morphology by X-ray analysis of seeds, Journal of the American Society for Horticultural Science (USA)*. [American Society for Horticultural Science]. Available at: http://agris.fao.org/agris-search/search.do?recordID=US9503558 (Accessed: 2 July 2019).

Butler, H. J. *et al.* (2016) 'Using Raman spectroscopy to characterize biological materials', *Nature Protocols*. Nature Publishing Group, 11(4), pp. 664–687. doi: 10.1038/nprot.2016.036.

Byrne, H. *et al.* (2015) 'Optimal Choice of Sample Substrate and Laser Wavelength for Raman Spectroscopic Analysis of Biological Specimen', *Analytical Methods*, 7, pp. 5041–5952. doi: 10.1039/b000000x.

С

Caballero-Guzman, A., Sun, T. and Nowack, B. (2015) 'Flows of engineered nanomaterials through the recycling process in Switzerland', *Waste Management (New York, N.Y.)*, 36, pp. 33–43. doi: 10.1016/j.wasman.2014.11.006.

Calderone, G. (2005) *Application des techniques isotopiques modernes pour la caractérisation de produits alimentaires et de boissons Institute for Health and Consumer Protection*. Available at: http://ihcp.jrc.cec.eu.int/ (Accessed: 27 June 2019).

Calisi, A. *et al.* (2016) 'Multibiomarker response in the earthworm Eisenia fetida as tool for assessing multiwalled carbon nanotube ecotoxicity', *Ecotoxicology (London, England)*, 25(4), pp. 677–687. doi: 10.1007/s10646-016-1626-x.

Canas, J. E. *et al.* (2008) 'Effects of Functionalized and Nonfunctionalized Single-Walled Carbon Nanotubes on Root Elongation of Select Crop Species.', *Environ. Toxicol. Chem.*, 27(9), pp. 1922–1931.

Cañas, J. E. *et al.* (2008) 'EFFECTS OF FUNCTIONALIZED AND NONFUNCTIONALIZED SINGLE-WALLED CARBON NANOTUBES ON ROOT ELONGATION OF SELECT CROP SPECIES', *Environmental Toxicology and Chemistry*. John Wiley & Sons, Ltd, 27(9), p. 1922. doi: 10.1897/08-117.1.

Cano, A. M. *et al.* (2016) 'Determination of uptake, accumulation, and stress effects in corn (Zea mays L.) grown in single-wall carbon nanotube contaminated soil', *Chemosphere*, 152, pp. 117–122. doi: 10.1016/j.chemosphere.2016.02.093.

Capacitor Electronics (2019) *Dielectric Properties of Materials Industry Applications/Products*. Available at: www.keysight.com (Accessed: 26 February 2019).

Castiglione, M. R. *et al.* (2011) 'The effects of nano-TiO2on seed germination, development and mitosis of root tip cells of Vicia narbonensis L. and Zea mays L', *Journal of Nanoparticle Research*, 13(6), pp. 2443–2449. doi: 10.1007/s11051-010-0135-8.

Castillo-Michel, H. A. *et al.* (2017) 'Practical review on the use of synchrotron based micro- and nano- X-ray fluorescence mapping and X-ray absorption spectroscopy to investigate the interactions between plants and engineered nanomaterials', *Plant Physiology and Biochemistry*. Elsevier Masson, 110, pp. 13–32. doi: 10.1016/J.PLAPHY.2016.07.018.

Cerrillo, C. *et al.* (2015) 'Ecotoxicity of multiwalled carbon nanotubes: Standardization of the dispersion methods and concentration measurements', *Environmental Toxicology and Chemistry*, 34(8), pp. 1854–1862. doi: 10.1002/etc.2999.

Chang, C.-M. and Liu, Y.-L. (2010) 'Functionalization of multi-walled carbon nanotubes with non-reactive polymers through an ozone-mediated process for the preparation of a wide range of high performance polymer/carbon nanotube composites', *Carbon*, 48(4), pp. 1289–1297. doi: 10.1016/j.carbon.2009.12.002.

Chartier, F., Isnard, H. and Nonell, A. (2014) 'Analyses isotopiques par spectrométrie de masse - Méthodes et applications', *Ref : TIP630WEB - 'Techniques d'analyse'*. Editions T.I. | Techniques de l'Ingénieur. Available at: https://www.techniques-ingenieur.fr/base-documentaire/mesures-analyses-th1/spectrometries-42390210/analyses-isotopiques-par-spectrometrie-de-masse-p3740/ (Accessed: 16 April 2019).

Chatterjee, C., Gopal, R. and Dube, B. K. (2006) 'Physiological and Biochemical Responses of French Bean to Excess Cobalt', *Journal of Plant Nutrition*. Taylor & Francis Group , 29(1), pp. 127–136. doi: 10.1080/01904160500416513.

Chen, G. *et al.* (2015) 'Carbon Nanotubes Act as Contaminant Carriers and Translocate within Plants', *Scientific Reports*. Nature Publishing Group, 5, pp. 1–9. doi: 10.1038/srep15682.

Chen, M. *et al.* (2018) 'Toxicity of carbon nanomaterials to plants, animals and microbes: Recent progress from 2015-present', *Chemosphere*. Pergamon, 206, pp. 255–264. doi: 10.1016/J.CHEMOSPHERE.2018.05.020.

Chen, M., Qin, X. and Zeng, G. (2017) 'Biodegradation of Carbon Nanotubes, Graphene, and Their Derivatives', *Trends in Biotechnology*. Elsevier Ltd, 35(9), pp. 836–846. doi: 10.1016/j.tibtech.2016.12.001.

Chen, S. *et al.* (2015) 'Mass spectrometry imaging reveals the sub-organ distribution of carbon nanomaterials', *Nature Nanotechnology*, 10(2), pp. 176–182. doi: 10.1038/nnano.2014.282.

Cheung, C. L., Hafner, J. H. and Lieber, C. M. (2000) 'Carbon nanotube atomic force microscopy tips: Direct

growth by chemical vapor deposition and application to high-resolution imaging', *Proceedings of the National Academy of Sciences*, 97(8), pp. 3809–3813.

Christian, P. *et al.* (2008) 'Nanoparticles: structure, properties, preparation and behaviour in environmental media', *Ecotoxicology*, 17(5), pp. 326–343. doi: 10.1007/s10646-008-0213-1.

Chung, H. *et al.* (2011a) 'The effect of multi-walled carbon nanotubes on soil microbial activity', *Ecotoxicology and Environmental Safety*, 74(4), pp. 569–575. doi: 10.1016/j.ecoenv.2011.01.004.

Chung, H. *et al.* (2011b) 'The effect of multi-walled carbon nanotubes on soil microbial activity', *Ecotoxicology and Environmental Safety*. Elsevier, 74(4), pp. 569–575. doi: 10.1016/j.ecoenv.2011.01.004.

Chylińska, M., Szymańska-Chargot, M. and Zdunek, A. (2016) 'FT-IR and FT-Raman characterization of noncellulosic polysaccharides fractions isolated from plant cell wall', *Carbohydrate Polymers*. Elsevier, 154, pp. 48–54. doi: 10.1016/J.CARBPOL.2016.07.121.

Clément, L., Hurel, C. and Marmier, N. (2013) 'Toxicity of TiO2 nanoparticles to cladocerans, algae, rotifers and plants – Effects of size and crystalline structure', *Chemosphere*. Pergamon, 90(3), pp. 1083–1090. doi: 10.1016/J.CHEMOSPHERE.2012.09.013.

Coats, A. W. and Redfern, J. P. (1963) 'Thermogravimetric Analysis A Review*', *Analyst*, 88(1053), p. 906. Available at: https://pubs.rsc.org/en/content/articlepdf/1963/an/an9638800906 (Accessed: 18 February 2019).

Commission Européenne (2016) COMMUNIQUES DE PRESSE - Communiqué de presse - Qu'est-ce qu'un «nanomatériau»?

Connolly, E. L. and Guerinot, M. (2002) 'Iron stress in plants.', *Genome biology*. BioMed Central, 3(8), p. REVIEWS1024. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12186653 (Accessed: 5 June 2019).

Cornelis, G. *et al.* (2014) 'Fate and Bioavailability of Engineered Nanoparticles in Soils: A Review', *Critical Reviews in Environmental Science and Technology*, 44(24), pp. 2720–2764. doi: 10.1080/10643389.2013.829767.

Costa, S. et al. (2008) Characterization of carbon nanotubes by Raman spectroscopy, Materials Science-Poland. Available at:

https://pdfs.semanticscholar.org/1ff1/537bc15f876ee6c63c662c82eafbf552a196.pdf (Accessed: 18 February 2019).

Cox, A. *et al.* (2016) 'Silver and titanium dioxide nanoparticle toxicity in plants: A review of current research', *Plant Physiology and Biochemistry*. Elsevier Masson, 107, pp. 147–163. doi: 10.1016/J.PLAPHY.2016.05.022.

CytoViva (no date) *Hyperspectral Microscopy* — *CytoViva, Inc.* Available at: http://cytoviva.com/products/hyperspectral-imaging-2/hyperspectral-imaging/ (Accessed: 20 May 2019).

Czarny, B. *et al.* (2014) 'Carbon Nanotube Translocation to Distant Organs after Pulmonary Exposure: Insights from in Situ 14C-Radiolabeling and Tissue Radioimaging', *ACS Nano*, 8(6), pp. 5715–5724. doi: 10.1021/nn500475u.

D

Dai, H. et al. (1996) 'Nanotubes as nanoprobes in scanning probe microscopy', Nature, 384(6605), pp. 147-

150. doi: 10.1038/384147a0.

Danish Consumer Council, The Ecological council, D. E. (2019) *Welcome to The Nanodatabase*. Available at: http://nanodb.dk/en/ (Accessed: 24 July 2018).

Dao, L., Beardall, J. and Heraud, P. (2017) 'Characterisation of Pb-induced changes and prediction of Pb exposure in microalgae using infrared spectroscopy', *Aquatic Toxicology*. Elsevier, 188(November 2016), pp. 33–42. doi: 10.1016/j.aquatox.2017.04.006.

Das, K. K., You, Y., *et al.* (2018) 'Development and application of a digestion-Raman analysis approach for studying multiwall carbon nanotube uptake in lettuce', *Environmental Science: Nano*. Royal Society of Chemistry, 5(3), pp. 659–668. doi: 10.1039/c7en01047h.

Das, K. K., Bancroft, L., *et al.* (2018) 'Digestion Coupled with Programmed Thermal Analysis for Quantification of Multiwall Carbon Nanotubes in Plant Tissues', *Environmental Science & Technology Letters*. American Chemical Society, 5(7), pp. 442–447. doi: 10.1021/acs.estlett.8b00287.

Das, K. K. *et al.* (2019) 'Emerging investigator series: quantification of multiwall carbon nanotubes in plant tissues with spectroscopic analysis', *Environmental Science: Nano*. The Royal Society of Chemistry, 6(2), pp. 380–387. doi: 10.1039/C8EN01252K.

Das, S., Sen, B. and Debnath, N. (2015) 'Recent trends in nanomaterials applications in environmental monitoring and remediation', *Environmental Science and Pollution Research*, 22(23), pp. 18333–18344. doi: 10.1007/s11356-015-5491-6.

Datsyuk, V. *et al.* (2009) 'Double-walled carbon nanotube dispersion via surfactant substitution', *Journal of Materials Chemistry*, 19, pp. 2729–2736.

Demšar, J. et al. (2013) Orange: Data Mining Toolbox in Python Tomaž Curk Matija Polajnar Laň Zagar,JournalofMachineLearningResearch.Availableat:http://www.jmlr.org/papers/volume14/demsar13a/demsar13a.pdf (Accessed: 15 February 2019).

Deng, Y. *et al.* (2017) 'Impact of multiwall carbon nanotubes on the accumulation and distribution of carbamazepine in collard greens (Brassica oleracea)', *Environmental Science: Nano.* doi: 10.1039/C6EN00419A.

