UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

INSTITUTO DE INVESTIGACIONES QUÍMICO BIOLÓGICAS

Laboratorio de Interacción Suelo Planta Microorganismo

“Evaluación de los efectos de los nanotubos de carbono naturales y sintéticos en la promoción del crecimiento vegetal”

TESIS

Para obtener el grado de Doctor en Ciencias en Biología Experimental

Presenta: M.**C. Gladys Juárez Cisneros**

Director: Dr. H. Javier A. Villegas Moreno
Co-director: Dr. Jesús Campos García

Morelia Michoacán. Septiembre 2020

INSTITUTO DE INVESTIGACIONES
QUÍMICO-BIOLÓGICAS
UMSNH

El presente trabajo de investigación plasmado en esta tesis, se llevó a cabo durante el periodo Marzo/2016 a Febrero/2020 en el laboratorio de Interacción Suelo Planta Microorganismo del Instituto de Investigaciones Químico Biológicas de la Universidad Michoacana de San Nicolás de Hidalgo. Fue financiado por la Coordinación de Investigación Científica (CIC) y el apoyo otorgado por el Consejo Nacional de Ciencia y Tecnología (CONACYT).

Agradecimientos:

En este camino, de infinitas posibilidades, llegar a hoy, es tener la certeza de la suma de voluntades que acompañaron mi viaje.

Gracias familia, gracias por sostenerme, por justificar mis ausencias, por siempre confiar en mi y por ser siempre el motor que mueve mi vida…

A mis amigos, gracias, porque el trabajo no existe donde se comparte el gusto por nuevos retos, porque un buen café con ustedes siempre reinició los días difíciles para hacerlos mejores, porque estuvieron ahí siempre…

Dr. Javier, gracias por este viaje, ha sido emocionante y un gusto sortearlo contigo, gracias por confiar en mí.

Dr. Jesús, gracias por su paciencia, por su tiempo, por poner siempre a prueba mi voluntad, porque su apoyo siempre incentivó mis esfuerzos…

A mi comité tutorial, gracias, por su valioso aporte a este trabajo de investigación y la atención que siempre mostraron por mi persona, aprecio su generoso apoyo durante todo este tiempo…

. . .

TABLA DE CONTENIDO

ÍNDICE DE FIGURAS

ACRÓNIMOS

Acrónimo	Significado
AIA	Ácido-indol-3-acético
ABA	Ácido abscísico
FTIR	Fourier transform infrared spectroscopy
DQV	Deposición química de vapor
GAs	Giberelinas
LC-MS/MS	Cromatografía de líquidos-espectrometría de masas-masas
HRTEM	Microscopía electrónica de transmisión de alta resolución
IBA	Ácido-indol-3- butírico
JA	Ácido jasmónico
Kt	Kinetina
MWCNTs	Multi-walled carbon nanotubes
NTC	Nanotubos de carbono
PI3K	Fosfatidil inositol-3-cinasa
ROS	Especies reactivas de oxígeno
S6K	S6 cinasa ribosomal
SA	Ácido salicílico
SWCNTs	Single-walled carbon nanotubes
TGA	Análisis termogravimétrico
TOR	Target of rapamicina

RESUMEN

Los nanotubos de carbono de pared múltiple (MWCNTs) se consideraban nanopartículas sintéticas, no obstante, recientemente se demostró la formación de estas nanopartículas en la naturaleza durante los incendios forestales. Los MWCNTs sintéticos se consideran nanopartículas promotoras del crecimiento vegetal; sin embargo, también se han registrado efectos fitotóxicos. El desarrollo vegetal depende de factores como la vía de señalización de TOR (Target of rapamycin), proteina-cinasa clave durante la progresión del ciclo celular y el desarrollo vegetal; sin embargo, se desconoce si los efectos de los MWCNTs en la promoción del crecimiento vegetal impactan esta vía. Por lo que en este trabajo se evaluó el efecto de las nanopartículas de MWCNTs de origen natural y sintéticos en el desarrollo de plantas tales como *Eysenhardtia polystachya* y *Arabidopsis thaliana*. Se realizó la caracterización de los MWCNTs tanto de origen natural como sintéticos, así como carbono amorfo (obtenido del mismo sitio de colecta que los MWCNTs naturales) y se evaluaron sus efectos en *E. polystachya* y *A. thaliana* mediante bioensayos dosis-respuesta. Los resultados mostraron que a dosis equivalentes los MWCNTs naturales promovieron el desarrollo de *E. Polystachya* y *A. thaliana* al inducir la emergencia temprana de la semilla y el porcentaje de germinación, el área foliar, la biomasa y la arquitectura radical. Por el contrario, los MWCNTs sintéticos afectaron el desarrollo de estas plantas. El efecto de los MWCNTs en la vía TOR se analizó en la planta transgénica *AtTOR/tor1::uidA* y mediante la fosforilación de la proteína S6K. Se observó que los MWCNTs naturales incrementaron tanto la expresión de la ß-glucuronidasa (*GUS*) en *AtTOR/tor1::uidA*, así como la fosforilación de la proteína S6K; efecto contrario fue obtenido con los MWCNTs sintéticos. Asimismo, se evaluó la expresión *GUS* en la línea *AtCycB1;1::uidA* un marcador clave de la división celular, observando que los MWCNTs naturales estimularon la expresión del marcador asociado a *CYCB*, mientras que los MWCNTs sintéticos la disminuyeron. Al adicionar el inhibidor AZD8055 (inhibidor de TOR) se observó una correlación entre la inducción de la división celular y la fosforilación de la proteina-cinasa TOR. Adicionalmente se determinó el efecto de los MWCNTs sobre la producción de auxinas utilizando la planta transgénica *DR5::uidA*. Los resultados mostraron que los MWCNTs de origen natural indujeron la expresión del marcador de respuesta a auxinas *DR5::uidA*; por el contrario, el inhibidor AZD8055 no afectó la expresión de *GUS*, sugiriendo que los MWCNTs naturales estimularon

la síntesis de auxinas de manera independiente de la actividad de TOR. En contraste, el efecto de los MWCNTs sintéticos sobre la actividad auxínica fue dependiente de la proteina-cinasa TOR. Por otro lado, ambas nanopartículas de MWCNTs, naturales y sintéticas, modificaron el balance de fitorreguladores en *A. thaliana*. Los MWCNTs naturales incrementaron el contenido de los fitorreguladores IBA, AIA y GA, pero disminuyeron la acumulación de Kt; mientras que los MWCNTs sintéticos incrementaron la acumulación de IBA, GAs, SA; pero disminuyendo Kt. Los resultados obtenidos indicaron que el efecto estimulador de los MWCNTs naturales sobre el crecimiento vegetal en *A. thaliana* involucra la inducción de la actividad de la proteina-cinasa TOR, a través del incremento en acumulación de los fitorreguladores principalmente de la vía auxínica; efecto contrario fue observado con los MWCNTs de origen sintético.

Palabras clave: Nanotubos de carbono naturales; TOR; S6K; fitorreguladores; promoción de crecimiento vegetal, toxicidad.

ABSTRACT

Multi-walled carbon nanotubes (MWCNT) are considered synthetic nanoparticles, however, the formation of these nanoparticles in nature was recently demonstrated during wildfires. Synthetic MWCNTs are considered plant growth promoting nanoparticles; however, phytotoxic effects have also been recorded. Plant growth depends on factors such as the signaling pathway of TOR (rapamycin target), a key protein kinase during cell cycle progression and plant development; however, it is unknown whether the effects of MWCNTs on plant growth promotion impact this pathway. Therefore, in this work the effect of nanoparticles of MWCNTs of natural and synthetic origin on the development of plants such as *Eysenhardtia polystachya* and *Arabidopsis thaliana* was evaluated. Characterization of both naturally originated and synthetic MWCNTs, as well as amorphous carbon (obtained from the same collection site as natural MWCNTs) was performed and their effects on *E. polystachya* and *A. thaliana* were evaluated using dose-response bioassays. The results obtained shown that at equivalent doses the MWCNTs promoted the development of *E. Polystachya* and *A. thaliana* by inducing early seed emergence and germination percentage, leaf area, biomass and root architecture. In contrast, synthetic MWCNTs affect the development of these plants. The effect of MWCNTs on the TOR pathway was analyzed in the *AtTOR/tor1::uid*A transgenic plant and by phosphorylation of the S6K protein. Natural MWCNTs showed to increase both β-glucuronidase (*GUS*) expression in *AtTOR/tor1::uid*A, as well as phosphorylation of the S6K protein. The opposite effect was obtained with the synthetic MWCNT. Likewise, *GUS* expression was evaluated in the line *AtCycB1;1::uid*A a key marker of cell division, observing that natural MWCNTs increased the expression of the marker associated with *CYCB*, while synthetic MWCNTs decreased it. By adding the inhibitor AZD8055 (TOR inhibitor), a correlation was detected between the induction of cell division and the phosphorylation of the TOR protein kinase. Furthermore, the effect of MWCNTs on the production of auxins was determined using the *DR5::uid*A transgenic plant. Results indicated that naturally occurring MWCNTs induced expression of the *DR5::uid*A auxin response marker; conversely, the AZD8055 inhibitor did not affect *GUS* expression, suggesting that natural MWCNTs stimulated auxin synthesis independent of TOR activity. In contrast, the effect of synthetic MWCNTs on auxinic activity was dependent on the TOR protein kinase. On the other hand, both natural and synthetic MWCNTs nanoparticles modified the balance of phytoregulators in *A. thaliana*. Natural MWCNTs increased the content of the IBA, AIA and GA phytoregulators, but decreased Kt accumulation; while synthetic MWCNTs increased the accumulation of IBA, GA, SA; but decreasing Kt. The results obtained

indicate the stimulating effect of natural MWCNTs on plant growth in *A. thaliana* involves the introduction of TOR protein kinase activity, via the increase in the accumulation of phytoregulators, mainly of the auxinic pathway; the opposite effect was observed with MWCNTs of synthetic origin.

Keywords: Natural carbon nanotubes; TOR; S6K; phytoregulators; plant growth promotion, toxicity.