Deng, Y., White, J. C. and Xing, B. (2014) 'Interactions between engineered nanomaterials and agricultural crops: implications for food safety', *Journal of Zhejiang University SCIENCE A*. Zhejiang University Press, 15(8), pp. 552–572. doi: 10.1631/jzus.A1400165.

Djanaguiraman, M. *et al.* (2018) 'Cerium Oxide Nanoparticles Decrease Drought-Induced Oxidative Damage in Sorghum Leading to Higher Photosynthesis and Grain Yield.', *ACS omega*. American Chemical Society, 3(10), pp. 14406–14416. doi: 10.1021/acsomega.8b01894.

Domínguez-Rodríguez, G. *et al.* (2016) 'Influence of Structural Defects on the Electrical Properties of Carbon Nanotubes and Their Polymer Composites**'. doi: 10.1002/adem.201600116.

Donaldson, K. *et al.* (2013) 'Pulmonary toxicity of carbon nanotubes and asbestos — Similarities and differences', *Advanced Drug Delivery Reviews*. (Carbon Nanotubes in Medicine and Biology: Therapy and Diagnostics & Safety and Toxicology), 65(15), pp. 2078–2086. doi: 10.1016/j.addr.2013.07.014.

Doudrick, K. *et al.* (2013) 'Extraction and Quantification of Carbon Nanotubes in Biological Matrices with Application to Rat Lung Tissue', *ACS Nano*, 7(10), pp. 8849–8856. doi: 10.1021/nn403302s.

Doudrick, K., Herckes, P. and Westerhoff, P. (2012) 'Detection of Carbon Nanotubes in Environmental Matrices Using Programmed Thermal Analysis', *Environmental science & technology*, 46(22), pp. 12246–12253. doi: 10.1021/es300804f.

Dragoman, M. *et al.* (2006) 'Experimental determination of microwave attenuation and electrical permittivity of double-walled carbon nanotubes', *Applied Physics Letters*. American Institute of Physics, 88(15), p. 153108. doi: 10.1063/1.2193464.

Dresselhaus, M. S. *et al.* (2002) 'Raman spectroscopy on isolated single wall carbon nanotubes', *Carbon*. Pergamon, 40(12), pp. 2043–2061. doi: 10.1016/S0008-6223(02)00066-0.

Dresselhaus, M. S. *et al.* (2005) 'Raman spectroscopy of carbon nanotubes', *Physics Reports*. North-Holland, 409(2), pp. 47–99. doi: 10.1016/J.PHYSREP.2004.10.006.

Dresselhaus, M. S., Dresselhaus, G. and Avouris, P. (2003) *Carbon Nanotubes: Synthesis, Structure, Properties, and Applications*. Springer Science & Business Media.

Duhan, J. S. *et al.* (2017) 'Nanotechnology: The new perspective in precision agriculture', *Biotechnology Reports*, 15, pp. 11–23. doi: 10.1016/j.btre.2017.03.002.

E

van der Ent, A. *et al.* (2018) 'X-ray elemental mapping techniques for elucidating the ecophysiology of hyperaccumulator plants', *New Phytologist*, 218(2), pp. 432–452. doi: 10.1111/nph.14810.

Eom, H.-J. *et al.* (2015) 'A systems toxicology approach on the mechanism of uptake and toxicity of MWCNT in Caenorhabditis elegans', *Chemico-Biological Interactions*, 239, pp. 153–163. doi: 10.1016/j.cbi.2015.06.031.

Ernst, F. *et al.* (2017) 'Noncovalent Stable Functionalization Makes Carbon Nanotubes Hydrophilic and Biocompatible', *The Journal of Physical Chemistry C.* American Chemical Society, 121(34), pp. 18887–18891. doi: 10.1021/acs.jpcc.7b03062.

El Euch, S. K., Bouajila, J. and Bouzouita, N. (2015) 'Chemical composition, biological and cytotoxic activities of Cistus salviifolius flower buds and leaves extracts', *Industrial Crops and Products*. Elsevier, 76, pp. 1100–1105. doi: 10.1016/J.INDCROP.2015.08.033.

European Commision (2011) 'Commission Recommendation of 18 October 2011 on the definition of nanomaterial Text with EEA relevance OJ L 275', pp. 38–40. Available at: http://data.europa.eu/eli/reco/2011/696/oj.

F

Fagerlund, G. (1973) 'Determination of specific surface by the BET method', *Matériaux et Construction*, 6(3), pp. 239–245. doi: 10.1007/BF02479039.

Fahrni, C. J. (2007) 'Biological applications of X-ray fluorescence microscopy: exploring the subcellular topography and speciation of transition metals', *Current Opinion in Chemical Biology*. Elsevier Current Trends, 11(2), pp. 121–127. doi: 10.1016/J.CBPA.2007.02.039.

Feijo, J. A. and Moreno, N. (2004) 'Imaging plant cells by two-photon excitation', *Protoplasma*. Springer-Verlag, 223(1), pp. 1–32. doi: 10.1007/s00709-003-0026-2.

Fiorito, S. *et al.* (2014) 'Redox active Double Wall Carbon Nanotubes show intrinsic anti-proliferative effects and modulate autophagy in cancer cells', *Carbon*, 78, pp. 589–600. doi: 10.1016/j.carbon.2014.07.046.

Flahaut, E. *et al.* (2002) 'CCVD Synthesis and Characterization of Cobalt-Encapsulated Nanoparticles', *American Chemical Society*. American Chemical Society, 14(6), pp. 2553–2558. doi: 10.1021/CM011287H.

Flahaut, E. *et al.* (2003) 'Gram-scale CCVD synthesis of double-walled carbon nanotubes.', *Chemical communications* (*Cambridge, England*), (12), pp. 1442–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12841282 (Accessed: 18 December 2018).

Fourati, E. *et al.* (2019) 'Nickel tolerance and toxicity mechanisms in the halophyte Sesuvium portulacastrum L. as revealed by Ni localization and ligand environment studies', *Environmental Science and Pollution Research*. doi: 10.1007/s11356-019-05209-8.

Fox, J. (2002) An R and S-Plus companion to applied regression. Sage Publications. (Accessed: 15 February 2019).

Fultz, B. (Brent) and Howe, J. M. (2013) *Transmission electron microscopy and diffractometry of materials*. Springer. (Accessed: 28 January 2019).

G

Gajewska, E. and Skłodowska, M. (2007) 'Effect of nickel on ROS content and antioxidative enzyme activities in wheat leaves', *BioMetals*, 20(1), pp. 27–36. doi: 10.1007/s10534-006-9011-5.

Gall, H. *et al.* (2015) 'Cell Wall Metabolism in Response to Abiotic Stress', *Plants*, 4(1), pp. 112–166. doi: 10.3390/plants4010112.

Le Gall, H. *et al.* (2015) 'Cell Wall Metabolism in Response to Abiotic Stress', *Plants*, 4(1), pp. 112–166. doi: 10.3390/plants4010112.

Gao, J. *et al.* (2013) 'Effects of nano-TiO2 on photosynthetic characteristics of Ulmus elongata seedlings', *Environmental Pollution*. Elsevier, 176, pp. 63–70. doi: 10.1016/J.ENVPOL.2013.01.027.

García-Hevia, L. *et al.* (2015) 'Inhibition of Cancer Cell Migration by Multiwalled Carbon Nanotubes', *Advanced Healthcare Materials*, 4(11), pp. 1640–1644. doi: 10.1002/adhm.201500252.

García-Sánchez, S., Bernales, I. and Cristobal, S. (2015) 'Early response to nanoparticles in the Arabidopsis transcriptome compromises plant defence and root-hair development through salicylic acid signalling', *BMC Genomics*, 16(1). doi: 10.1186/s12864-015-1530-4.

Gashgari, R. *et al.* (2018) 'Comparison between Growing Plants in Hydroponic System and Soil Based System', *Chemical, and Material Engineering*. doi: 10.11159/icmie18.131.

Gautam, R. *et al.* (2015) 'Review of multidimensional data processing approaches for Raman and infrared spectroscopy', *EPJ Techniques and Instrumentation*. SpringerOpen, 2(1), p. 8. doi: 10.1140/epjti/s40485-015-0018-6.

Ge, C. *et al.* (2011) 'Significance and systematic analysis of metallic impurities of carbon nanotubes produced by different manufacturers.', *Journal of nanoscience and nanotechnology*, 11(3), pp. 2389–97. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21449398 (Accessed: 19 June 2019).

Ge, C. et al. (2012) 'The contributions of metal impurities and tube structure to the toxicity of carbon

nanotube materials', *NPG Asia Materials*. Nature Publishing Group, 4(12), pp. e32–e32. doi: 10.1038/am.2012.60.

Ge, Y. *et al.* (2016) 'Long-Term Effects of Multiwalled Carbon Nanotubes and Graphene on Microbial Communities in Dry Soil', *Environmental Science & Technology*, 50(7), pp. 3965–3974. doi: 10.1021/acs.est.5b05620.

Ghosh, M., Bandyopadhyay, M. and Mukherjee, A. (2010) 'Genotoxicity of titanium dioxide (TiO2) nanoparticles at two trophic levels: Plant and human lymphocytes', *Chemosphere*. Pergamon, 81(10), pp. 1253–1262. doi: 10.1016/J.CHEMOSPHERE.2010.09.022.

Gierlinger, N. (2018) 'New insights into plant cell walls by vibrational microspectroscopy', *Applied Spectroscopy Reviews*. Taylor & Francis, 53(7), pp. 517–551. doi: 10.1080/05704928.2017.1363052.

Giraldo, J. P. *et al.* (2014) 'Plant nanobionics approach to augment photosynthesis and biochemical sensing', *Nature Materials*, 13(4), pp. 400–408. doi: 10.1038/nmat3890.

Giraudoux, P. *et al.* (2018) 'Spatial Analysis and Data Mining for Field Ecologists [R package pgirmess version 1.6.9]', *Journal of Statistical Software*. Comprehensive R Archive Network (CRAN). Available at: https://cran.r-project.org/web/packages/pgirmess/index.html (Accessed: 15 February 2019).

Global market Insights (2017) *Carbon Nanotubes Market to hit USD 8.1bn by 2024: Global Market Insights, Inc. | Markets Insider*. Available at: https://markets.businessinsider.com/news/stocks/carbon-nanotubes-market-to-hit-usd-8-1bn-by-2024-global-market-insights-inc-1007881711 (Accessed: 10 April 2019).

Gogos, A., Knauer, K. and Bucheli, T. D. (2012) 'Nanomaterials in plant protection and fertilization: Current state, foreseen applications, and research priorities', *Journal of Agricultural and Food Chemistry*, 60(39), pp. 9781–9792. doi: 10.1021/jf302154y.

Gopal, R. *et al.* (2003) 'Cobalt Toxicity Effects on Growth and Metabolism of Tomato', *Communications in Soil Science and Plant Analysis*. Taylor & Francis Group , 34(5–6), pp. 619–628. doi: 10.1081/CSS-120018963.

Gottschalk, F. *et al.* (2009) 'Modeled Environmental Concentrations of Engineered Nanomaterials (TiO ₂, ZnO, Ag, CNT, Fullerenes) for Different Regions', *Environmental Science & Technology*. American Chemical Society, 43(24), pp. 9216–9222. doi: 10.1021/es9015553.

Gottschalk, F. *et al.* (2015) 'Modeling flows and concentrations of nine engineered nanomaterials in the Danish environment', *International Journal of Environmental Research and Public Health*, 12(5), pp. 5581–5602. doi: 10.3390/ijerph120505581.

Gottschalk, F., Sun, T. and Nowack, B. (2013) 'Environmental concentrations of engineered nanomaterials: review of modeling and analytical studies', *Environmental Pollution (Barking, Essex: 1987)*, 181, pp. 287–300. doi: 10.1016/j.envpol.2013.06.003.

Graves, S., Piepho, H.-P. and Selzer, L. (2015) *Package 'multcompView' Visualizations of Paired Comparisons*. Available at: http://202.90.158.4/pub/pub/R/web/packages/multcompView/multcompView.pdf (Accessed: 15 February 2019).

Grenier, K. *et al.* (2010) 'Resonant based microwave biosensor for biological cells discrimination', in 2010 *IEEE Radio and Wireless Symposium (RWS)*. IEEE, pp. 523–526. doi: 10.1109/RWS.2010.5434223.

Gui, X. et al. (2011) 'Recyclable carbon nanotube sponges for oil absorption', Acta Materialia, 59(12), pp.

4798-4804. doi: 10.1016/j.actamat.2011.04.022.

Gupta, S. M. and Tripathi, M. (2011) 'A review of TiO2 nanoparticles', *Chinese Science Bulleton*, 56(16), pp. 1639–1657.

Gupta, V. and Trivedi, P. (2018) 'In vitro and in vivo characterization of pharmaceutical topical nanocarriers containing anticancer drugs for skin cancer treatment', *Lipid Nanocarriers for Drug Targeting*. William Andrew Publishing, pp. 563–627. doi: 10.1016/B978-0-12-813687-4.00015-3.

Η

Hamdi, H. *et al.* (2015) 'Impact of non-functionalized and amino-functionalized multiwall carbon nanotubes on pesticide uptake by lettuce (Lactuca sativa L.)', *Nanotoxicology*, 9(2), pp. 172–180. doi: 10.3109/17435390.2014.907456.

Hanna, S. K., Miller, R. J. and Lenihan, H. S. (2014) 'Deposition of carbon nanotubes by a marine suspension feeder revealed by chemical and isotopic tracers', *Journal of Hazardous Materials*. Elsevier, 279, pp. 32–37. doi: 10.1016/J.JHAZMAT.2014.06.052.

Hao, Y. *et al.* (2016) 'Carbon Nanotubes Filled with Different Ferromagnetic Alloys Affect the Growth and Development of Rice Seedlings by Changing the C:N Ratio and Plant Hormones Concentrations', *PloS one*, 11(6), p. e0157264. doi: 10.1371/journal.pone.0157264.

Hao, Y. *et al.* (2018) 'Carbon nanomaterials alter plant physiology and soil bacterial community composition in a rice-soil-bacterial ecosystem', *Environmental Pollution*. Elsevier Ltd, 232(September), pp. 123–136. doi: 10.1016/j.envpol.2017.09.024.

Hatami, M. and Ghorbanpour, M. (2014) 'Nano-anatase TiO2 modulates the germination behavior and
seedling vigority of the five commercially important medicinal and aromatic plants', Journal of Biology and
Environmental Sciences, 22(8), pp. 53–59. Available at:
https://www.researchgate.net/publication/271270095 (Accessed: 15 March 2019).

Hatami, M., Hadian, J. and Ghorbanpour, M. (2017) 'Mechanisms underlying toxicity and stimulatory role of single-walled carbon nanotubes in Hyoscyamus niger during drought stress simulated by polyethylene glycol', *Journal of Hazardous Materials*. Elsevier B.V., 324, pp. 306–320. doi: 10.1016/j.jhazmat.2016.10.064.

Hatami, M., Kariman, K. and Ghorbanpour, M. (2016) 'Engineered nanomaterial-mediated changes in the metabolism of terrestrial plants', *Science of The Total Environment*, 571, pp. 275–291. doi: 10.1016/j.scitotenv.2016.07.184.

He, H. and Pan, B. (2009) 'Studies on structural defects in carbon nanotubes', *Frontiers of Physics in China*. SP Higher Education Press, 4(3), pp. 297–306. doi: 10.1007/s11467-009-0021-y.

He, Y. *et al.* (2019) 'Rapid and versatile pre-treatment for quantification of multi-walled carbon nanotubes in the environment using microwave-induced heating', *Environmental Science and Pollution Research*. Springer Berlin Heidelberg, 26(14), pp. 13999–14012. doi: 10.1007/s11356-019-04229-8.

Herrero-Latorre, C. *et al.* (2015) 'Characterization of carbon nanotubes and analytical methods for their determination in environmental and biological samples: A review', *Analytica Chimica Acta*. Elsevier B.V., 853(1), pp. 77–94. doi: 10.1016/j.aca.2014.10.008.

Hiroi, T., Ata, S. and Shibayama, M. (2016) 'Transitions of Aggregation States for Concentrated Carbon

Nanotube Dispersion', *The Journal of Physical Chemistry C*, 120(10), pp. 5776–5782. doi: 10.1021/acs.jpcc.5b12464.

Hodges, D. M. *et al.* (1999) 'Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds', *Planta*. Springer-Verlag, 207(4), pp. 604–611. doi: 10.1007/s004250050524.

Houston, K. *et al.* (2016) 'The Plant Cell Wall: A Complex and Dynamic Structure As Revealed by the Responses of Genes under Stress Conditions', *Frontiers in Plant Science*. Frontiers, 7, p. 984. doi: 10.3389/fpls.2016.00984.

Hu, C. *et al.* (2013) 'Toxicological effects of multi-walled carbon nanotubes adsorbed with nonylphenol on earthworm Eisenia fetida', *Environmental Science. Processes & Impacts*, 15(11), pp. 2125–2130. doi: 10.1039/c3em00376k.

Hu, C. W. *et al.* (2014) 'Evaluation of the combined toxicity of multi-walled carbon nanotubes and sodium pentachlorophenate on the earthworm Eisenia fetida using avoidance bioassay and comet assay', *Soil Biology and Biochemistry*, 70, pp. 123–130. doi: 10.1016/j.soilbio.2013.12.018.

Huang, P. . (2008) 'Soil Physicochemical and Biological Interfacial Interactions in the Rhizosphere: Impacts on Food Security and Ecosystem Integrity', *Revista de la ciencia del suelo y nutrición vegetal*. Sociedad Chilena de la Ciencia del Suelo, 8(especial), pp. 23–27. doi: 10.4067/S0718-27912008000400005.

Huang, Y., Zhao, L. and Keller, A. A. (2017) 'Interactions, Transformations, and Bioavailability of Nano-Copper Exposed to Root Exudates', *Environmental Science & Technology*. American Chemical Society, 51(17), pp. 9774–9783. doi: 10.1021/acs.est.7b02523.

Hull, M. *et al.* (2012) 'Moving beyond Mass: The Unmet Need to Consider Dose Metrics in Environmental Nanotoxicology Studies', *Environmental Science & Technology*. American Chemical Society, 46(20), pp. 10881–10882. doi: 10.1021/es3035285.

Hull, M. S. *et al.* (2009) 'Release of Metal Impurities from Carbon Nanomaterials Influences Aquatic Toxicity', *Environmental Science & Technology*. American Chemical Society, 43(11), pp. 4169–4174. doi: 10.1021/es802483p.

Hussein, M. M. and Abou-Baker, N. H. (2018) 'The contribution of nano-zinc to alleviate salinity stress on cotton plants.', *Royal Society open science*. The Royal Society, 5(8), p. 171809. doi: 10.1098/rsos.171809.

Ι

lavicoli, I. *et al.* (2017) *Nanotechnology in agriculture: Opportunities, toxicological implications, and occupational risks, Toxicology and Applied Pharmacology.* Elsevier Inc. doi: 10.1016/j.taap.2017.05.025.

lijima, S. (1991) 'Helical microtubules of graphitic carbon', *Nature*. Nature Publishing Group, 354(6348), pp. 56–58. doi: 10.1038/354056a0.

Irin, F. *et al.* (2012) 'Detection of carbon nanotubes in biological samples through microwave-induced heating', *Carbon*, 50, pp. 4441–4449. doi: 10.1016/j.carbon.2012.05.022.

J

Jackson, P. *et al.* (2013) 'Bioaccumulation and ecotoxicity of carbon nanotubes', *Chemistry Central Journal*, 7(1), pp. 1–21. doi: 10.1186/1752-153X-7-154.

Jain, N. *et al.* (2017) 'Does seed size and surface anatomy play role in combating phytotoxicity of nanoparticles?', *Ecotoxicology*. Springer US, 26(2), pp. 238–249. doi: 10.1007/s10646-017-1758-7.

Jaisi, D. P. and Elimelech, M. (2009) 'Single-walled carbon nanotubes exhibit limited transport in soil columns.', *Environmental science & technology*, 43(24), pp. 9161–9166. doi: 10.1021/es901927y.

Jakubek, L. M. *et al.* (2009) 'The inhibition of neuronal calcium ion channels by trace levels of yttrium released from carbon nanotubes.', *Biomaterials.* NIH Public Access, 30(31), pp. 6351–7. doi: 10.1016/j.biomaterials.2009.08.009.

Jamin, E., Martin, F. and Martin, G. G. (2004) 'Determination of the 13C/12C ratio of ethanol derived from fruit juices and maple syrup by isotope ratio mass spectrometry: collaborative study.', *Journal of AOAC International*, 87(3), pp. 621–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15287660 (Accessed: 27 June 2019).

Jaurand, M.-C. F., Renier, A. and Daubriac, J. (2009) 'Mesothelioma: Do asbestos and carbon nanotubes pose the same health risk?', *Particle and Fibre Toxicology*, 6, p. 16. doi: 10.1186/1743-8977-6-16.

Jeevanandam, J. *et al.* (2018) 'Review on nanoparticles and nanostructured materials: history, sources, toxicity and regulations', *Beilstein Journal of Nanotechnology*. Beilstein-Institut, 9(1), pp. 1050–1074. doi: 10.3762/bjnano.9.98.

Jena, P. V. *et al.* (2016) 'Photoluminescent carbon nanotubes interrogate the permeability of multicellular tumor spheroids', *Carbon*. (BIOMEDICAL APPLICATIONS OF CARBON NANOMATERIALS), 97, pp. 99–109. doi: 10.1016/j.carbon.2015.08.024.

Jin, L. *et al.* (2013) 'High concentrations of single-walled carbon nanotubes lower soil enzyme activity and microbial biomass', *Ecotoxicology and Environmental Safety*. Elsevier, 88, pp. 9–15. doi: 10.1016/j.ecoenv.2012.10.031.

Jin, L. *et al.* (2014) 'Single-walled carbon nanotubes alter soil microbial community composition', *The Science of the Total Environment*, 466–467, pp. 533–538. doi: 10.1016/j.scitotenv.2013.07.035.

Joshi, A. *et al.* (2018a) 'Multi-walled carbon nanotubes applied through seed-priming influence early germination, root hair, growth and yield of bread wheat (*Triticum aestivum* L.)', *Journal of the Science of Food and Agriculture*, 98(8), pp. 3148–3160. doi: 10.1002/jsfa.8818.

Joshi, A. *et al.* (2018b) 'Multi-walled carbon nanotubes applied through seed-priming influence early germination, root hair, growth and yield of bread wheat (*Triticum aestivum* L.)', *Journal of the Science of Food and Agriculture*. John Wiley & Sons, Ltd, 98(8), pp. 3148–3160. doi: 10.1002/jsfa.8818.

\mathbf{K}

Kahru, A. and Dubourguier, H.-C. (2010) 'From ecotoxicology to nanoecotoxicology', *Toxicology*. (Potential Hazard of Nanoparticles: From Properties to Biological & Environmental Effects), 269(2–3), pp. 105–119. doi: 10.1016/j.tox.2009.08.016.

Karami Mehrian, S., Heidari, R. and Rahmani, F. (2015) 'Effect of silver nanoparticles on free amino acids content and antioxidant defense system of tomato plants', *Indian Journal of Plant Physiology*. Springer India, 20(3), pp. 257–263. doi: 10.1007/s40502-015-0171-6.

Kasai, T. *et al.* (2015) 'Thirteen-week study of toxicity of fiber-like multi-walled carbon nanotubes with whole-body inhalation exposure in rats', *Nanotoxicology*, 9(4), pp. 413–422. doi:

10.3109/17435390.2014.933903.