1. INTRODUCCIÓN Y ANTECEDENTES

1.1. Nanopartículas

La nanotecnología es una ciencia emergente con creciente interés multidisciplinario, ha revolucionado el desarrollo industrial, tecnológico y científico [1]; y surge como tecnología de vanguardia en la física de materiales para la manufactura de polímeros, sensores o producción de dispositivos eléctricos y electrónicos [2]. El uso de nanomateriales también converge ampliamente en el estudio en áreas ambientales, energías renovables, en la nanomedicina para el desarrollo de nuevos fármacos [3,4], en la industria cosmética y alimentaria, y en el área vegetal para el desarrollo de insumos agrícolas más eficientes [5–8]. Una nanopartícula tiene un tamaño en el rango entre 1-100 nm y una morfología superficial típica [9]. De acuerdo a su naturaleza química, estas se clasifican en dos grupos: 1) Nanopartículas inorgánicas como el ZnO, FeO, TiO_2, CeO_2, SiO_2, In_2O_3, Nd_2O_3, CoO, Co_2O_3,Co_3O_4, etc., o metales como el Au, Ag, Fe, Pd, Cu y Cd [6,8,10]; y 2) nanopartículas orgánicas o de carbono, como los fullerenos, nanodots, fluorescent carbon dots, nanohorns, fullerol, óxido de grafeno, carbon nanohorns, nanocarbon sol, water-soluble carbon nano-onions y nanotubos de carbono (NTC), (la figura 1 muestra un esquema representativo de la diversidad de nanopartículas en la actualidad) [9,11].

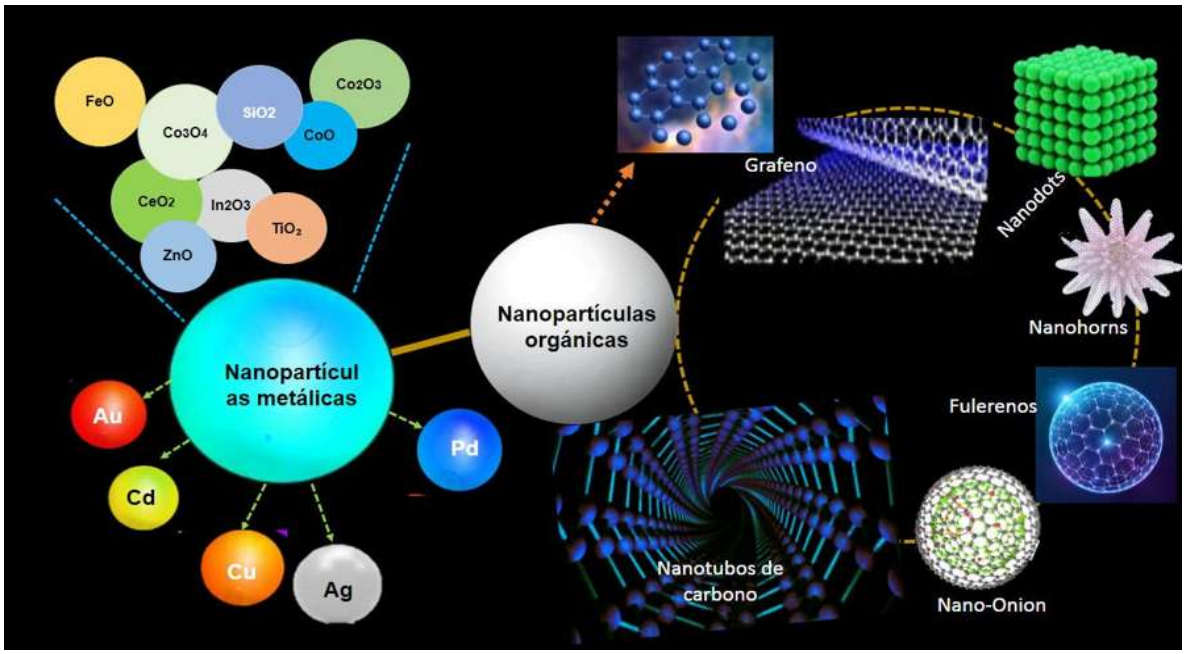


Figura 1. Esquema general de nanopartículas según su naturaleza química: metálicas y orgánicas.

1.2. Los nanotubos de carbono (NTC)

Entre las nanopartículas de carbono, los fullerenos y los NTC son los más ampliamente estudiados al presentar propiedades físico-químicas únicas debido a su tamaño, estructura química y morfología [12–14]. Los NTC, son nanopartículas cilíndricas constituidas por una o varias capas de carbono con un átomo de espesor (grafeno) unidos por enlaces covalentes, densamente empaquetados en una red cristalina [15]. De acuerdo al número de capas de grafeno que los constituye, los NTC se han clasificado principalmente en SWCNTs (por sus siglas en inglés, Single-walled carbon nanotubes) si tienen una sola capa, y MWCNTs (por sus siglas en inglés, multi-walled carbon nanotubes) o nanotubos de carbono de pared múltiple si contienen varias capas de grafeno [16] (La figura 2 muestra la estructura típica de los SWCNTs y MWCNTs).

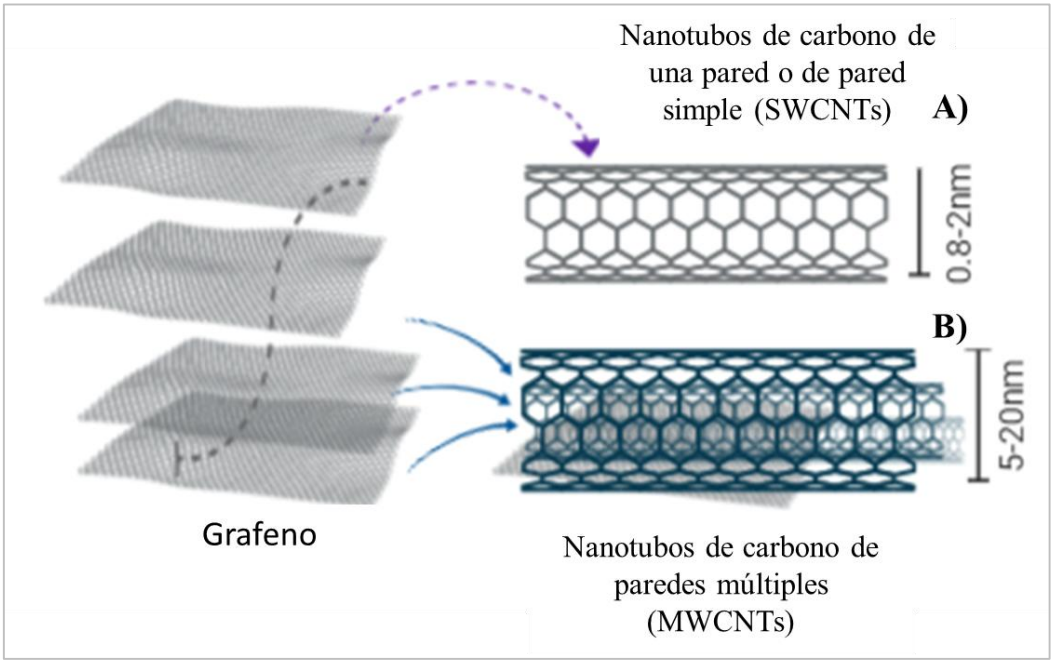


Figura 2. Estructura de los nanotubos de carbono: A) SWCNTs (Single-walled carbon naotube) y B) MWCNTs (Multi-walled carbon nanotubes).

Los MWCNTs se componen de varios nanotubos concéntricos de pared simple o varias capas de grafeno, unidas entre sí por fuerzas van der Waals [2,13]. Desde el descubrimiento de estas nanopartículas por Lijima (1991) [17], los MWCNTs han sido de creciente interés multidisciplinario. En general, los MWCNTs poseen diámetros interno y externo con dimensiones que oscilan entre 5-20nm [18]. Son nanomateriales con alta estabilidad estructural, 50 veces mayor

que el acero [19]. Son materiales ligeros y presentan alta conductividad eléctrica (comparable al cobre), por lo que se clasifican dentro del rango de los materiales metálicos o semiconductores [15]. Estas y otras propiedades les confieren múltiples usos, especialmente en áreas de investigación de nanomateriales, ingeniería eléctrica, mecánica, biomédica [20] y en el desarrollo biotecnológico para la mejora de los cultivos vegetales [21,22].

1.3. Métodos de síntesis de MWCNTs

Los métodos más comunes para la obtención de MWCNTs es por ablación con láser, descarga de arco y la deposición química de vapor (DQV) [23]. La síntesis de MWCNTs por DQV requiere una fuente volátil de carbono como el metano, hexano, acetileno, etileno; un catalizador como el Fe, Ni, Co o Mo de forma individual o combinados; y temperaturas ≥ 600°C [24]. Asimismo, para la síntesis de estos nanomateriales se ha sustituido la fuente de carbono sintético por precursores de origen biótico, como el aceite de trementina [25], eucalipto, alcanfor o sustratos resinosos ricos en α-pineno [26], también sustitutos de ferroceno u otros materiales inorgánicos de Fe, por catalizadores naturales disponibles en organismos como el hongo *Auricularia auricula-judae* y la semilla de sésamo negro, ambos caracterizados por su alto contenido de este metal [27].

1.3.2. Los MWCNTs de origen natural

La obtención de MWCNTs desde su descubrimiento en 1991 y hasta el 2017, era por métodos sintéticos. En 2017, Lara-Romero y colaboradores [28] mostraron evidencia fehaciente de la presencia de MWCNTs generados de forma espontánea en madera calcinada de especies de pino resinoso después de los incendios forestales. En dicho estudio, la caracterización de estos nanomateriales se realizó mediante un análisis termogravimétrico (TGA) cuantificándose la cantidad de MWCNTs de la madera quemada. El análisis reveló que las muestras de madera de *Pinus oocarpa* y *Pinus pseudostrobus* contenían entre 0.1% y 2.8% (p/p) de estas nanopartículas respectivamente. Asimismo, mediante espectroscopía Raman detectaron las bandas características de los MWCNTs. Además, por microscopía electrónica de transmisión de alta resolución (HRTEM), observaron que los MWCNTs encontrados estaban constituidos de ~10 capas de grafeno, con diámetros interno de ~2.52 nm y diámetro externo de ~12–15 nm, y longitudes de entre 2.5–20 µm. Finalmente, por dispersión de rayos X (SDS), detectaron la presencia de Fe, y sugirieron que podía ser utilizado como un potencial catalizador para la formación de los nanotubos

[28]. Dichos hallazgos, generaron interrogantes sobre el posible impacto eco-fisiológico que los MWCNTs naturales pueden presentar en poblaciones autóctonas de ecosistemas forestales después un incendio forestal.