Kasel, D. *et al.* (2013) 'Limited transport of functionalized multi-walled carbon nanotubes in two natural soils', *Environmental Pollution*, 180, pp. 152–158. doi: 10.1016/j.envpol.2013.05.031.

Kassambara, A. and Mundt, F. (2017) 'Factoextra: extract and visualize the results of multivariate data analyses', *Journal of Statistical Software*.

Kaur, I. *et al.* (2017) 'Dispersion of Nanomaterials in Aqueous Media: Towards Protocol Optimization', *Journal of Visualized Experiments*, (130). doi: 10.3791/56074.

Keller, A. A. and Lazareva, A. (2014) 'Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local', *Environmental Science & Technology Letters*. American Chemical Society, 1(1), pp. 65–70. doi: 10.1021/ez400106t.

Kennedy *et al.* (2009) 'Influence of nanotube preparation in Aquatic Bioassays', *Environmental Toxicology and Chemistry*, 28(9), pp. 1930–1938. Available at: http://dx.doi.org/10.1897/09-024.1.

Kennedy, A. J. *et al.* (2008) 'Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment', *Environmental Toxicology and Chemistry*. John Wiley & Sons, Ltd, 27(9), p. 1932. doi: 10.1897/07-624.1.

Kesten, C., Menna, A. and Sánchez-Rodríguez, C. (2017) 'Regulation of cellulose synthesis in response to stress', *Current Opinion in Plant Biology*. Elsevier Current Trends, 40, pp. 106–113. doi: 10.1016/J.PBI.2017.08.010.

Khan, Z. and Upadhyaya, H. (2019) 'Impact of Nanoparticles on Abiotic Stress Responses in Plants: An Overview', *Nanomaterials in Plants, Algae and Microorganisms*. Academic Press, pp. 305–322. doi: 10.1016/B978-0-12-811488-9.00015-9.

Khodakovskaya, M., Dervishi, E., Mahmood, M., Xu, Y., Li, Z., Watanabe, F. and Biris, A. S. (2009) 'Carbon Nanotubes Are Able To Penetrate Plant Seed Coat and Dramatically Affect Seed Germination and Plant Growth (Retracted article. See vol. 6, pg. 7541, 2012)', *Acs Nano*, 3(10), pp. 3221–3227. doi: 10.1021/nn900887m.

Khodakovskaya, M. V. *et al.* (2011) 'Complex genetic, photothermal, and photoacoustic analysis of nanoparticle-plant interactions', *Proceedings of the National Academy of Sciences of the United States of America*, 108(3), pp. 1028–1033. doi: 10.1073/pnas.1008856108.

Khodakovskaya, M. V. *et al.* (2012) 'Carbon nanotubes induce growth enhancement of tobacco cells', *ACS Nano*, 6(3), pp. 2128–2135. doi: 10.1021/nn204643g.

Khodakovskaya, M. V. *et al.* (2013) 'Carbon Nanotubes as Plant Growth Regulators: Effects on Tomato Growth, Reproductive System, and Soil Microbial Community', *Small*, 9(1), pp. 115–123. doi: 10.1002/smll.201201225.

Kibbey, T. C. G. and Strevett, K. A. (2019) 'The effect of nanoparticles on soil and rhizosphere bacteria and plant growth in lettuce seedlings', *Chemosphere*. Pergamon, 221, pp. 703–707. doi: 10.1016/J.CHEMOSPHERE.2019.01.091.

Kolosnjaj-Tabi, J. *et al.* (2015) 'Anthropogenic Carbon Nanotubes Found in the Airways of Parisian Children', *EBioMedicine*, 2(11), pp. 1697–1704. doi: 10.1016/j.ebiom.2015.10.012.

Kotchey, G. P. et al. (2012) 'A Natural Vanishing Act: The Enzyme-Catalyzed Degradation of Carbon

Nanomaterials', Accounts of Chemical Research, 45(10), pp. 1770–1781. doi: 10.1021/ar300106h.

Kuo, J. (2014) 'Processing Plant Tissues for Ultrastructural Study', in. Humana Press, Totowa, NJ, pp. 39– 55. doi: 10.1007/978-1-62703-776-1_3.

Kwak, S.-Y. *et al.* (2019) 'Chloroplast-selective gene delivery and expression in planta using chitosancomplexed single-walled carbon nanotube carriers', *Nature Nanotechnology*, 14(5), pp. 447–455. doi: 10.1038/s41565-019-0375-4.

L

De La Torre-Roche, R., Hawthorne, J., Deng, Y., Xing, B., Cai, W., Newman, L. a, *et al.* (2013) 'Multiwalled Carbon Nanotubes and C 60 Fullerenes Di ff erentially Impact the Accumulation of Weathered Pesticides in Four Agricultural Plants', *Environmental science & technology*, 47, pp. 12539–12547. doi: 10.1021/es4034809.

De La Torre-Roche, R., Hawthorne, J., Deng, Y., Xing, B., Cai, W., Newman, L. A., *et al.* (2013) 'Multiwalled Carbon Nanotubes and C60 Fullerenes Differentially Impact the Accumulation of Weathered Pesticides in Four Agricultural Plants', *Environmental Science & Technology*, 47(21), pp. 12539–12547. doi: 10.1021/es4034809.

Lagier, L. *et al.* (2017) 'Surface area of carbon-based nanoparticles prevails on dispersion for growth inhibition in amphibians', *Carbon*. Pergamon, 119, pp. 72–81. doi: 10.1016/J.CARBON.2017.04.016.

Lahiani, M. H. *et al.* (2013) 'Impact of carbon nanotube exposure to seeds of valuable crops', ACS Applied Materials and Interfaces, 5(16), pp. 7965–7973. doi: 10.1021/am402052x.

Lahiani, M. H. *et al.* (2015) 'Interaction of carbon nanohorns with plants: Uptake and biological effects', *Carbon*. Elsevier Ltd, 81(1), pp. 607–619. doi: 10.1016/j.carbon.2014.09.095.

Lahiani, M. H. *et al.* (2016) 'Comparative study of plant responses to carbon-based nanomaterials with different morphologies', *Nanotechnology*. IOP Publishing, 27(26), pp. 1–13. doi: 10.1088/0957-4484/27/26/265102.

Lahiani, M. H. *et al.* (2016) 'Comparative study of plant responses to carbon-based nanomaterials with different morphologies', *Nanotechnology*, 27(26), p. 265102. doi: 10.1088/0957-4484/27/26/265102.

Lahiani, M. H. *et al.* (2018) 'Assessment of Effects of the Long-Term Exposure of Agricultural Crops to Carbon Nanotubes', *Journal of Agricultural and Food Chemistry*, 66(26), pp. 6654–6662. doi: 10.1021/acs.jafc.7b01863.

Lahiani, M. H. *et al.* (2019) 'The impact of tomato fruits containing multi-walled carbon nanotube residues on human intestinal epithelial cell barrier function and intestinal microbiome composition.', *Nanoscale*. doi: 10.1039/c8nr08604d.

Lamprecht, C. *et al.* (2009) 'AFM imaging of functionalized carbon nanotubes on biological membranes', *Nanotechnology*, 20(43), p. 434001. doi: 10.1088/0957-4484/20/43/434001.

Landois, P. (2008) 'Synthèse, fonctionnalisation et impact sur l'environnement de nanotubes de carbone', *http://www.theses.fr*. Toulouse 3. Available at: http://www.theses.fr/2008TOU30175 (Accessed: 22 January 2019).

Largo-Gosens, A. et al. (2014) 'Fourier transform mid infrared spectroscopy applications for monitoring

the structural plasticity of plant cell walls', *Frontiers in Plant Science*. Frontiers, 5, p. 303. doi: 10.3389/fpls.2014.00303.

Larkin, P. (Peter J. . (2018) *Infrared and Raman spectroscopy : principles and spectral interpretation*. 2nd Editio. Edited by Elsevier.

Larue, C. (2011) 'Impact de nanoparticules de TiO2 et de nanotubes de carbone sur les végétaux'. AgroParisTech. Available at: https://pastel.archives-ouvertes.fr/pastel-00765312 (Accessed: 15 April 2019).

Larue, C. *et al.* (2012) 'Quantitative evaluation of multi-walled carbon nanotube uptake in wheat and rapeseed', *Journal of Hazardous Materials*. Elsevier B.V., 227–228, pp. 155–163. doi: 10.1016/j.jhazmat.2012.05.033.

Larue, C. *et al.* (2014) 'Fate of pristine TiO 2 nanoparticles and aged paint-containing TiO 2 nanoparticles in lettuce crop after foliar exposure', *Journal of Hazardous Materials*, 273, pp. 17–26. doi: 10.1016/j.jhazmat.2014.03.014.

Laurent, C. *et al.* (1998) 'Metal nanoparticles for the catalytic synthesis of carbon nanotubes', *New Journal of Chemistry*, 22(n° 11), pp. 1229–1237. doi: 10.1039/a801991f.

Lê, S. *et al.* (2008) *FactoMineR: An R Package for Multivariate Analysis, JSS Journal of Statistical Software.* Available at: http://www.jstatsoft.org/ (Accessed: 15 February 2019).

Lefebvre, J. *et al.* (2004) 'Photoluminescence from an individual single-walled carbon nanotube', *Physical Review B*. American Physical Society, 69(7), p. 075403. doi: 10.1103/PhysRevB.69.075403.

Lenth, R. V. (2016) 'Least-Squares Means: The *R* Package **Ismeans**', *Journal of Statistical Software*, 69(1), pp. 1–33. doi: 10.18637/jss.v069.i01.

Leonardo, T. *et al.* (2014) 'Determination of elemental distribution in green micro-algae using synchrotron radiation nano X-ray fluorescence (SR-nXRF) and electron microscopy techniques – subcellular localization and quantitative imaging of silver and cobalt uptake by Coccomyxa actinabiotis', *Metallomics*. The Royal Society of Chemistry, 6(2), p. 316. doi: 10.1039/c3mt00281k.

Lewis, J. A. and Lewis, J. A. (2014) 'Instrumentation', *Forensic Document Examination*. Academic Press, pp. 69–79. doi: 10.1016/B978-0-12-416693-6.00006-0.

Li, S. *et al.* (2013) 'Determination of multi-walled carbon nanotube bioaccumulation in earthworms measured by a microwave-based detection technique', *Science of The Total Environment*, 445–446, pp. 9–13. doi: 10.1016/j.scitotenv.2012.12.037.

Lichtenthaler, H. K. (1987) '[34] Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes', *Methods in Enzymology*. Academic Press, 148, pp. 350–382. doi: 10.1016/0076-6879(87)48036-1.

Lim, D.-H. *et al.* (2012) 'The effects of sub-lethal concentrations of silver nanoparticles on inflammatory and stress genes in human macrophages using cDNA microarray analysis', *Biomaterials*, 33(18), pp. 4690–4699. doi: 10.1016/j.biomaterials.2012.03.006.

Lin, C. *et al.* (2009) 'Studies on toxicity of multi-walled carbon nanotubes on Arabidopsis T87 suspension cells', *Journal of Hazardous Materials*, 170(2–3), pp. 578–583. doi: 10.1016/j.jhazmat.2009.05.025.

Lin, D. and Xing, B. (2007) 'Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth', *Environmental Pollution*, 150(2), pp. 243–250. doi: 10.1016/j.envpol.2007.01.016.

Lin Guo, † *et al.* (2007) 'Iron Bioavailability and Redox Activity in Diverse Carbon Nanotube Samples'. American Chemical Society . doi: 10.1021/CM062691P.

Lin, S. *et al.* (2009) 'Uptake, translocation, and transmission of carbon nanomaterials in rice plants', *Small*, 5(10), pp. 1128–1132. doi: 10.1002/smll.200801556.

Liné, C. and Larue, C. (no date) 'Utilisation des nanoparticules en remédiation et implications écotoxicologiques', *Bulletin de Veille Scientifique, ANSES*.

Liné, C., Larue, C. and Flahaut, E. (2017) 'Carbon nanotubes: Impacts and behaviour in the terrestrial ecosystem - A review', *Carbon*, 123. doi: 10.1016/j.carbon.2017.07.089.

Liu, J., Reid, R. J. and Smith, F. A. (2000) 'The mechanism of cobalt toxicity in mung beans', *Physiologia Plantarum*. John Wiley & Sons, Ltd (10.1111), 110(1), pp. 104–110. doi: 10.1034/j.1399-3054.2000.110114.x.

Liu, L. *et al.* (2010) 'Microwave Dielectric Properties of Carbon Nanotube Composites', in *Carbon Nanotubes*. InTech. doi: 10.5772/39420.

Liu, S. *et al.* (2009) 'Sharper and Faster "Nano Darts" Kill More Bacteria: A Study of Antibacterial Activity of Individually Dispersed Pristine Single-Walled Carbon Nanotube', *ACS Nano*, 3(12), pp. 3891–3902. doi: 10.1021/nn901252r.

Llamas, A., Ullrich, C. I. and Sanz, A. (2008) 'Ni2+ toxicity in rice: Effect on membrane functionality and plant water content', *Plant Physiology and Biochemistry*, 46(10), pp. 905–910. doi: 10.1016/j.plaphy.2008.05.006.

Longobardi, F. *et al.* (2015) 'Discrimination of geographical origin of lentils (Lens culinaris Medik.) using isotope ratio mass spectrometry combined with chemometrics', *Food Chemistry*, 188, pp. 343–349. doi: 10.1016/j.foodchem.2015.05.020.

Lu, P.-J. *et al.* (2015) 'Analysis of titanium dioxide and zinc oxide nanoparticles in cosmetics', *Journal of Food and Drug Analysis*, 23(3), pp. 587–594. doi: 10.1016/j.jfda.2015.02.009.

Lu, Y., Yang, K. and Lin, D. (2014) 'Transport of surfactant-facilitated multiwalled carbon nanotube suspensions in columns packed with sized soil particles', *Environmental Pollution*. Elsevier Ltd, 192, pp. 36–43. doi: 10.1016/j.envpol.2014.05.008.

Lutsyk, P. *et al.* (2016) 'A sensing mechanism for the detection of carbon nanotubes using selective photoluminescent probes based on ionic complexes with organic dyes', *Light: Science & Applications*, 5(2), p. e16028. doi: 10.1038/lsa.2016.28.

Lyng, F., Gazi, E. and Gardner, P. (2011) *Preparation of Tissues and Cells for Infrared and Raman Spectroscopy and Imaging in D. Moss (ed) Biomedical Applications of Synchrotron Infrared Microspectroscopy, RSC Analytical Spectroscopy Monographs.* Available at: https://arrow.dit.ie/radrep (Accessed: 16 June 2019).

Μ

Maes, H. M. *et al.* (2014) 'Accumulation and Distribution of Multiwalled Carbon Nanotubes in Zebrafish (*Danio rerio*)', *Environmental Science & Technology*, 48(20), pp. 12256–12264. doi: 10.1021/es503006v.

Mariotti, A. (1991) Le carbone 13 en abondance naturelle, traceur de la dynamique de la matière organique

des sols et de l'évolution des paléoenvironnements continentaux. Available at: http://horizon.documentation.ird.fr/exl-doc/pleins_textes/cahiers/PTP/39450.PDF (Accessed: 4 July 2019).

MarketsandMarkets[™] (2019) Carbon Nanotubes (CNT) Market by Type (Single, Multi Walled), Method (Chemical Vapor Deposition, Catalytic Chemical Vapor Deposition, High Pressure Carbon Monoxide), Application (Electronics, Chemical, Batteries, Energy, Medical) - Global Forecast to 2023. Available at: https://www.marketsandmarkets.com/Market-Reports/carbon-nanotubes-139.html (Accessed: 24 May 2019).

Martínez-Ballesta, M. C. *et al.* (2016) 'Multiwalled carbon nanotubes enter broccoli cells enhancing growth and water uptake of plants exposed to salinity', *Journal of Nanobiotechnology*, 14, p. 42. doi: 10.1186/s12951-016-0199-4.

Maynard, A. D. *et al.* (2006) 'Safe handling of nanotechnology', *Nature*, 444(7117), pp. 267–269. doi: 10.1038/444267a.

McGehee, D. L. *et al.* (2017) 'Multiwalled Carbon Nanotubes Dramatically Affect the Fruit Metabolome of Exposed Tomato Plants', *ACS Applied Materials & Interfaces*, 9(38), pp. 32430–32435. doi: 10.1021/acsami.7b10511.

McGrath, S. P. *et al.* (2010) 'Predicting molybdenum toxicity to higher plants: Influence of soil properties', *Environmental Pollution*, 158(10), pp. 3095–3102. doi: 10.1016/j.envpol.2010.06.027.

Meyer, J. N. *et al.* (2010) 'Intracellular uptake and associated toxicity of silver nanoparticles in Caenorhabditis elegans', *Aquatic Toxicology*, 100(2), pp. 140–150. doi: 10.1016/j.aquatox.2010.07.016.

Miralles, P. *et al.* (2012) 'Multiwalled carbon nanotubes in alfalfa and wheat: Toxicology and uptake', *Journal of the Royal Society Interface*, 9(77), pp. 3514–3527. doi: 10.1098/rsif.2012.0535.

Mittler, R. (2006) 'Abiotic stress, the field environment and stress combination', *Trends in Plant Science*, 11(1), pp. 15–19. doi: 10.1016/j.tplants.2005.11.002.

Mohammadi, H., Esmailpour, M. and Gheranpaye, A. (2016) 'Effects of TiO 2 nanoparticles and waterdeficit stress on morpho-physiological characteristics of dragonhead (Dracocephalum moldavica L.) plants', *Acta agriculturae Slovenica*, 107, pp. 385–396. doi: 10.14720/aas.2016.107.2.11.

Moll, J. *et al.* (2016) 'Effect of nanoparticles on red clover and its symbiotic microorganisms', *Journal of Nanobiotechnology*. BioMed Central, 14(1), pp. 1–8. doi: 10.1186/s12951-016-0188-7.

Mondal, A. *et al.* (2011) 'Beneficial role of carbon nanotubes on mustard plant growth: An agricultural prospect', *Journal of Nanoparticle Research*, 13(10), pp. 4519–4528. doi: 10.1007/s11051-011-0406-z.

Monthioux, M. *et al.* (2007) 'Introduction to Carbon Nanotubes', in Prof, B. B. (ed.) *Springer Handbook of Nanotechnology*. Springer Berlin Heidelberg, pp. 43–112.

Monthioux, M. *et al.* (2014) 'Properties of Carbon Nanotubes', in *Handbook of Nanomaterials Properties*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 1–49. doi: 10.1007/978-3-642-31107-9_24.

Morales, M. I. *et al.* (2013) 'Toxicity Assessment of Cerium Oxide Nanoparticles in Cilantro (Coriandrum sativum L.) Plants Grown in Organic Soil'. doi: 10.1021/jf401628v.

Mortimer, M. et al. (2014) 'Potential of Hyperspectral Imaging Microscopy for Semi-quantitative Analysis of Nanoparticle Uptake by Protozoa', *Environmental Science & Technology*. American Chemical Society,

48(15), pp. 8760–8767. doi: 10.1021/es500898j.

Mottier, A. *et al.* (2016) 'Surface Area of Carbon Nanoparticles: A Dose Metric for a More Realistic Ecotoxicological Assessment', *Nano Letters*, 16(6), pp. 3514–3518. doi: 10.1021/acs.nanolett.6b00348.

Mukherjee, A. *et al.* (2016) 'Carbon Nanomaterials in Agriculture: A Critical Review', *Frontiers in Plant Science*, 7(February), pp. 1–16. doi: 10.3389/fpls.2016.00172.

Murthy, S. K. (2007) 'Nanoparticles in modern medicine: state of the art and future challenges.', *International journal of nanomedicine*. Dove Press, 2(2), pp. 129–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17722542 (Accessed: 24 July 2018).

Ν

Nagajyoti, P. C., Lee, K. D. and Sreekanth, T. V. M. (2010) 'Heavy metals, occurrence and toxicity for plants: a review', *Environmental Chemistry Letters*. Springer-Verlag, 8(3), pp. 199–216. doi: 10.1007/s10311-010-0297-8.

Ncibi, M. C. and Sillanpää, M. (2017) 'Optimizing the removal of pharmaceutical drugs Carbamazepine and Dorzolamide from aqueous solutions using mesoporous activated carbons and multi-walled carbon nanotubes', *Journal of Molecular Liquids*. Elsevier, 238, pp. 379–388. doi: 10.1016/J.MOLLIQ.2017.05.028.

Nguyen, C. V., Ye, Q. and Meyyappan, M. (2005) 'Carbon nanotube tips for scanning probe microscopy: fabrication and high aspect ratio nanometrology', *Measurement Science and Technology*, 16(11), p. 2138. doi: 10.1088/0957-0233/16/11/003.

Niu, Y. and Xiang, Y. (2018) 'An Overview of Biomembrane Functions in Plant Responses to High-Temperature Stress.', *Frontiers in plant science*. Frontiers Media SA, 9, p. 915. doi: 10.3389/fpls.2018.00915.

Nowack, B. *et al.* (2013) 'Potential release scenarios for carbon nanotubes used in composites', *Environment International*. The Authors, 59, pp. 1–11. doi: 10.1016/j.envint.2013.04.003.

0

Oheim, M. *et al.* (2006) 'Principles of two-photon excitation fluorescence microscopy and other nonlinear imaging approaches', *Advanced Drug Delivery Reviews*. Elsevier, 58(7), pp. 788–808. doi: 10.1016/J.ADDR.2006.07.005.

Oleszczuk, P., Jośko, I. and Xing, B. (2011) 'The toxicity to plants of the sewage sludges containing multiwalled carbon nanotubes', *Journal of Hazardous Materials*, 186(1), pp. 436–442. doi: 10.1016/j.jhazmat.2010.11.028.

Ρ

Pang, L. S. K., Saxby, J. D. and Chatfield, S. P. (1993) *Thermogravimetric Analysis of Carbon Nanotubes and Nanoparticles*. Available at: https://pubs.acs.org/sharingguidelines (Accessed: 18 February 2019).

Park, S. and Ahn, Y.-J. (2016) 'Multi-walled carbon nanotubes and silver nanoparticles differentially affect seed germination, chlorophyll content, and hydrogen peroxide accumulation in carrot (Daucus carota L.)', *Biocatalysis and Agricultural Biotechnology*, 8, pp. 257–262. doi: 10.1016/j.bcab.2016.09.012.

Park, S. and Ahn, Y. J. (2016) 'Multi-walled carbon nanotubes and silver nanoparticles differentially affect

seed germination, chlorophyll content, and hydrogen peroxide accumulation in carrot (Daucus carota L.)', *Biocatalysis and Agricultural Biotechnology*. Elsevier, 8(July), pp. 257–262. doi: 10.1016/j.bcab.2016.09.012.

Parks, A. N. *et al.* (2013) 'Bioaccumulation and toxicity of single-walled carbon nanotubes to benthic organisms at the base of the marine food chain', *Environmental Toxicology and Chemistry*, 32(6), pp. 1270–1277. doi: 10.1002/etc.2174.

Passardi, F., Penel, C. and Dunand, C. (2004) 'Performing the paradoxical: how plant peroxidases modify the cell wall', *Trends in Plant Science*. Elsevier Current Trends, 9(11), pp. 534–540. doi: 10.1016/J.TPLANTS.2004.09.002.

Peigney, A. *et al.* (2001) 'Specific surface area of carbon nanotubes and bundles of carbon nanotubes', *Carbon*. Pergamon, 39(4), pp. 507–514. doi: 10.1016/S0008-6223(00)00155-X.