1.4. MWCNTs y las plantas

El concepto de Nanoagricultura surgió en la última década, debido a los efectos potenciales documentados de los MWCNTs sintéticos y otras nanopartículas en la promoción del crecimiento de diversas especies vegetales [29,30]. El estudio de estos nanomateriales se ha centrado en el desarrollo de estrategias innovadoras que mejoren el crecimiento de las plantas de interés agrícola principalmente, para ello, se explora la generación de sistemas de monitoreo de la calidad nutricional del suelo-planta y elaboración de nanofertilizantes para mejorar el desarrollo vegetal [31–33]; reducir los efectos negativos causados por el estrés ambiental así como los efectos perniciosos ocasionados por plagas y enfermedades, esto último mediante la elaboración de nanopesticidas [34,35] y en general, utilizar estas nanopartículas como vehículos para mejorar el desarrollo de productos agrícolas de liberación lenta más eficientes [36,37].

- Efecto de los MWCNTs en la promoción del crecimiento vegetal

Si bien, los efectos de los MWCNTs de origen sintético en el desarrollo vegetal han sido ampliamente documentado. La evidencia científica sobre el uso de MWCNTs de origen natural, y sus efectos potenciales en sistemas biológicos ha sido escasamente documentada, debido a su relativo reciente descubrimiento. Resultados obtenidos por Lara-Romero *et al.(*2017) [28] demostraron que el uso de MWCNTs sintéticos con características estructurales parecidas a las de los MWCNTs naturales, en plantas de origen forestal promovieron la emergencia temprana de las semillas, incrementaron el porcentaje de germinación, el crecimiento vegetal en follaje y la raíz, así como la biomasa de dichas plantas. En plantas de interés agrícola los efectos de los MWCNTs sintéticos ha sido más ampliamente explorado [32], se ha atribuido a estas nanopartículas efectos positivos en la emergencia temprana de semillas e incrementos en el porcentaje de germinación, por ejemplo en *Zea mays* [38], híbridos de *Glycine max*, y *Hordeum vulgare* [31]. Además, mejoraron la elongación y ramificación de la raíz en *Brassica oleracea, Daucus carota, Cucumis sativus, Allium* [21] y *Cicer arietinum* [39]. Los MWCNTs sintéticos en tomate también

incrementaron la absorción de agua al interior de la semilla y la planta, favoreciendo el porcentaje de germinación y emergencia temprana de la radícula, además de generar incrementos significativos en la biomasa total [22]; en un bioensayo independiente, también en tomate, se reportó que los MWCNTs generaron un incremento en el número de hojas, y se duplicó el número de flores, frutos y semillas en cada fruto [40].

Existe suficiente evidencia sobre los efectos benéficos que los MWCNTs sintéticos causan en las plantas, sin embargo, a pesar de que se han propuesto varios mecanismos fisiológicos y moleculares, aún no es claro cómo dichos nanomateriales participan en el desarrollo vegetal. Hasta ahora, la emergencia temprana de la radícula y los incrementos del porcentaje de germinación de las semillas, se han asociado a la formación de nuevos poros que los MWCNTs causan en la testa de la semillas al penetrarlas, incrementando la captación de agua [35,39]; esta mayor absorción de agua hacia las semillas y hacia las plantas en general, también se asocian a una mayor expresión de genes reguladores de acuaporinas [32]. Durante otros estadios de desarrollo fisiológico de las plantas, se ha reportado que los MWCNTs promueven la elongación celular en el xilema y el floema, lo que facilita el transporte de agua y nutrientes [41,42]. Asimismo, se reportaron incrementos significativos en el contenido de clorofila y mayor actividad fotosintética [42]. Además, se ha reportado una mayor expresión de *CYCB*, marcador clave en la progresión de la mitosis [43].

Los efectos de los MWCNTs en general se ven reflejados en la formación de un sistema radical más extenso y diámetros mayores en el xilema, lo que mejora el desarrollo y biomasa de estas plantas [41,42]. En la última década, además, se reportó que los MWCNTs afectan el metabolismo secundario de las plantas, por ejemplo en tomate, se reportó un aumento en la expresión de genes que participan en la regulación de su metabolismo secundario [44]. Como otro ejemplo, en *S. Khuzestanica* la adición de MWCNTs sintéticos favorecieron la síntesis de compuestos fenólicos, flavonoides, ácido rosmárico y ácido cafeico [45]. En *Hibiscus sabdariffa* L. también se reportó que estas nanopartículas modificaron el balance de metabolitos secundarios [44]. (La figura 3

muestra algunos de los efectos positivos atribuidos a la adición de MWCNTs de origen sintético en el desarrollo vegetal)

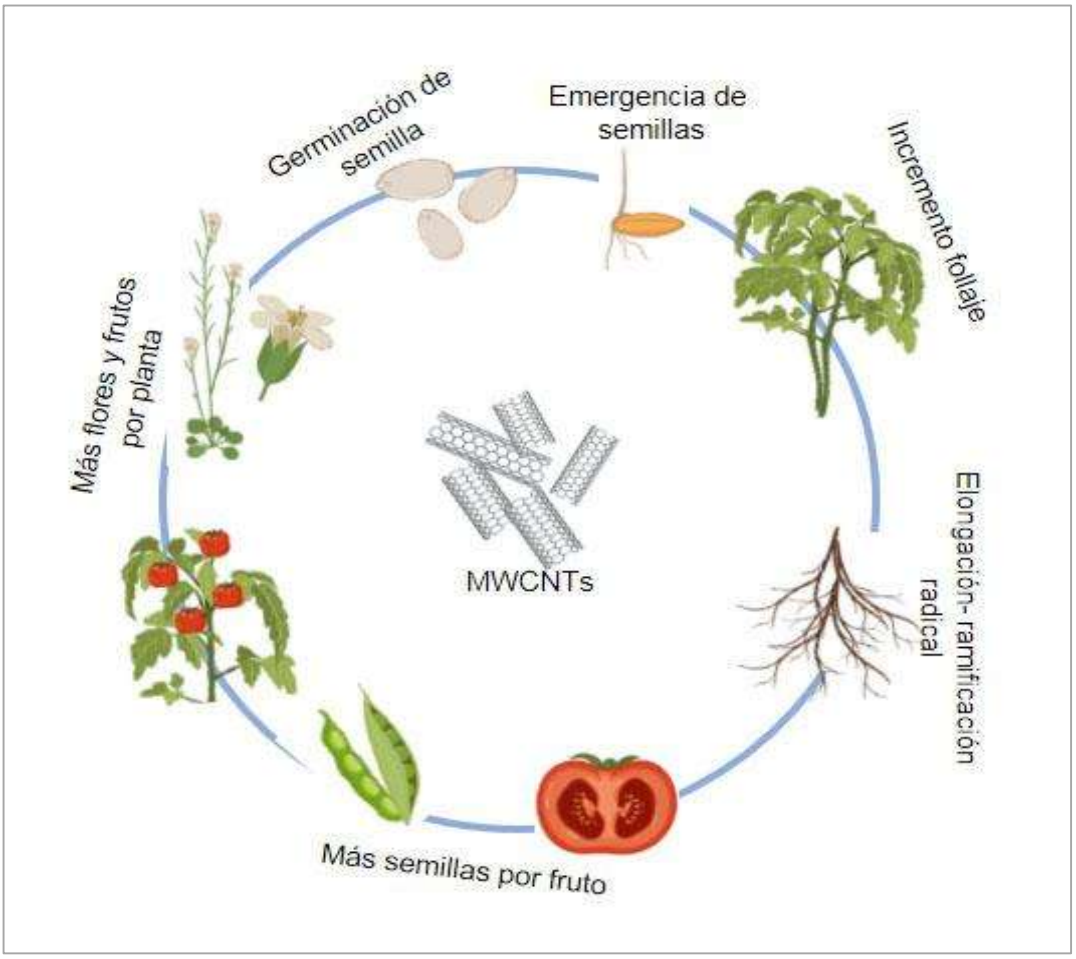


Figura 3. Efectos positivos atribuidos a los MWCNTs de origen sintético en el desarrollo vegetal

- Efecto fitotóxico de los MWCNTs

Si bien se han reportado ampliamente los efectos benéficos de los MWCNTs en las plantas, también existe evidencia suficiente en la cual estas nanopartículas tienen efectos tóxicos en varias especies vegetales [1,29]. Estos efectos contrastantes se han asociado con las propiedades intrínsecas de las nanopartículas, especialmente su forma, dimensiones, conductividad eléctrica, estabilidad y su limitada solubilidad [46], así como a la dosis aplicada de nanopartículas y las características de cada modelo biológico utilizado [47]. Por ejemplo, en plantas como *Cucurbita pepo* (calabacín), la adición de MWCNTs sintéticos afectaron la germinación de las semillas y su biomasa en un sistema hidropónico [48]; en un bioensayo independiente en esta misma planta, también se reportó una reducción en el porcentaje de germinación, en la longitud de la raíz y en los brotes, la acumulación

de biomasa y el vigor de la planta [49]. En *Lactuca sativa* L., los MWCNTs inhibieron la germinación y limitaron el crecimiento y la biomasa vegetal al inducir la muerte celular [50]. De manera similar, el uso de SWCNTs en plantas de tomate y espinacas, afectaron la elongación radical de ambas plantas [21]. Mientras que en especies de interés agronómico como en *Lactuca sativa* [50], *Amaranthus tricolor* L. y *Cucumis sativus* [51] también se han reportado efectos fitotóxicos por estos nanomateriales sintéticos.

Los mecanismos relacionados con la fitotoxicidad de los MWCNTs sintéticos en las plantas no se han dilucidado en detalle; sin embargo, se han reportado un aumento en el estrés oxidativo vegetal generado por una sobreproducción y acumulación de especies reactivas de oxígeno (ROS), daño a la membrana celular [49,52] y la alteración de la actividad de enzimas como la superóxido dismutasa, catalasa y peroxidasa [49] cuando los MWCNTs están en contacto con los organelos en la célula vegetal [53], colateralmente la proliferación celular se ve afectada y causan la muerte celular [54]. Sin embargo, es de gran importancia profundizar en los posibles efectos negativos de los MWCNTs en las plantas, ya que estas nanopartículas se han localizado en frutos y tejidos vegetales de gran importancia alimentaria y comercial [51]. La figura 4, muestra un esquema general de los efectos contrastantes causados por los MWCNTs de origen sintético y natural en el desarrollo vegetal.