Pérez-de-Luque, A. (2017) 'Interaction of Nanomaterials with Plants: What Do We Need for Real Applications in Agriculture?', *Frontiers in Environmental Science*. Frontiers, 5, p. 12. doi: 10.3389/fenvs.2017.00012.

Petersen, E. J. *et al.* (2008) 'Ecological uptake and depuration of carbon nanotubes by Lumbriculus variegatus.', *Environmental health perspectives*. National Institute of Environmental Health Science, 116(4), pp. 496–500. doi: 10.1289/ehp.10883.

Petersen, E. J. *et al.* (2009) 'Influence of Carbon Nanotubes on Pyrene Bioaccumulation from Contaminated Soils by Earthworms', *Environmental Science & Technology*, 43(11), pp. 4181–4187. doi: 10.1021/es803023a.

Petersen, E. J. *et al.* (2011) 'Effects of polyethyleneimine-mediated functionalization of multi-walled carbon nanotubes on earthworm bioaccumulation and sorption by soils', *Environmental Science & Technology*, 45(8), pp. 3718–3724. doi: 10.1021/es103004r.

Petersen, E. J. (2014) *Ecotoxicological effects of carbon nanotubes: Test methods and current research, Health and Environmental Safety of Nanomaterials: Polymer Nancomposites and Other Materials Containing Nanoparticles.* Woodhead Publishing Limited. doi: 10.1533/9780857096678.3.175.

Petersen, E. J. *et al.* (2016) 'Quantification of Carbon Nanotubes in Environmental Matrices: Current Capabilities, Case Studies, and Future Prospects', *Environmental Science and Technology*, 50(9), pp. 4587–4605. doi: 10.1021/acs.est.5b05647.

Petersen, E. J. *et al.* (2019) 'Strategies for robust and accurate experimental approaches to quantify nanomaterial bioaccumulation across a broad range of organisms', *Environmental Science: Nano*. The Royal Society of Chemistry. doi: 10.1039/C8EN01378K.

Petersen, E. J. and Henry, T. B. (2012) 'Methodological considerations for testing the ecotoxicity of carbon nanotubes and fullerenes: Review', *Environmental Toxicology and Chemistry*, 31(1), pp. 60–72. doi: 10.1002/etc.710.

Petersen, E. J., Huang, Q. and Weber, W. J. (2008) 'Bioaccumulation of radio-labeled carbon nanotubes by Eisenia foetida', *Environmental Science & Technology*, 42(8), pp. 3090–3095.

Phan, D. C. *et al.* (2018) 'Biodegradability of carbon nanotube/polymer nanocomposites under aerobic mixed culture conditions', *Science of The Total Environment*, 639, pp. 804–814. doi: 10.1016/j.scitotenv.2018.05.137.

Prasek, J. *et al.* (2011) 'Methods for carbon nanotubes synthesis - Review', *Journal of Materials Chemistry*, 21(40), pp. 15872–15884. doi: 10.1039/c1jm12254a.

Pumera*, M. (2007) 'Carbon Nanotubes Contain Residual Metal Catalyst Nanoparticles even after Washing with Nitric Acid at Elevated Temperature Because These Metal Nanoparticles Are Sheathed by Several Graphene Sheets'. American Chemical Society . doi: 10.1021/LA070088V.

Pumera, M., Ambrosi, A. and Chng, E. L. K. (2012) 'Impurities in graphenes and carbon nanotubes and their influence on the redox properties', *Chemical Science*. The Royal Society of Chemistry, 3(12), p. 3347. doi: 10.1039/c2sc21374e.

Pumera, M. and Miyahara, Y. (2009) 'What amount of metallic impurities in carbon nanotubes is small enough not to dominate their redox properties?', *Nanoscale*. The Royal Society of Chemistry, 1(2), p. 260. doi: 10.1039/b9nr00071b.

Punshon, T., Guerinot, M. Lou and Lanzirotti, A. (2009) 'Using synchrotron X-ray fluorescence microprobes in the study of metal homeostasis in plants', *Annals of Botany*. Narnia, 103(5), pp. 665–672. doi: 10.1093/aob/mcn264.

R

R-Nano.fr (2016).

Rahimi, D. *et al.* (2016) 'Increasing drought resistance of Alnus subcordata C.A. Mey. seeds using a nano priming technique with multi-walled carbon nanotubes', *JOURNAL OF FOREST SCIENCE*, 62(6), pp. 269–278. doi: 10.17221/15/2016-JFS.

Rahman, H. *et al.* (2005) 'Effects of Nickel on Growth and Composition of Metal Micronutrients in Barley Plants Grown in Nutrient Solution', *Journal of Plant Nutrition*. Taylor & Francis Group , 28(3), pp. 393–404. doi: 10.1081/PLN-200049149.

Rane, T. D. and Armani, A. M. (2016) 'Two-Photon Microscopy Analysis of Gold Nanoparticle Uptake in 3D Cell Spheroids.', *PloS one*. Public Library of Science, 11(12), p. e0167548. doi: 10.1371/journal.pone.0167548.

Ratnikova, T. A. *et al.* (2015) 'Tomato Seed Coat Permeability to Selected Carbon Nanomaterials and Enhancement of Germination and Seedling Growth', *Scientific World Journal*, 2015. doi: 10.1155/2015/419215.

Ray, P. C., Yu, H. and Fu, P. P. (2009) 'Toxicity and environmental risks of nanomaterials: challenges and future needs.', *Journal of environmental science and health. Part C, Environmental carcinogenesis & ecotoxicology reviews*. NIH Public Access, 27(1), pp. 1–35. doi: 10.1080/10590500802708267.

Reddy, P. V. L. *et al.* (2016) 'Lessons learned: Are engineered nanomaterials toxic to terrestrial plants?', *Science of The Total Environment*. Elsevier, 568, pp. 470–479. doi: 10.1016/J.SCITOTENV.2016.06.042.

Remy, E. *et al.* (2015) 'Quantitative investigation of mineral impurities of HiPco SWCNT samples: Chemical mechanisms for purification and annealing treatments', *Carbon*, 93, pp. 933–944. doi: 10.1016/j.carbon.2015.06.015.

Rico, C. M. *et al.* (2011) 'Interaction of Nanoparticles with Edible Plants and Their Possible Implications in the Food Chain', *Journal of Agricultural and Food Chemistry*. American Chemical Society, 59(8), pp. 3485–3498. doi: 10.1021/jf104517j.

Rico, C. M. *et al.* (2013) 'Effect of Cerium Oxide Nanoparticles on the Quality of Rice (Oryza sativa L.) Grains'. doi: 10.1021/jf404046v.

Rodrigues, D. F., Jaisi, D. P. and Elimelech, M. (2013) 'Toxicity of functionalized single-walled carbon nanotubes on soil microbial communities: implications for nutrient cycling in soil', *Environmental Science* & *Technology*, 47(1), pp. 625–633. doi: 10.1021/es304002q.

Roth, G. A. *et al.* (2015) 'Hyperspectral microscopy as an analytical tool for nanomaterials', *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*. John Wiley & Sons, Ltd, 7(4), pp. 565–579. doi: 10.1002/wnan.1330.

Roxbury, D. *et al.* (2015) 'Hyperspectral Microscopy of Near-Infrared Fluorescence Enables 17-Chirality Carbon Nanotube Imaging', *Scientific Reports*. Nature Publishing Group, 5(1), p. 14167. doi: 10.1038/srep14167.

Rubart, M. (2004) 'Two-Photon Microscopy of Cells and Tissue', *Circulation Research*. Lippincott Williams & Wilkins, 95(12), pp. 1154–1166. doi: 10.1161/01.RES.0000150593.30324.42.

S

Saifuddin, N., Raziah, A. Z. and Junizah, A. R. (2013) 'Carbon Nanotubes: A Review on Structure and Their Interaction with Proteins', *Journal of Chemistry*. Hindawi, 2013, pp. 1–18. doi: 10.1155/2013/676815.

Saito, R. *et al.* (2011) 'Raman spectroscopy of graphene and carbon nanotubes', *Advances in Physics*. Taylor & Francis , 60(3), pp. 413–550. doi: 10.1080/00018732.2011.582251.

Salieri, B. *et al.* (2017) 'Key physicochemical properties of nanomaterials in view of their toxicity: an exploratory systematic investigation for the example of carbon-based nanomaterial', *Journal of Nanoparticle Research*. Journal of Nanoparticle Research, 19(3). doi: 10.1007/s11051-017-3748-3.

De Santiago-Martín, A. *et al.* (2015) *Bioavailability of Engineered Nanoparticles in Soil Systems*. Available at: http://tarjomefa.com/wp-content/uploads/2017/02/6033-English-TarjomeFa.pdf (Accessed: 14 June 2019).

Sattelmacher, B. and Horst, W. J. (eds) (2007) *The Apoplast of Higher Plants: Compartment of Storage, Transport and Reactions*. Dordrecht: Springer Netherlands. doi: 10.1007/978-1-4020-5843-1.

Schierz, A. *et al.* (2014) 'Fate of single walled carbon nanotubes in wetland ecosystems', *Environ. Sci.: Nano*. The Royal Society of Chemistry, 1(6), pp. 574–583. doi: 10.1039/C4EN00063C.

Schlagenhauf, L. *et al.* (2012) 'Release of Carbon Nanotubes from an Epoxy-Based Nanocomposite during an Abrasion Process', *Environmental Science & Technology*, 46(13), pp. 7366–7372. doi: 10.1021/es300320y.

Schwab, F. *et al.* (2013) 'Diuron Sorbed to Carbon Nanotubes Exhibits Enhanced Toxicity to Chlorella vulgaris', *Environmental Science & Technology*. American Chemical Society, 47(13), pp. 7012–7019. doi: 10.1021/es304016u.

Schwab, F. *et al.* (2015) 'Barriers, pathways and processes for uptake, translocation and accumulation of nanomaterials in plants – Critical review', *Nanotoxicology*, pp. 1–22. doi: 10.3109/17435390.2015.1048326.

Schweikert, C., Liszkay, A. and Schopfer, P. (2002) 'Polysaccharide degradation by Fenton reaction- or

peroxidase-generated hydroxyl radicals in isolated plant cell walls', *Phytochemistry*, 61(1), pp. 31–35. doi: 10.1016/S0031-9422(02)00183-8.

Scott-Fordsmand, J. J. *et al.* (2008) 'The toxicity testing of double-walled nanotubes-contaminated food to Eisenia veneta earthworms', *Ecotoxicology and Environmental Safety*, 71(3), pp. 616–619. doi: 10.1016/j.ecoenv.2008.04.011.

Serag, M. F. *et al.* (2011) 'Trafficking and Subcellular Localization of Multiwalled Carbon Nanotubes in Plant Cells', *ACS Nano*, 5(1), pp. 493–499. doi: 10.1021/nn102344t.

Serag, M. F., Kaji, N., Tokeshi, M. and Baba, Y. (2012) 'Introducing carbon nanotubes into living walled plant cells through cellulase-induced nanoholes', *RSC Advances*, 2(2), pp. 398–400. doi: 10.1039/c1ra00760b.

Serag, M. F., Kaji, N., Tokeshi, M., Bianco, A., *et al.* (2012) 'The plant cell uses carbon nanotubes to build tracheary elements', *Integrative Biology*, 4(2), pp. 127–131. doi: 10.1039/c2ib00135g.

Serag, M. F. *et al.* (2013) 'Nanobiotechnology meets plant cell biology: Carbon nanotubes as organelle targeting nanocarriers', *RSC Advances*, 3(15), pp. 4856–4862. doi: 10.1039/c2ra22766e.

Servin, A. *et al.* (2015) 'A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield', *Journal of Nanoparticle Research*. Springer Netherlands, 17(2), p. 92. doi: 10.1007/s11051-015-2907-7.