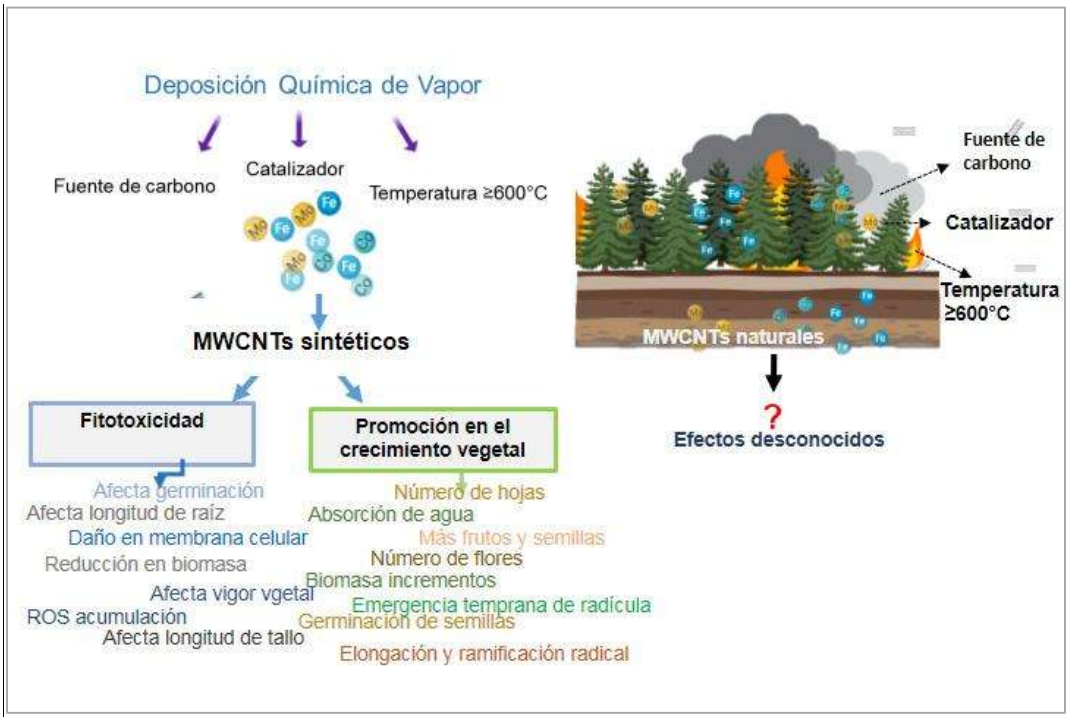


Figura 4. Efectos de los MWCNTs de origen sintético y natural en plantas

Crecimiento vegetal

Las plantas son eucariontes fotoautótrofos sésiles, que durante su crecimiento y desarrollo en condiciones naturales, son afectados positiva o negativamente por factores bióticos como las interacciones microbianas, insectos y nematodos [55], y abióticos, principalmente la temperatura, intensidad de la luz o la disponibilidad de agua, minerales esenciales y la homeostasis de fitorreguladores [56,57].

1.5. La vía de señalización TOR

En las plantas superiores, la vía de señalización de la proteína blanco de la rapamicina TOR (por su nombre en inglés, Target of rapamycin), juega un papel clave durante la progresión del ciclo celular y durante todas las etapas fisiológicas del desarrollo vegetal, al integrar señales que regulan la asimilación de nutrientes, energía, respuesta al estrés biótico o abiótico y fitorreguladores [58].

La proteína TOR es una ser/thr cinasa (approx. 280 kDa) de la familia de la fosfatidil inositol-3-cinasa (PI3K), es una proteína evolutivamente estructural y funcionalmente conservada en eucariontes [59] y constituye el componente central del complejo TOR. Dos genes TOR se han identificado en levaduras y otros organismos, sin embargo, a la fecha en plantas como *A. thaliana* solamente un gen se ha reportado [59], dando origen al complejo multiprotéico TORC1 [60]. En plantas, en las reacciones del complejo TORC1 "rio abajo", se han identificado diferentes proteínas blanco, entre ellas la cinasa de la proteína ribosómica S6 (S6K), el factor de transcripción E2Fa y TAP46, una subunidad reguladora de la proteína fosfatasa tipo 2A (PP2A), que participa en regular el crecimiento de las plantas y la autofagia [61]. Este complejo multiprotéico está constituido por las proteínas TOR, Regulatory Associated Protein of TOR (RAPTOR) y Lethal with SEC13 protein 8 (LST8) [62], donde RAPTOR juega un papel clave en la regulación de la S6K ya que se une directamente con ella, por lo que actúa como un andamio para facilitar la unión de otras proteínas a TOR. S6K puede recibir la señal directa de TOR [63], mientras que la LST8 estabiliza el complejo [61,64]. TORC1 en plantas está regulado por la disponibilidad de nutrientes, la cantidad de luz, la captación de CO_2, la concentración de fotosintatos como la glucosa y sacarosa, o la presencia de NO_3^-. De acuerdo con Shi et al., (2018), TORC1 en plantas regula la proliferación y elongación celular, la embriogénesis, el desarrollo de cloroplastos, la senescencia, la floración, la proliferación celular en meristemos y la formación de pelos radicales (Figuras 5 y 6). Asimismo, participa en la regulación de la embriogénesis ribosomal, la reprogramación transcripcional de genes que regulan

el metabolismo central y secundario, en la respuesta vegetal al estrés [65] y en la defensa contra patógenos [60].

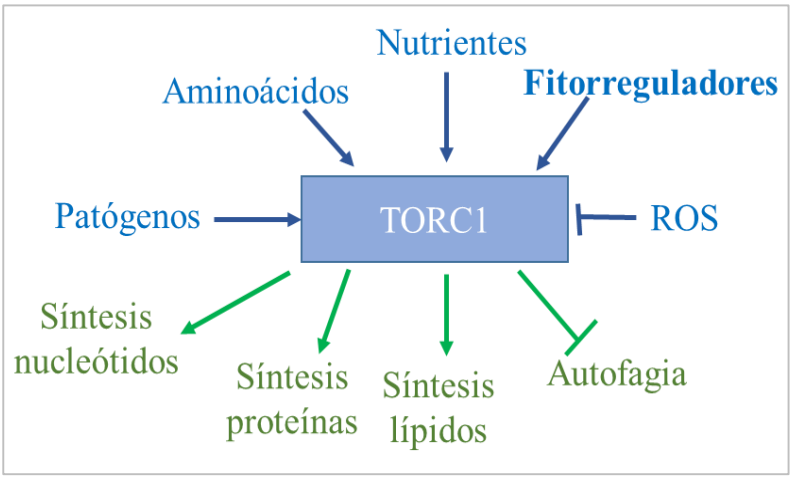


Figura 5. Factores que regulan la vía de señalización TOR y su efecto en procesos bioquímicos durante el desarrollo vegetal.

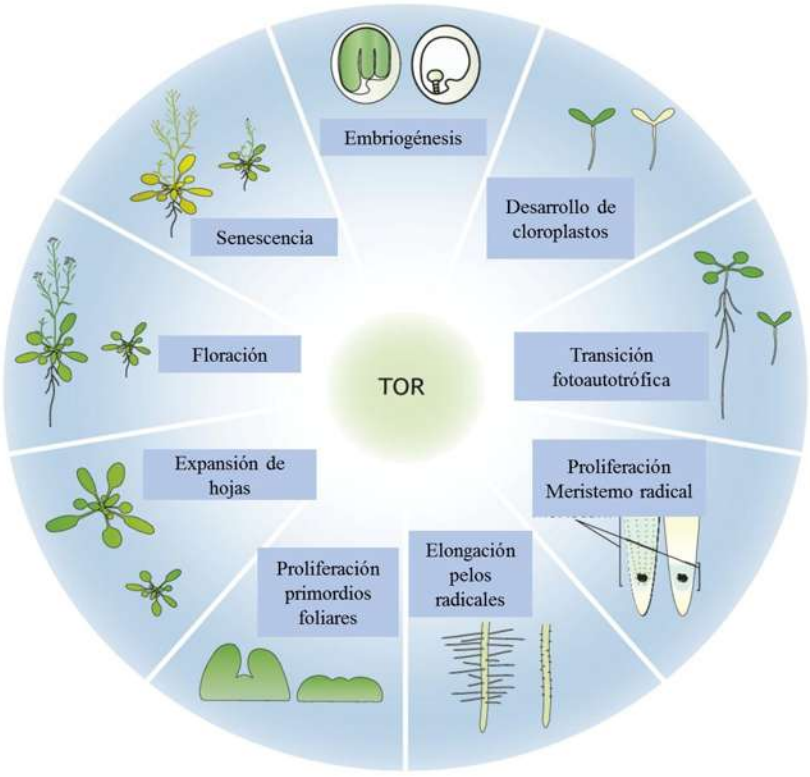


Figura 6. Rol de la vía de señalización TOR en el desarrollo vegetal [66]

- La Proteína S6k

El complejo TOR regula el crecimiento de las plantas a través de la fosforilación de proteínas ubicadas rio abajo, como la cinasa de la proteína ribosomal S6 (S6K), de la familia de ser/thr de 70 kDa [67]. Presenta las isoforma citosólica p70 S6K1 y nuclear p85 S6K1 [68] y su actividad es modulada en respuesta a factores de crecimiento, homeostasis de citocininas o nutrientes [69]; está involucrada en regular el tamaño celular y homeostasis de la glucosa. Se ha reportado que la sobreexpresión de S6K genera aumentos en el tamaño celular [70] y estimula la síntesis de proteínas [71]. Esta cinasa a su vez fosforila eIF3h para mantener la traducción a través de uORF en polisomas [72], por lo que se deduce que esta vía TOR representa un punto de convergencia de las vías de nutrientes, energía y auxinas para regular el crecimiento [73]. Las auxinas son un regulador positivo de la división celular que activa y promueve la carga de TOR en los polisomas, TOR activo fosforila S6K1, e inicia una cascada de señalización que facilita la reiniciación de la traducción [72].

- La vía de señalización de TOR y su relación con los fitorreguladores.

El complejo TOR también es dependiente de la homeostasis de fitorreguladores como las auxinas y las citocininas [60,67] principalmente, fitorreguladores clave en la progresión del ciclo celular, la actividad meristemática, la organización de la arquitectura de la raíz y el dominio apical, así como la organogénesis vegetal [74–76]. Se ha documentado que las auxinas estimulan la actividad de TOR e inducen la reiniciación de la traducción de ARNm a través de S6K1, mientras que la deficiencia en la señalización de TOR deteriorada el gravitropismo de raíz mediado por auxinas [72]. Además, Yunting *et al*., (2017) sugieren que las auxinas interactúan con moléculas de respuesta al estrés en las plantas a través de la regulación de la actividad de TOR [61]. Recientemente, se ha reportado que TOR desempeña un papel antagónico con fitorreguladores como el ácido jasmónico y el ácido salicílico [65,77,78].

El siguiente esquema muestra un modelo propuesto de la vía de señalización de TOR y la contribución de los MWCNTs como mecanismo de regulación del desarrollo en las plantas (figura 6).