Servin, A. D. *et al.* (2012) 'Synchrotron Micro-XRF and Micro-XANES Confirmation of the Uptake and Translocation of TiO ₂ Nanoparticles in Cucumber (*Cucumis sativus*) Plants', *Environmental Science & Technology*. American Chemical Society, 46(14), pp. 7637–7643. doi: 10.1021/es300955b.

Settharaksa, S. *et al.* (2014) 'Effect of solvent types on phenolic, flavonoid contents and antioxidant activities of Syzygium Gratum (Wight) S.N', *International Journal of Pharmacy and Pharmaceutical Sciences*, 6(2), pp. 114–116. Available at: https://innovareacademics.in/journal/ijpps/Vol6Issue2/8545.pdf (Accessed: 15 February 2019).

Shahzad, B. *et al.* (2018) 'Nickel; whether toxic or essential for plants and environment - A review', *Plant Physiology and Biochemistry*. Elsevier Masson, 132, pp. 641–651. doi: 10.1016/J.PLAPHY.2018.10.014.

Shan, J. *et al.* (2015a) 'Biochar, activated carbon, and carbon nanotubes have different effects on fate of 14 C-catechol and microbial community in soil', *Scientific Reports*. Nature Publishing Group, 5(March), pp. 1–11. doi: 10.1038/srep16000.

Shen, C.-X. *et al.* (2010) 'Induction of programmed cell death in Arabidopsis and rice by single-wall carbon nanotubes', *American Journal of Botany*, 97(10), pp. 1602–1609. doi: 10.3732/ajb.1000073.

Shrestha, B. *et al.* (2013) 'An evaluation of the impact of multiwalled carbon nanotubes on soil microbial community structure and functioning', *Journal of Hazardous Materials*. Elsevier B.V., 261, pp. 188–197. doi: 10.1016/j.jhazmat.2013.07.031.

Shrestha, B. *et al.* (2015) 'The influence of multiwalled carbon nanotubes on polycyclic aromatic hydrocarbon (PAH) bioavailability and toxicity to soil microbial communities in alfalfa rhizosphere', *Ecotoxicology and Environmental Safety*, 116, pp. 143–149. doi: 10.1016/j.ecoenv.2015.03.005.

Shrivastava, M. *et al.* (2019) 'Monitoring of engineered nanoparticles in soil-plant system: A review', *Environmental Nanotechnology, Monitoring & Management*. Elsevier, 11, p. 100218. doi: 10.1016/J.ENMM.2019.100218.

Siegwolf, R. T. W. *et al.* (2001) 'Stable isotope analysis reveals differential effects of soil nitrogen and nitrogen dioxide on the water use efficiency in hybrid poplar leaves', *New Phytologist*. John Wiley & Sons, Ltd (10.1111), 149(2), pp. 233–246. doi: 10.1046/j.1469-8137.2001.00032.x.

Siesler, H. W. (2002) Near-infrared spectroscopy : principles, instruments, applications. Wiley-VCH. Available at:

https://books.google.fr/books?hl=fr&lr=&id=U7vqrf2YqmcC&oi=fnd&pg=PP2&dq=raman+spectroscopy+ principle&ots=R0IZ4TUhtH&sig=bF5im2UoHMpC0vZ3TLfWGPCbVE4#v=onepage&q=raman spectroscopy principle&f=false (Accessed: 18 February 2019).

Simon-Deckers, A. *et al.* (2009) 'Size-, composition- and shape-dependent toxicological impact of metal oxide nanoparticles and carbon nanotubes toward bacteria', *Environmental Science and Technology*, 43(21), pp. 8423–8429. doi: 10.1021/es9016975.

Simon, F. *et al.* (2005) 'Isotope Engineering of Carbon Nanotube Systems', *Physical Review Letters*, 95(1), p. 017401. doi: 10.1103/PhysRevLett.95.017401.

Simon, M. *et al.* (2011) 'Titanium dioxide nanoparticles induced intracellular calcium homeostasis modification in primary human keratinocytes. Towards an *in vitro* explanation of titanium dioxide nanoparticles toxicity', *Nanotoxicology*. Taylor & Francis, 5(2), pp. 125–139. doi: 10.3109/17435390.2010.502979.

Singh, A. L. *et al.* (2010) 'Toxicities and Tolerance of Mineral Elements Boron, Cobalt, Molybdenum and Nickel in Crop Plants', *Plant stress*, 4(2), pp. 31–56. Available at: http://www.globalsciencebooks.info/Online/GSBOnline/images/2010/PS_4(SI2)/PS_4(SI2)31-560.pdf (Accessed: 5 June 2019).

Smirnova, E. *et al.* (2012) 'Uptake and accumulation of multiwalled carbon nanotubes change the morphometric and biochemical characteristics of Onobrychis arenaria seedlings', *Frontiers of Chemical Science and Engineering.* SP Higher Education Press, 6(2), pp. 132–138. doi: 10.1007/s11705-012-1290-5.

Smith, B. R. *et al.* (2014) 'Selective uptake of single-walled carbon nanotubes by circulating monocytes for enhanced tumour delivery', *Nature Nanotechnology*, 9(6), pp. 481–487. doi: 10.1038/nnano.2014.62.

Solé, V. A. *et al.* (2007) 'A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra', *Spectrochimica Acta Part B: Atomic Spectroscopy*. Elsevier, 62(1), pp. 63–68. doi: 10.1016/J.SAB.2006.12.002.

Song, B. *et al.* (2017) 'Effect of multi-walled carbon nanotubes on phytotoxicity of sediments contaminated by phenanthrene and cadmium', *Chemosphere*. Elsevier B.V., 172, pp. 449–458. doi: 10.1016/j.chemosphere.2017.01.032.

Sophia A., C. and Lima, E. C. (2018) 'Removal of emerging contaminants from the environment by adsorption', *Ecotoxicology and Environmental Safety*. Academic Press, 150, pp. 1–17. doi: 10.1016/J.ECOENV.2017.12.026.

Sravan Kumar, S., Manoj, P. and Giridhar, P. (2015) 'Fourier transform infrared spectroscopy (FTIR) analysis, chlorophyll content and antioxidant properties of native and defatted foliage of green leafy vegetables', *Journal of Food Science and Technology*, 52(12), pp. 8131–8139. doi: 10.1007/s13197-015-1959-0.

Srivastava, A. and Rao, D. P. (2014) 'Enhancement of seed germination and plant growth of wheat, maize, peanut and garlic using multiwalled carbon nanotubes', *Chem. Bull*, 3(5), pp. 502–504. doi:

10.17628/ECB.2014.3.502.

Stampoulis, D., Jet, N. and Haven, N. (2009) 'Assay-Dependent Phytotoxicity of Nanoparticles to Plants Assay-Dependent Phytotoxicity of Nanoparticles to Plants', *Environmental Science & Technology*, 43, pp. 9473–9479. doi: 10.1021/es901695c.

Stampoulis, D., Sinha, S. K. and White, J. C. (2009) 'Assay-Dependent Phytotoxicity of Nanoparticles to Plants', *Environmental Science & Technology*, 43(24), pp. 9473–9479. doi: 10.1021/es901695c.

Stefov, V., Najdoski, M. and Bogoeva-gaceva, G. (2015) 'Thermal analysis of multi-walled carbon nanotubes material obtained by catalytic pyrolysis of polyethylene THERMAL ANALYSIS OF MULTI-WALLED CARBON NANOTUBES MATERIAL', (November). doi: 10.20450/mjcce.2015.620.

Stoeger, T. *et al.* (2006) 'Instillation of Six Different Ultrafine Carbon Particles Indicates a Surface Area Threshold Dose for Acute Lung Inflammation in Mice', *Environmental Health Perspectives*, 114(3), pp. 328–333. doi: 10.1289/ehp.8266.

Sun, T. Y. *et al.* (2016) 'Dynamic Probabilistic Modeling of Environmental Emissions of Engineered Nanomaterials', *Environmental Science & Technology*, 50(9), pp. 4701–4711. doi: 10.1021/acs.est.5b05828.

Szymanska-Chargot, M. and Zdunek, A. (2013) 'Use of FT-IR Spectra and PCA to the Bulk Characterization of Cell Wall Residues of Fruits and Vegetables Along a Fraction Process', *Food Biophysics*. Springer US, 8(1), pp. 29–42. doi: 10.1007/s11483-012-9279-7.

Т

Taha, R. A. *et al.* (2016) 'Carbon nanotubes impact on date palm in vitro cultures', *Plant Cell, Tissue and Organ Culture*. Springer Netherlands, 127(2), pp. 525–534. doi: 10.1007/s11240-016-1058-6.

Tan, X.-M. and Fugetsu, B. (2007) 'Multi-Walled Carbon Nanotubes Interact with Cultured Rice Cells: Evidence of a Self-Defense Response', *Journal of Biomedical Nanotechnology*, 3(3), pp. 285–288. doi: 10.1166/jbn.2007.035.

Tan, X. ming, Lin, C. and Fugetsu, B. (2009) 'Studies on toxicity of multi-walled carbon nanotubes on suspension rice cells', *Carbon*. Elsevier Ltd, 47(15), pp. 3479–3487. doi: 10.1016/j.carbon.2009.08.018.

Tcherkez, G., Mahé, A. and Hodges, M. (2011) '12C/13C fractionations in plant primary metabolism', *Trends in Plant Science*. Elsevier Current Trends, 16(9), pp. 499–506. doi: 10.1016/J.TPLANTS.2011.05.010.

Team, Rs. (2015) 'RStudio: Integrated Development Environment for R'. Boston, MA. Available at: http://www.rstudio.com/.

Tenhaken, R. (2014) 'Cell wall remodeling under abiotic stress.', *Frontiers in plant science*. Frontiers Media SA, 5, p. 771. doi: 10.3389/fpls.2014.00771.

Terrones, M. (2004) 'Carbon nanotubes: synthesis and properties, electronic devices and other emerging applications', *International Materials Reviews*, 49(6), pp. 325–377. doi: 10.1179/174328004X5655.

Tewes, L. J. *et al.* (2018) 'Metal hyperaccumulation in the Brassicaceae species Arabidopsis halleri reduces camalexin induction after fungal pathogen attack', *Environmental and Experimental Botany*. Elsevier, 153, pp. 120–126. doi: 10.1016/J.ENVEXPBOT.2018.05.015.

ThermoFishher Scientific (no date) $OMNIC^{TM}$ Series Software. Available at:

https://www.thermofisher.com/order/catalog/product/INQSOF018 (Accessed: 24 April 2019).

Thumanu, K. *et al.* (2015) 'Use of infrared microspectroscopy to determine leaf biochemical composition of cassava in response to Bacillus subtilis CaSUT007', *Journal of Plant Interactions*, 10(1), pp. 270–279. doi: 10.1080/17429145.2015.1059957.

Tiwari, D. K. *et al.* (2014) 'Interfacing carbon nanotubes (CNT) with plants: enhancement of growth, water and ionic nutrient uptake in maize (Zea mays) and implications for nanoagriculture', *Applied Nanoscience*, 4(5), pp. 577–591. doi: 10.1007/s13204-013-0236-7.

Tiwari, M. K. *et al.* (2013) 'The ID21 Scanning X-ray Microscope at ESRF A microprobe-XRF Beamline on Indus-2 Synchrotron Light Source Recent citations The ID21 Scanning X-ray Microscope at ESRF', *J. Phys.: Conf. Ser*, 425, p. 182004. doi: 10.1088/1742-6596/425/18/182004.

Tong, Z. *et al.* (2012) 'Response of soil microorganisms to as-produced and functionalized single-wall carbon nanotubes (SWNTs)', *Environmental Science and Technology*, 46(24), pp. 13471–13479. doi: 10.1021/es303251r.

Trevisan, J. *et al.* (2012) 'Extracting biological information with computational analysis of Fourier-transform infrared (FTIR) biospectroscopy datasets: current practices to future perspectives', *The Analyst*, 137(14), p. 3202. doi: 10.1039/c2an16300d.