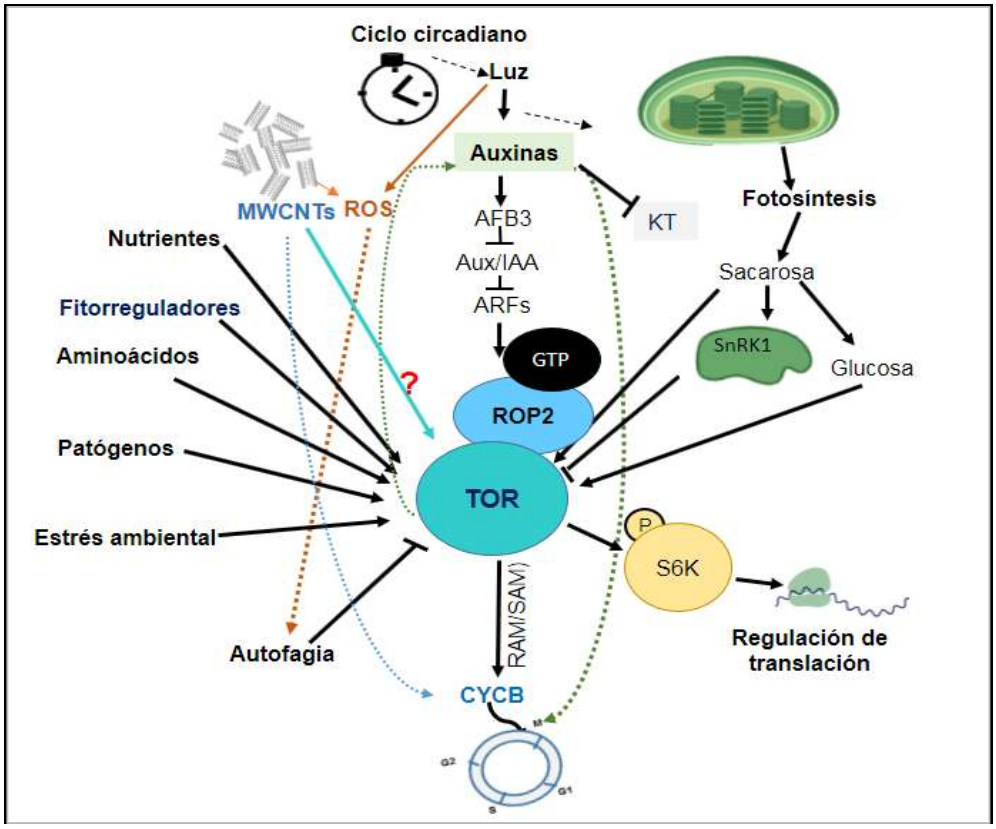


Figura 7. Esquema propuesto sobre la vía de señalización de TOR y su interacción con MWCNTs

*Root and shot apical meristem RAM/SAM

1.6. Fitorreguladores

Son metabolitos de origen sintético o endógeno que controlan el desarrollo vegetal, dependiente de procesos fisiológicos, morfológicos y/o de desarrollo y defensa vegetal [79]. El balance de estos fitorreguladores coordina el crecimiento y organogénesis vegetal, desde la embriogénesis hasta la senescencia al controlar la actividad meristemática mediante la división celular, tanto en los ápices de los brotes como en las raíces [80–82]. Los principales fitorreguladores hasta ahora reportados son las auxinas, las citocininas, las giberelinas, el ácido abcíscico, los brasinoesteroides, el etileno, el jasmonato y las estrigolactonas [80].

- Auxinas

La vía de señalización de auxinas contribuye a la regulación del crecimiento y desarrollo durante todo su ciclo biológico de las plantas, participa en la formación embrionaria del eje apical-basal, el desarrollo vascular, la organogénesis postembrionaria, el desarrollo del fruto, la respuesta al estrés,

la senescencia y el dominio apical [83–85]. Las auxinas, son un grupo de moléculas estructuralmente heterogéneas entre sí [86], incluyen compuestos naturales como el ácido indol-3-acético (AIA), ácido-indol-3-butírico (IBA, por sus siglas en inglés), 4-cloro-indol-acético (4-Cl-IAA) [87], ácido fenilacético o sintéticos como ácido-1-naftalenacético y ácido-2,4-diclorofenóxiacetico [88]. El AIA, fue la primera auxina reportada (1930s), esta a la fecha es considerada la auxina endógena más activa en las plantas superiores [89] y juega un papel central en la progresión del desarrollo vegetal y a respuestas ambientales, como el gravitropismo, el fototropismo y el desarrollo de la plasticidad radical[90]. La síntesis de AIA, se origina principalmente en hojas jóvenes, sin embargo, se ha documentado que en plántulas de *Arabidopsis* este fitorregulador se sintetiza prácticamente en todos los tejidos. La síntesis de auxinas puede ser por vías dependiente o independiente del triptófano, uno de los precursores más importantes [91]. La síntesis y actividad auxínica es dependiente de un gradiente de concentración en los tejidos vegetales, de su transporte y de su conjugación con aminoácidos, péptidos o azúcares y su catabolismo [92].

El IBA, es otra de las auxinas endógenas en plantas, es considerado una forma más estable de almacenamiento y un precursor del AIA, en plántulas de *Arabidopsis* se ha reportado que este fitorregulador constituye hasta 25-30% del total de las auxinas presentes [93]. En maíz, la síntesis de IBA fue detectado en raíces, en el coleóptilo y hojas [94]. El IBA, participa en procesos de iniciación de la raíz, la curvatura del tallo, la epinastia de la hoja, en el alargamiento de los pelos radiculares, en la expansión de las células de cotiledón, en la regulación del tamaño del meristemo apical radical, en el desarrollo de raíces laterales y la formación de raíces adventicias [92,95,96].

- Citocininas

Las citocininas, "se definen como sustancias que inducen la citocinesis en presencia de auxina"[97]. Son fitorreguladores derivados de la adenina, sustituidos en su posición N6 con una cadena lateral isoprenoide o aromática [98]. Estructuralmente en plantas superiores, las principales citocininas isoprenoides endógenas son la N6-(Δ2-isopentenil)- adenina, zinetina, zeatina (más abundante citocinina en plantas) y la dihidrozeatina, esta última puede orientarse en una configuración trans o cis que representa trans-zeatina o cis-zeatina, respectivamente [99]. Las citocininas, en interacción con otros fitorreguladores intervienen durante todo el desarrollo vegetal, por ejemplo, controlan la división y el ciclo celular, la organogénesis, latencia y la germinación de las semillas, la

senescencia, la liberación de brotes del dominio apical, la estimulación de la expansión de las hojas, la promoción de la germinación de semillas y en la formación de cloroplastos [100–102]. Se considera que regulan positivamente el meristemo apical en tallos a través de la estimulación de la división celular y negativamente el meristemo apical de la raíz a través de la promoción de la diferenciación celular [101]. Los niveles de citocininas activas se modulan mediante la conjugación con azúcares como la glucosa, o mediante la degradación irreversible por las citocininas oxidasas/deshidrogenasa. Estas se sintetizan en diversos tipos de células tanto en raíces como en los brotes en el tallo [100,103]. Su transporte es desde la raíz a los brotes a través del xilema, y de los brotes a la raíz a través del floema [97,104]. De acuerdo a la literatura, las auxinas y citocininas juegan un papel antagónico entre sí durante el desarrollo vegetal, y el balance de estas fitohormonas es crítico para asegurar el crecimiento y desarrollo vegetal en general, al regular el tamaño y actividad meristemática, la elongación y diferenciación celular, la arquitectura de la raíz y la dominancia apical, así como la organogénesis vegetal [74–76].

- Giberelinas (GAs)

Son ácidos carboxílicos diterpenoides tetracíclicos que en interacción con fitorreguladores como las auxinas [105], el ácido abscísico (ABA) [106], el etileno[107], los epibrazinoides [108] o el jasmonato [109,110] regulan procesos fisiológicos importantes del desarrollo vegetal, como la dormancia y germinación de las semillas, el alargamiento de brotes y la transición floral [111], la expansión de las hojas, en la maduración y crecimiento de los frutos [107]. Asimismo, estas regulan la fertilidad vegetal, al permitir el alargamiento y desarrollo del estambre, la liberación, germinación y el crecimiento del tubo de polen [112].

A la fecha, se han reportado más de 130 tipos de giberelinas en plantas, hongos y bacterias [113], sin embargo, pocas son biológicamente activas en plantas superiores [111], por ejemplo GA1, GA3, GA4 y GA7, de estas, GA1 y GA4 tienen una abundancia relativamente alta, mientras que GA3 y GA7 son menos abundantes en diversas especies vegetales [114]. Entre las giberelinas, la GA3 o ácido giberélico, es la más conocida, es una fitohormona endógena en plantas superiores, aunque para su uso comercial, esta giberelina se obtiene del hongo *Fusarium fujikuroi*. Los niveles más altos de giberelinas se han detectado en órganos en crecimiento, por ejemplo, en hojas y entrenudos en expansión, yemas apicales y zarcillos [115]. En semillas en desarrollo se han detectado niveles muy altos y heterogéneos de giberelinas, la mayoría de estructuras de esta fitohormona se

identificaron a partir de semillas inmaduras, mientras que en semillas cerca de la madurez se detectaron altos niveles de GAs con actividad inactivadora, asegurando una regulación correcta del proceso de germinación [112].

- Ácido jasmónico (JA)

Citando las palabras de John Browse: “Para muchas personas, el aroma de las flores de jazmín es un seductor perfume exótico, para las plantas, es un saxofón estridente que hace sonar una advertencia de un enemigo en puerta, o la más melodiosa orquesta durante la madurez sexual de las plantas”. El metil jasmonato, es el componente principal del aroma de jazmín, este fitorregulador ayuda a regular diversos procesos fisiológicos de las plantas, en respuesta al estrés y su desarrollo [116]. Pertenece químicamente a las oxilipinas, su síntesis se deriva de la peroxidación del ácido linolénico y otros ácidos grasos poliinsaturados de la membrana celular [116,117]. El metil jasmonato así como el ácido jasmónico, participa en procesos clave del desarrollo vegetal: la senescencia, la maduración de frutos, el desarrollo de polen viable y el crecimiento radical [118]. Además, juega un papel clave durante la respuesta al estrés biótico (causado por insectos y microorganismos patógenos), y abiótico como daños mecánicos, déficit hídrico y la radiación UV [116]. El nivel de ácido jasmónico en las plantas es dependientes del tipo de tejido vegetal, su etapa fisiológica de desarrollo y estímulos ambientales. Los niveles más altos de este fitorregulador se han detectado en el hipocótilo, en la zona de división celular y plántulas más jóvenes; también en flores y tejidos de pericarpio de estructuras reproductivas en desarrollo [118], este fitorregulador es clave en la señalización para atracción de polinizadores, la síntesis de proteínas de defensa y otras sustancias químicas que protegen a los tejidos reproductivos contra el ataque de insectos y patógenos [116]. El ácido jasmónico se acumula en zonas con heridas causadas por patógenos o daño mecánico [116], también en células con cloroplastos, donde se puede almacenar a medida que se sintetiza, este es catabolizado para formar al metil jasmonato, sus conjugados y catabolitos con actividad biológica [119].