Tripathi, D. K. *et al.* (2017) 'An overview on manufactured nanoparticles in plants: Uptake, translocation, accumulation and phytotoxicity', *Plant Physiology and Biochemistry*. Elsevier Ltd, 110, pp. 2–12. doi: 10.1016/j.plaphy.2016.07.030.

Tripathi, S., Sonkar, S. K. and Sarkar, S. (2011) 'Growth stimulation of gram (Cicer arietinum) plant by water soluble carbon nanotubes', *Nanoscale*, 3(3), pp. 1176–1181. doi: 10.1039/c0nr00722f.

Tucker, I. M. *et al.* (2015) 'Laser Doppler Electrophoresis applied to colloids and surfaces', *Current Opinion in Colloid & Interface Science*. Elsevier, 20(4), pp. 215–226. doi: 10.1016/J.COCIS.2015.07.001.

Türker-Kaya, S. and Huck, C. W. (2017) 'A Review of Mid-Infrared and Near-Infrared Imaging: Principles, Concepts and Applications in Plant Tissue Analysis', *Molecules*, 22(1). doi: 10.3390/molecules22010168.

U

Upadhyayula, V. K. K. *et al.* (2009) 'Application of carbon nanotube technology for removal of contaminants in drinking water: A review', *Science of the Total Environment*, 408(1), pp. 1–13. doi: 10.1016/j.scitotenv.2009.09.027.

Upadhyayula, V. K. K. *et al.* (2012) 'Life cycle assessment as a tool to enhance the environmental performance of carbon nanotube products: a review', *Journal of Cleaner Production*, 26, pp. 37–47. doi: 10.1016/j.jclepro.2011.12.018.

USEPA (2003) *Ecological Soil Screening Level for Iron Interim Final*. Available at: https://rais.ornl.gov/documents/eco-ssl_iron.pdf (Accessed: 5 June 2019).

Vance, M. E. *et al.* (2015) 'Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory', *Beilstein Journal of Nanotechnology*, 6(1), pp. 1769–1780. doi: 10.3762/bjnano.6.181.

Vasefi, F., MacKinnon, N. and Farkas, D. L. (2016) 'Hyperspectral and Multispectral Imaging in Dermatology', *Imaging in Dermatology*. Academic Press, pp. 187–201. doi: 10.1016/B978-0-12-802838-

4.00016-9.

Verma, S. K. *et al.* (2019) 'Applications of carbon nanomaterials in the plant system: A perspective view on the pros and cons', *Science of The Total Environment*. Elsevier, 667, pp. 485–499. doi: 10.1016/J.SCITOTENV.2019.02.409.

Verneuil, L. (2015) 'Toxicité environnementale et écotoxicité de nanotubes de carbone chez des diatomées benthiques : de la cellule au biofilm', *http://www.theses.fr*. Toulouse, INPT. Available at: http://www.theses.fr/2015INPT0006 (Accessed: 28 June 2019).

Villagarcia, H. *et al.* (2012) 'Surface chemistry of carbon nanotubes impacts the growth and expression of water channel protein in tomato plants', *Small*, 8(15), pp. 2328–2334. doi: 10.1002/smll.201102661.

Vithanage, M. *et al.* (2017) 'Contrasting effects of engineered carbon nanotubes on plants: a review', *Environmental Geochemistry and Health*. Springer Netherlands, 39(6), pp. 1421–1439. doi: 10.1007/s10653-017-9957-y.

Voigt, C. A. (2014) 'Callose-mediated resistance to pathogenic intruders in plant defense-related papillae', *Frontiers in Plant Science*, 5, p. 168. doi: 10.3389/fpls.2014.00168.

De Volder, M. F. L. *et al.* (2013) 'Carbon Nanotubes: Present and Future Commercial Applications', *Science*, 339(6119), pp. 535–539. doi: 10.1126/science.1222453.

W

Wang, C. *et al.* (2019) 'The Smaller the Leaf Is, the Faster the Leaf Water Loses in a Temperate Forest.', *Frontiers in plant science*. Frontiers Media SA, 10, p. 58. doi: 10.3389/fpls.2019.00058.

Weir, A. *et al.* (2012) 'Titanium Dioxide Nanoparticles in Food and Personal Care Products', *Environmental Science & Technology*, 46(4), pp. 2242–2250. doi: 10.1021/es204168d.

Wenseleers, W. *et al.* (2004) 'Efficient Isolation and Solubilization of Pristine Single-Walled Nanotubes in Bile Salt Micelles', *Advanced Functional Materials*, 14(11), pp. 1105–1112. doi: 10.1002/adfm.200400130.

Wickham, H. (2009) *Ggplot2* : *elegant graphics for data analysis*. Springer.

Wild, E. and Jones, K. C. (2009) 'Novel Method for the Direct Visualization of in Vivo Nanomaterials and Chemical Interactions in Plants', *Environmental Science & Technology*, 43(14), pp. 5290–5294. doi: 10.1021/es900065h.

Wu, J. and Kong, L. (2004) 'High microwave permittivity of multiwalled carbon nanotube composites', *Applied Physics Letters*. American Institute of Physics, 84(24), pp. 4956–4958. doi: 10.1063/1.1762693.

Xu, R. (2008) 'Progress in nanoparticles characterization: Sizing and zeta potential measurement', *Particuology*. Elsevier, 6(2), pp. 112–115. doi: 10.1016/J.PARTIC.2007.12.002.

X

Xu, Z., Fang, F. and Dong, S. (2011) 'Carbon Nanotube AFM Probe Technology'. doi: 10.5772/17350.

Y

Yan, S. et al. (2016) 'Single-wall and multi-wall carbon nanotubes promote rice root growth by eliciting the

similar molecular pathways and epigenetic regulation', *IET Nanobiotechnology*, 10(4), pp. 222–229. doi: 10.1049/iet-nbt.2015.0046.

Yang, F. *et al.* (2006) 'Influences of nano-anatase TiO2on the nitrogen metabolism of growing spinach', *Biological Trace Element Research*, 110(2), pp. 179–190. doi: 10.1385/BTER:110:2:179.

Yang, S., Akkus, O. and Creasey, D. (2017) '1064-nm Raman: The Right Choice for Biological Samples?', *Spectroscopy*, 32(6), pp. 46–54. Available at: http://www.spectroscopyonline.com/1064-nm-raman-right-choice-biological-samples?pageID=2 (Accessed: 18 June 2019).

Yang, W. *et al.* (2007) 'Carbon nanotubes for biological and biomedical applications', *Nanotechnology*, 18(41), p. 412001. doi: 10.1088/0957-4484/18/41/412001.

Yao, Z., Kane, C. L. and Dekker, C. (2000) 'High-Field Electrical Transport in Single-Wall Carbon Nanotubes', *Physical Review Letters*, 84(13), pp. 2941–2944. doi: 10.1103/PhysRevLett.84.2941.

Yu, null *et al.* (2000) 'Strength and breaking mechanism of multiwalled carbon nanotubes under tensile load', *Science (New York, N.Y.)*, 287(5453), pp. 637–640.

Yuan, Z. *et al.* (2017) 'Novel impacts of functionalized multi-walled carbon nanotubes in plants: promotion of nodulation and nitrogenase activity in the rhizobium-legume system.', *Nanoscale*, 9(28), pp. 9921–9937. doi: 10.1039/c7nr01948c.

Z

Zaytseva, O., Wang, Z. and Neumann, G. (2017) 'Phytotoxicity of carbon nanotubes in soybean as determined by interactions with micronutrients', *Journal of Nanoparticle Research*. Journal of Nanoparticle Research, 19(2). doi: 10.1007/s11051-016-3722-5.

Zejian, L. *et al.* (2006) 'Carbon Nanotube Based Microfocus Field Emission X-Ray Source for Microcomputed Tomography', *Applied Physics Letters*, 89(10). doi: 10.1063/1.2345829.

Zhai, G. *et al.* (2015a) 'Charge, Size, and Cellular Selectivity for Multiwall Carbon Nanotubes by Maize and Soybean', *Environmental Science & Technology*, 49(12), pp. 7380–7390. doi: 10.1021/acs.est.5b01145.

Zhai, G. *et al.* (2015b) 'Charge, Size, and Cellular Selectivity for Multiwall Carbon Nanotubes by Maize and Soybean', *Environmental Science and Technology*, 49(12), pp. 7380–7390. doi: 10.1021/acs.est.5b01145.

Zhai, P., Isaacs, J. A. and Eckelman, M. J. (2016) 'Net energy benefits of carbon nanotube applications', *Applied Energy*. Elsevier Ltd, 173, pp. 624–634. doi: 10.1016/j.apenergy.2016.04.001.

Zhang, H. *et al.* (2017) 'Influence of multiwalled carbon nanotubes and sodium dodecyl benzene sulfonate on bioaccumulation and translocation of pyrene and 1-methylpyrene in maize (Zea mays) seedlings', *Environmental Pollution (Barking, Essex: 1987)*, 220(Pt B), pp. 1409–1417. doi: 10.1016/j.envpol.2016.10.093.

Zhang, H. *et al.* (2017) 'Physiological Effects of Single- and Multi-Walled Carbon Nanotubes on Rice Seedlings', *IEEE Transactions on Nanobioscience*, 16(7), pp. 563–570. doi: 10.1109/TNB.2017.2715359.

Zhang, L. *et al.* (2012) 'Interactions of 14C-labeled multi-walled carbon nanotubes with soil minerals in water', *Environmental Pollution*. Elsevier, 166, pp. 75–81. doi: 10.1016/J.ENVPOL.2012.03.008.

Zhang, L. *et al.* (2014) 'Acute toxicity of multi-walled carbon nanotubes, sodium pentachlorophenate, and their complex on earthworm Eisenia fetida', *Ecotoxicology and Environmental Safety*, 103, pp. 29–35. doi:

10.1016/j.ecoenv.2014.01.041.

Zhang, Y. *et al.* (2015) 'Reduction in toxicity of wastewater from three wastewater treatment plants to alga (Scenedesmus obliquus) in northeast China', *Ecotoxicology and Environmental Safety*, 119, pp. 132–139. doi: 10.1016/j.ecoenv.2015.04.034.

Zhao, L. *et al.* (2014) 'CeO 2 and ZnO Nanoparticles Change the Nutritional Qualities of Cucumber (Cucumis sativus)'. doi: 10.1021/jf405476u.

Zhao, Q. *et al.* (2017) 'Quantitative evaluation of multi-wall carbon nanotube uptake by terrestrial plants', *Carbon*. Elsevier Ltd, 114, pp. 661–670. doi: 10.1016/j.carbon.2016.12.036.

Zheng, L. *et al.* (2005) 'Effect of Nano-TiO<SUB>2</SUB> on Strength of Naturally Aged Seeds and Growth of Spinach', *Biological Trace Element Research*, 104(1), pp. 083–092. doi: 10.1385/BTER:104:1:083.

Zheng, X. *et al.* (2014) 'Carboxyl-modified single-walled carbon nanotubes negatively affect bacterial growth and denitrification activity', *Scientific Reports*, 4, p. 5653. doi: 10.1038/srep05653.

Zhu, J.-K. (2016) 'Abiotic Stress Signaling and Responses in Plants.', *Cell*. Elsevier, 167(2), pp. 313–324. doi: 10.1016/j.cell.2016.08.029.

Zhu, Z.-J. *et al.* (2012) 'Effect of Surface Charge on the Uptake and Distribution of Gold Nanoparticles in Four Plant Species', *Environmental Science & Technology*. American Chemical Society, 46(22), pp. 12391–12398. doi: 10.1021/es301977w.

Zuverza-Mena, N. *et al.* (2016) 'Effects of Silver Nanoparticles on Radish Sprouts: Root Growth Reduction and Modifications in the Nutritional Value.', *Frontiers in plant science*. Frontiers Media SA, 7, p. 90. doi: 10.3389/fpls.2016.00090.