- Ácido abscísico (ABA)

Históricamente, este fitorregulador ha sido considerado un inhibidor del crecimiento vegetal [120], es un sesquiterpeno ($C_{15}H_{20}O_4$)[121] con un C-1′ quiral activo [121], en interacción con otros fitorreguladores el ABA desempeña un rol clave durante la formación de la semilla (almacén de

almidón de reserva, prevención de germinación en etapas tempranas, tolerancia a la desecación) e inducción de la dormancia [122]; en tallo retarda su desarrollo pero promueve positivamente el desarrollo de hojas y raíces [123,124], causa la represión de la actividad del meristemo del brote [125], además regula el tamaño y cierre de los estomas [124]. Asimismo, participa en la iniciación de raíces laterales [126], y en la maduración embrionaria, la inducción floral y es uno de los fitorreguladores más importantes en respuesta al estrés hídrico, la salinidad, el frío, la redición UV o por fitopatógenos [123]. En *Arabidopsis*, la acumulación de ABA se ha detectado principalmente en la endodermis y centro quiescente de la raíz [120,127].

- Ácido salicílico (SA)

El ácido 2-hydroxybenzoico o ácido salicílico, es un compuesto fenólico (un anillo aromático unido a un grupo funcional hidroxilo) presente naturalmente en las plantas, y ha sido de gran importancia farmacológica durante miles de años para la salud humana [128]. El uso del SA se remonta a mucho antes de que los salicilatos fueran químicamente identificados [129,130]. La primera evidencia documentada hasta ahora sobre el uso del SA con fines analgésicos fue de un análisis de restos de corteza de álamo obtenidos de la placa dental de fósiles de Neandertales de la cueva El Sidrón, lo que sugirió que estos individuos masticaban esta planta para aliviar el dolor causado por los abscesos dentales [131]. En cultivos vegetales, el SA es un fitorregulador endógeno que juega un papel clave en respuesta a la resistencia contra patógenos biotróficos y hemibiotróficos [132] , y contra necrotróficos en interacción con el JA y etileno [117,133]. Asimismo, este mitiga el estrés abiótico como el causado por presencia de metales como el cadmio [134], participa en la termorregulación [129] y contrarresta el efecto negativo de altas concentraciones de NaCl [135]. Además, participa en procesos fisiológicos importantes, por ejemplo, la germinación de semillas [135,136], la regulación de la expresión génica durante la senescencia [137,138], la inducción de la floración [139], el crecimiento celular, la apertura de los estomas, la respiración [140], y en leguminosas interviene en el proceso de nodulación [128].

.

2. JUSTIFICACIÓN

A la fecha, los MWCNTs han sido considerados nanopartículas sintéticas que afectan positiva o negativamente el desarrollo vegetal. Un estudio reciente demostró que estas nanopartículas también se producen en la naturaleza durante los incendios forestales. Dichos hallazgos, generaron interrogantes sobre el impacto eco-fisiológico que los MWCNTs naturales desempeñan en estos ambientes y en sus poblaciones autóctonas; sin embargo, se desconoce el impacto de estos nanomateriales en los sistemas biológicos subyacente a los efectos de los MWCNTs naturales y sintéticos en las plantas. Se han propuestos diversos mecanismos fisiológicos y moleculares para explicar los efectos de estos nanomateriales sobre el crecimiento vegetal, sin embargo, no se ha explorado los efectos de estas nanopartículas en la vía de señalización a nivel molecular, ni se han contrastado los efectos de estos sobre vías celulares de señalización involucradas en crecimiento vegetal como lo es la vía TOR. A pesar de que TOR es una vía que desempeña un papel crítico durante la progresión del ciclo celular y durante el desarrollo vegetal al integrar señales que regulan la asimilación de nutrientes, energía, respuesta al estrés y homeostasis de fitorreguladores. Por lo que en este trabajo nos planteamos comparar los efectos de los MWCNTs naturales y sintéticos en la promoción del crecimiento vegetal y su relación con la vía de señalización TOR y la acumulación de fitorreguladores utilizando como modelo de estudio las plantas de *E. polystachya* y *A. thaliana*.

3. HIPÓTESIS

Los MWCNTs naturales y sintéticos promueven el crecimiento y desarrollo vegetal a través de la vía de señalización dependiente de la proteína-cinasa TOR.

4. OBJETIVOS

4.1. OBJETIVO GENERAL

Evaluar el efecto de los MWCNTs naturales y sintéticos en el desarrollo y crecimiento de *Eysenhardtia polystachya* y *Arabidopsis thaliana*, así como analizar la participación de la vía de señalización de la proteína-cinasa TOR.

4.2. OBJETIVOS PARTICULARES

- Caracterizar la estructura de los MWCNTs sintéticos y los naturales obtenidos de *P. oocarpa* de muestras carbonizadas durante los incendios forestales.
- Evaluar los efectos de los MWCNTs sintéticos y naturales sobre variables morfofisiológicas de *E. polystachya* y *A. thaliana*.
- Evaluar los efectos de MWCNTs sintéticos y naturales sobre la inducción de la vía de señalización del complejo TOR en *A. thaliana* y su efecto en el balance de fitorreguladores.

5. ESTRATEGIA EXPERIMENTAL

Fase I: Preparación y caracterización de materiales

1. Obtención de MWCNTs naturales y carbono amorfo
2. Caracterización de MWCNTs de origen natural y sintético
3. Cuantificación de MWCNTs naturales

Actividades

1. Colecta de muestras de *P. oocarpa* y *P. montezumae* carbonizadas durante un incendio forestal.
2. Análisis comparativo de las muestras de MWCNTs naturales, sintéticos y carbono amorfo mediante espectroscopía Raman, microscopía FTIR, EDS.
3. Cuantificar MWCNTs naturales y descartar presencia de material cristalino mediante TGA.

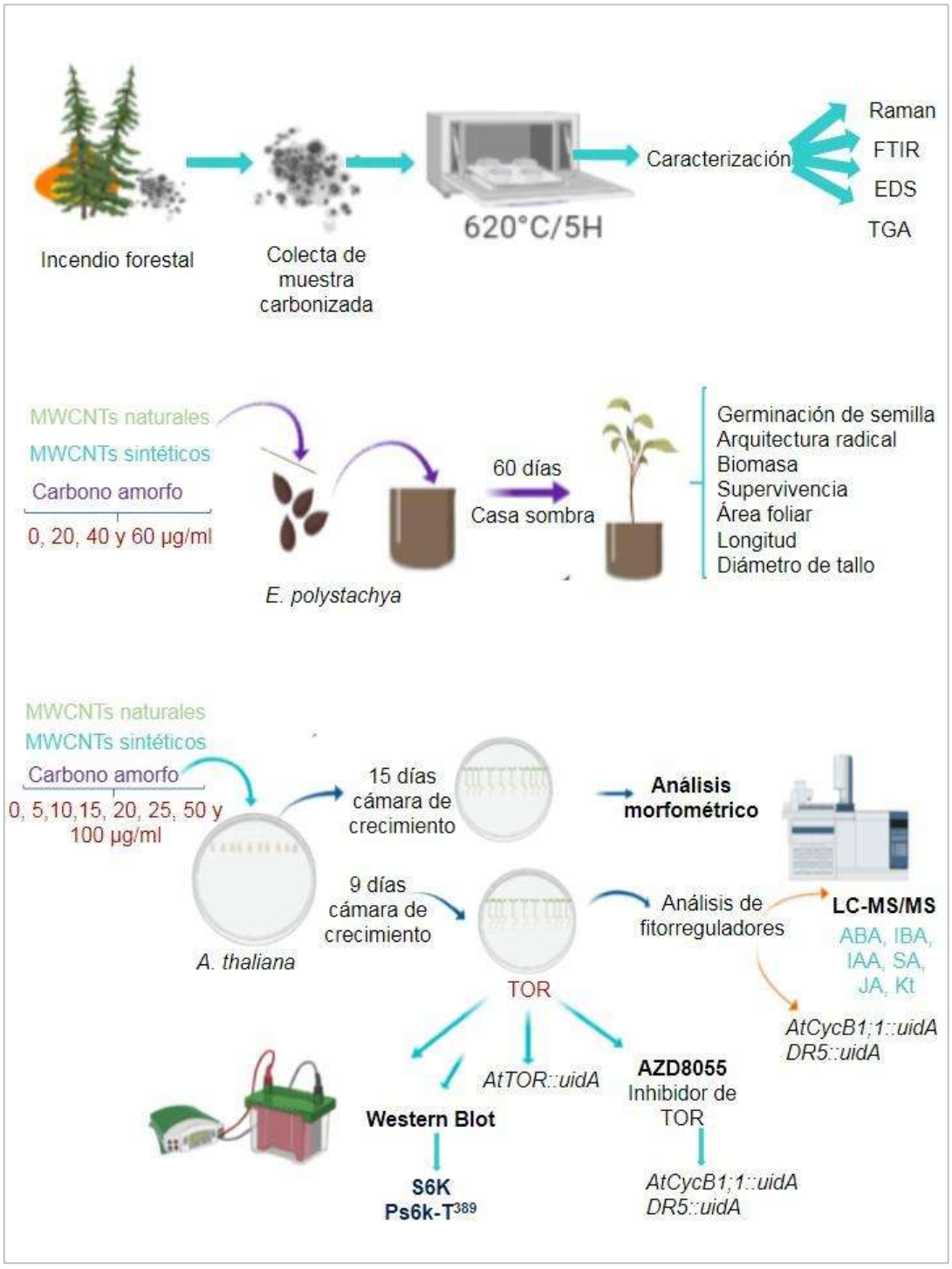
Fase II: Efecto del carbono amorfo, MWCNTs naturales y sintéticos en *E. polystachya*

1. Establecimiento de bioensayo dosis respuesta de *E. Polystachya* con carbono amorfo, MWCNTs naturales o MWCNTs sintéticos en casa sombra.
2. Evaluación de germinación, supervivencia, evaluación de variables morfométricas (follaje- arquitectura radical) y biomasa.

Fase III: Establecimiento de bioensayo dosis respuesta de *A. thaliana* con carbono amorfo, MWCNTs naturales o MWCNTs sintéticos *in vitro*

1. Análisis de los efectos de los MWCNTs en la germinación, variables morfométricas (follaje- arquitectura radical) y biomasa en bioensayos dosis-respuesta.
2. Análisis del efecto de MWCNTs naturales o MWCNTs sintéticos en la vía de señalización TOR.
3. Análisis del efecto de MWCNTs naturales o MWCNTs sintéticos en la abundancia de fitorreguladores.
4. Análisis del efecto de MWCNTs naturales o MWCNTs sintéticos en la interacción de la vía de señalización TOR- fitorreguladores.

6. DIAGRAMA GENERAL



7. RESULTADOS

Los resultados obtenidos durante el desarrollo de este trabajo se presentan en los siguientes capítulos:

Capítulo 1. Multi-walled carbon nanotubes produced after forest fires improve germination and development of *Eysenhardtia polystachya*. *PeerJ* https://doi.org/10.7717/peerj.8634.

En este capítulo, aportamos el primer reporte sobre los efectos de los MWCNTs de origen natural sobre el desarrollo vegetal, los comparamos con los efectos de MWCNTs sintéticos y también con los generados por carbono amorfo obtenido del mismo sitio de colecta que los MWCNTs de origen natural. Se utilizó como modelo de estudio *E. polystachya*, debido a que es una leguminosa arbustiva de interés forestal en México, sometida con frecuencia a disturbios por incendios.

Capítulo 2. Natural multi-walled carbon nanotubes produced after forest fires improved seed germination and plant growth in *Arabidopsis thaliana* through activating the TOR signaling pathway.

En nuestro grupo de investigación recientemente se demostró que los MWCNTs de origen natural pueden ser nanopartículas promotoras del crecimiento vegetal en plantas como *E. polystachya*, aunque se desconocen los mecanismos fisiológicos y moleculares que participan en la promoción del crecimiento vegetal, o si estos mecanismos son similares a los reportados con el uso de MWCNTs de síntesis química. En este capítulo aportamos la primera evidencia de que los efectos positivos de los MWCNTs naturales en el desarrollo de *A. thaliana* involucran como mecanismo de promoción vegetal la estimulación de la vía de señalización de la proteína-cinasa TOR y balance de fitorreguladores.

Capítulo 3. Synthetic multi-walled carbon nanotubes in *Arabidopsis thaliana* affected the plant growth through the repression of the TOR signaling pathway.

Los MWCNTs se han considerado nanopartículas con aplicaciones potenciales en la agricultura al promover el crecimiento vegetal. Sin embargo, también se han documentado sus efectos fitotóxicos, y se busca elucidar los mecanismos fisiológicos o moleculares involucrados. En este capítulo aportamos evidencia de que los efectos negativos de los MWCNTs sintéticos en el desarrollo de *A. thaliana* involucran a la vía de señalización de TOR y el balance de fitorreguladores en esta planta usada como modelo de estudio.

CAPÍTULO 1.

Multi-walled carbon nanotubes produced after forest fires improve germination and development of *Eysenhardtia polystachya*

Multi-walled carbon nanotubes produced after forest fires improve germination and development of *Eysenhardtia polystachya*

Gladys Juárez-Cisneros[1], Mariela Gómez-Romero[2], Homero Reyes de la Cruz[1], Jesús Campos-García[1] and Javier Villegas[1]

[1] Instituto de Investigaciones Químico Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México

[2] Cátedras CONACYT-Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México

ABSTRACT

Background. Multi-walled carbon nanotubes (MWCNTs) are nanoparticles with countless applications. MWCNTs are typically of synthetic origin. However, recently, the formation of MWCNTs in nature after forest fires has been documented. Previous reports have demonstrated the positive effects of synthetic MWCNTs on the germination and development of species of agronomic interest; nevertheless, there is practically no information on how synthetic or natural MWCNTs affect forest plant development. In this report, based on insights from dose-response assays, we elucidate the comparative effects of synthetic MWCNTs, amorphous carbon, and natural MWCNTs obtained after a forest fire on Eysenhardtia polystachya plant.

Methods. E. polystachya seeds were sown in peat moss-agrolite substrate and conserved in a shade house. Germination was recorded daily up to 17 days after sowing, and plant development (manifested in shoot and root length, stem diameter, foliar area, and root architecture parameters) was recorded 60 days after sowing.

Results. The treatments with natural MWCNTs accelerated the emergence and improved the germination of this plant, thus while untreated seeds achieve 100% of germination within 16th day, seeds supplemented with natural MWCNTs at doses of 20 µg/mL achieve the above percentage within the 4th day. Natural MWCNTs also promoted fresh and dry biomass in all applied treatments, especially at doses of 40 µg/mL where natural MWCNTs significantly promoted leaf number, root growth, and the dry and fresh weights of shoots and roots of seedlings. Seeds supplemented with doses between 20 and 40 µg/mL of amorphous carbon achieving 100% of germination within the 6th day; however, seeds supplemented either with doses of 60 µg/mL of the above carbon or with synthetic MWCNTs at all the tested concentrations could achieve at most 80 % and 70% of germination respectively within the 17 days. Finally, neither treatments added with amorphous carbon nor those added with synthetic MWCNTs, showed significant increases in the fresh and dry biomass of the tested plant. Likewise, the survival of seedlings was reduced between 10 and 20 % with 40 and 60 µg/mL of amorphous carbon, and with synthetic MWCNTs in all the doses applied was reduced at 30% of survival plants.

Conclusions. These findings indicate that MWCNTs produced by wildfire act as plant growth promoters, contributing to the germination and development of adapted to fire-prone conditions species such as *E. polystachya*.

Submitted 8 October 2019
Accepted 26 January 2020
Published 23 April 2020

Corresponding author
Javier Villegas, vmoreno@umich.mx

Academic editor
Walter de Azevedo Jr.

Additional Information and Declarations can be found on page 10

DOI 10.7717/peerj.8634

OPEN ACCES

Subjects Biochemistry, Ecology, Plant Science, Soil Science, Forestry

Keywords Natural multi-walled carbon nanotubes, Nanomaterials, Plant growth promotion, amorphous carbon.

INTRODUCTION

Multi-walled carbon nanotubes (MWCNTs) are nanoparticles with unique physico-chemical properties that have recently been the focus of scientific, commercial, and biotechnological interest (De Volder et al., 2013; Zhu et al., 2013). In the last two decades, the applications of MWCNTs in different plant species of agronomic interest have been explored. The results documented so far show that MWCNTs promote plant growth. The capacity of MWCNTs to promote early emergence of seeds and increase the percentage of germination has been demonstrated in corn (Tiwari et al., 2014), soybean, barley, and corn hybrids (Lahiani et al., 2013). It has also been reported that synthetic MWCNTs promote elongation and root branching in *Brassica oleracea*, *Daucus carota*, *Cucumis sativus*, *Allium* sp. (Cañas et al., 2008), and *Cicer arietinum* (Tripathi, Sonkar & Sarkar, 2011). However, the phytotoxic effects of MWCNTs have also been reported in several plant species (Vithanage et al., 2017). For example, in lettuce (*Lactuca sativa* L.) (Ikhtiari et al., 2013), MWCNTs inhibited germination, and limited growth and biomass by inducing cell death. Similarly, in tomato and spinach, single-walled carbon nanotubes (SWCNTs) were shown to inhibit radical elongation (Cañas et al., 2008), while in *Cucurbita pepo* L., exposure to MWCNTs significantly decreased the germination percentage, root and shoot length, and biomass accumulation (Hatami, 2017). Contrasting effects of these nanoparticles have been associated with intrinsic characteristics, such as their shape, dimensions, electrical conductivity, stability, and limited solubility (Scown, Van Aerle & Tyler, 2010), as well as the concentration of nanoparticles and the plant species used as the test model (Jackson et al., 2013). To date, MWCNTs have been considered to be synthetic nanoparticles (Liu et al., 2014), obtained principally by arc-discharge, laser ablation, and chemical vapor deposition methods (Zaytseva & Neumann, 2016). However, Lara-Romero et al. (2017) demonstrated the presence of MWCNTs with ~10 layers of graphene in the calcined wood of resinous pine species after forest fire events. Furthermore, the authors performed a thermogravimetric analysis (TGA) to determinate the amount of MWCNTs in the burned wood. The analysis revealed that the wood samples of Pinus oocarpa contained ~2.8% (w/w) of these nanomaterials. These findings raise questions about the eco-physiological impacts of natural MWCNTs on the plant populations of these ecosystems. There is practically no information about the effects of MWCNTs on indigenous plant populations; nevertheless, these nanoparticles may play a significant role in the growth and development of such plant species.

Eysenhardtia polystachya is a leguminous shrub, characteristic of pine forests in Mexico subjected to fire disturbance. Owing to it is rapid growth and abundant seed production, it is an interesting candidate to test the effects of MWCNTs. The objective of this study was to evaluate and compare the effects of amorphous carbon and MWCNTs of natural and synthetic origin on the germination and morphological seedling variables of *E. polystachya*.

MATERIALS & METHODS

MWCNTs and amorphous carbon specifications

Synthetic MWCNTs used in this study had an outer diameter of 6–13 nm, the internal diameter of 2.0–4.0 nm, length of 2.5–20 µm, an average wall thickness of 7–13 graphene layers, and purity >98% (Aldrich). Natural MWCNTs were obtained from carbonized *P. oocarpa* wood samples collected six weeks after a forest fire in Huashan mountain in Nahuatzen Michoacán, Mexico, as described by Lara-Romero et al. (2017). The samples were first sieved using a 0.2-micron mesh to homogenize the particle size, and then calcined at 620 °C for three hours to mineralize up to 98% of organic matter from amorphous sources (amorphous carbon). Non-crystalline carbon samples from *Pinus montezumae* (rich in amorphous carbon) were also collected from the same site and at the same time, as mentioned previously.

Nanomaterial solutions were prepared by adding natural MWCNTs, synthetic MWCNTs, and amorphous carbon individually to sterile distilled water. For each nanomaterial, solutions with three different concentrations: 20, 40, and 60 µg/mL were prepared. These solutions were sonicated to facilitate the carbon material dispersion, 60 min before the seed treatments. The above concentrations were chosen in the range of 10–60 µg/mL, based on previous studies Lara-Romero et al. (2017), that used synthetic MWCNTs (with structural features similar to those found in the natural samples) to evaluate growth and development *E. polystachya.*

Seed germination and plant growth

Seeds of E. polystachya were collected from Cerro del Punhuato, Michoacán, Mexico. Seeds were disinfected with 10% (v/v) H_2O_2 for 20 min in Brandson 5510 sonicator. Subsequently, each seed was planted in a polypropylene container with peat moss (PREMIER)-agrolite substrate (1:2) that had been previously sterilized (Gómez-Romero et al., 2013). A total of 1.0 mL of the suspension containing the carbon materials at the prepared concentrations were then added to the seeds. The experiments were performed using a completely randomized experimental design. Treatments consisted in: (I) natural MWCNTs, (II) synthetic MWCNTs, and (III) amorphous carbon, each with three different levels (concentration: 20, 40, 60 µg/mL), replicated eight times in polypropylene containers each with one seed. Treatments were compared with the control (concentration: 0 µg/mL of carbon source supplemented) with the same number of seeded polypropylene container replicates.

The seeded containers were then placed in a shade house, and watered three times a week, maintaining field capacity during the experiment.

Treatments were evaluated at 18 different time intervals; germination was recorded daily up to 17 days after sowing, and plant development was recorded at the end of the trial, i.e., 60 days after sowing. To record its development, plants were removed from the containers, and the roots were washed with running water to remove the adhering substrate residues. The percentage of survival was registered, after which the plants were cut from the base of the stem, and shoot and root length, stem diameter, and foliar area were measured.

Variables of root architecture, such as primary root length, lateral roots, tertiary roots, and root volume, were also recorded using the WinRHIZO software coupled to an

EPSON Expression 11000XL scanner (Régent Instruments Inc., Québec, CA). Finally, the shoot and the root were weighed separately, then placed in paper bags and allowed to dry at room temperature before being weighed again to obtain the dry weight.

Statistical analysis

Germination cumulated data, available for 17 days, were analyzed using a generalized linear model (GLM) with a binomial distribution and Cox analysis, to determine the behavior of the germination curves between treatments over time.

Growth data were analyzed using one-way ANOVA, and the means were compared using Tukey's tests with $P < 0.05$, in GraphPad software. The analyses were performed using eight repetitions to balance out the effect of non-germinated seeds.

RESULTS

Seed germination and survival of *E. polystachya*

Natural MWCNTs accelerated the germination of this legume; at the end of the germination test, Cox's proportional hazards test indicated that the germination rates during the test period were significantly different ($X^2 = 17.04$, $P = 0.01$). *E. polystachya* seeds exposed to different carbon sources showed different germination rates. Three days after sowing, 60–90% germination was recorded in seeds treated with natural MWCNTs compared with 40% those kept as control. While six days after sowing, seeds treated with natural MWCNTs had reached 100% germination in all the doses applied, compared with 90% of germination in control, an 80%–100% germination in seeds treated with amorphous carbon and 70–80 with synthetic MWCNTs (Table 1). Furthermore, the control seeds took 16 days to reach 100% germination, and it was evident that synthetic MWCNTs slowed down seed germination, which reached a maximum of 90% in the same period.

E. polystachya plant observed sixty days after sowing (Table 1), showed 100% survival in the control group and groups treated with natural MWCNTs (all doses) or 20 µg/mL of amorphous carbon. In contrast, seeds treated with 40 and 60 µg/mL of amorphous carbon showed 90% and 80% survival, respectively, indicating that an increase in amorphous carbon concentration resulted in a decreased survival percentage. The addition of synthetic MWCNTs also negatively affected *E. polystachya* survival. We obtained 70% survival with all the doses applied of synthetic MWCNTs.

Table 1 **Effect of synthetic MWCNTs, carbon amorphous and natural MWCNTs on Germination and survival of *Eysenhardtia polystachya*.** Seeds of *E. polystachya* were supplemented with 1.0 mL suspension containing either 0 (control), 20, 40, or 60 µg/mL of the different carbon materials. Germination was recorded daily up to 17 days after sowing, and survival was recorded at the end of the trial, 60 days after sowing. The results represent the mean of three independent assays with $n = 8$. The germination was analyzed through a generalized linear model (GLM) for the data, with a binomial distribution and a Cox analysis.

Treatment	Carbon source (µg/seed)	Days after planting								Survival (%)
		3	4	5	6	14	15	16	17	
		% of germination								
Control	0	40	70	80	90	90	90	100	100	100
Natural MWCNTs	20	60	80	90	100	100	100	100	100	100
	40	90	100	100	100	100	100	100	100	100
	60	90	90	100	100	100	100	100	100	100
Amorphous carbon	20	40	60	80	100	100	100	100	100	100
	40	50	70	80	100	100	100	100	100	90
	60	50	70	70	80	80	80	80	80	80
Synthetic MWCNTs	20	20	70	70	70	70	70	70	70	70
	40	50	50	80	80	80	90	90	90	70
	60	60	60	80	80	80	80	80	80	70

Aerial growth of *E. polystachya*

The effects of natural MWCNTs, amorphous carbon, and synthetic MWCNTs at concentrations of 0, 20, 40, and 60 µg/mL on the seeds of *E. polystachya* grown in shade house conditions sixty days after sowing are shown in the Figs. 1 and 2. We observed that treatment with 40 µg/mL of natural MWCNTs significantly promoted leaf formation, when compared with treatment with synthetic MWCNTs and control (Fig. 2A), but no significant difference was observed in other treatments (Tukey test with $P < 0.05$).

Furthermore, treatments containing natural MWCNTs significantly increased the foliar area at all concentrations tested, while amorphous carbon and synthetic MWCNTs did not have any significant effect (Fig. 2B). In addition, no significant differences were observed in the height of E. polystachya plants treated with natural MWCNTs or amorphous carbon and those kept as controls (Fig. 2C) according with Tukey test ($P < 0.05$). However, treatments with synthetic MWCNTs negatively affected plant height at concentrations of 60 µg/m. The aerial dry weight of plants treated with 40 µg/mL of natural MWCNTs was significantly higher, while plants under other treatments did not show any difference with respect to the control (Fig. 2D).

Root architecture of *E. polystachya*

The effects of natural and synthetic MWCNTs and amorphous carbon on root architecture of *E. polystachya* were evaluated 60 days after sowing (Fig. 3). It was observed that the primary root length showed significant increases in treatments with natural MWCNTs, compared to the control plants (Fig. 4A); however, the number of secondary roots did not show significant differences between the treatments containing the tested materials and the control (Fig. 4C). It was evident that treatments with 40 and 60 µg/mL of natural MWCNTs modified the root architecture by promoting the formation of tertiary roots

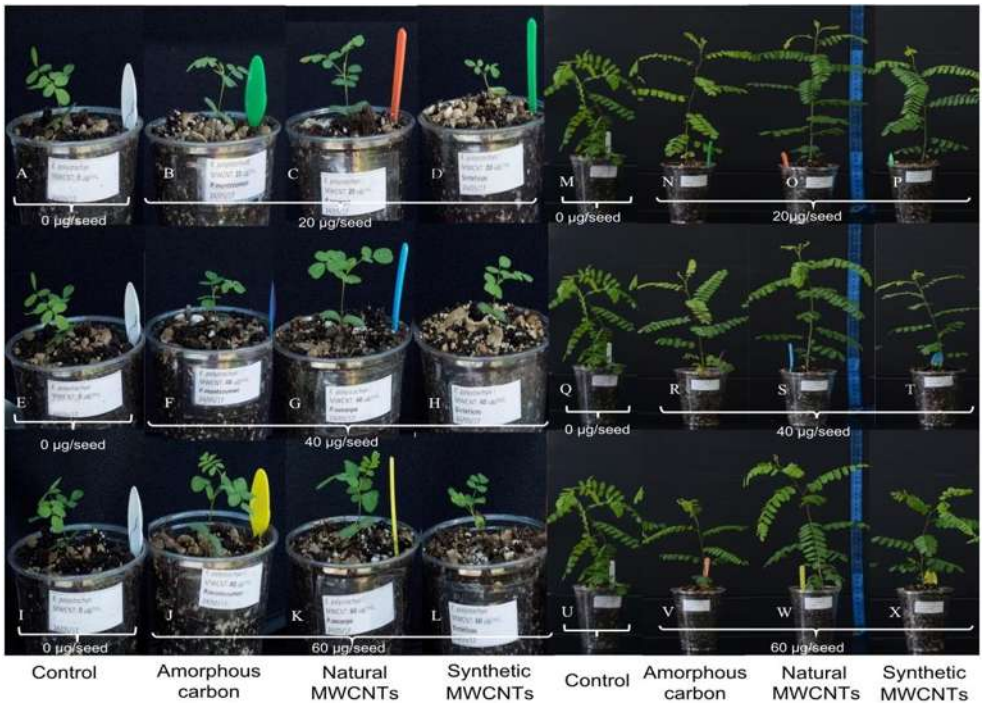


Figure 1 Images showing the effect of synthetic MWCNTs, carbon amorphous and natural MWCNTs on growth of *Eysenhardtia polystachya*. Seeds of E. polystachya were planted in containers with peat moss-agrolite substrate and supplemented with 1.0 mL suspension containing either 0 (control), 20, 40, or r 60 µg/mL of the different carbon materials. A–L and M–X correspond to plants after 20 and 60 days of growth, respectively. DOI: 10.7717/peerj.8634/fig-1

(Fig. 4B), significantly increases in the root volume were observed in plants treated with 40 and 60 µg/mL of natural MWCNTs compared to the control group and treatments containing synthetic MWCNTs or amorphous carbon (Fig. 4D) according to with Tukey test ($P < 0.05$). Furthermore, the fresh and dry root weights of *E. polystachya* seeds treated with natural MWCNTs at concentrations higher than 40 µg/mL were significantly increased (Figs. 4E and 4F) compared to the weights recorded in other treatments. Conversely, the addition of amorphous carbon and synthetic MWCNTs significantly decreased the dry root weight at concentrations above 20 and 40 µg/mL according to with Tukey test ($P < 0.05$).

DISCUSSION

The use of synthetic MWCNTs as plant growth promoters has been reported in several crop plants in the two last decades (Khodakovskaya et al., 2012; Khodakovskaya et al., 2013; Lahiani et al., 2015). The scientific findings report both positive (Joshi et al., 2018a; Joshi et al., 2018b) and negative (Ikhtiari et al., 2013; McGehee et al., 2017) effects of synthetic MWCNTs on plants species. However, to date, the effects of naturally occurring MWCNTs are poorly known. Thus, in the present study, we evaluated the effects of natural and synthetic MWCNTs as well as amorphous carbon on the germination and development of *E. polystachya* plants grown in shade house conditions

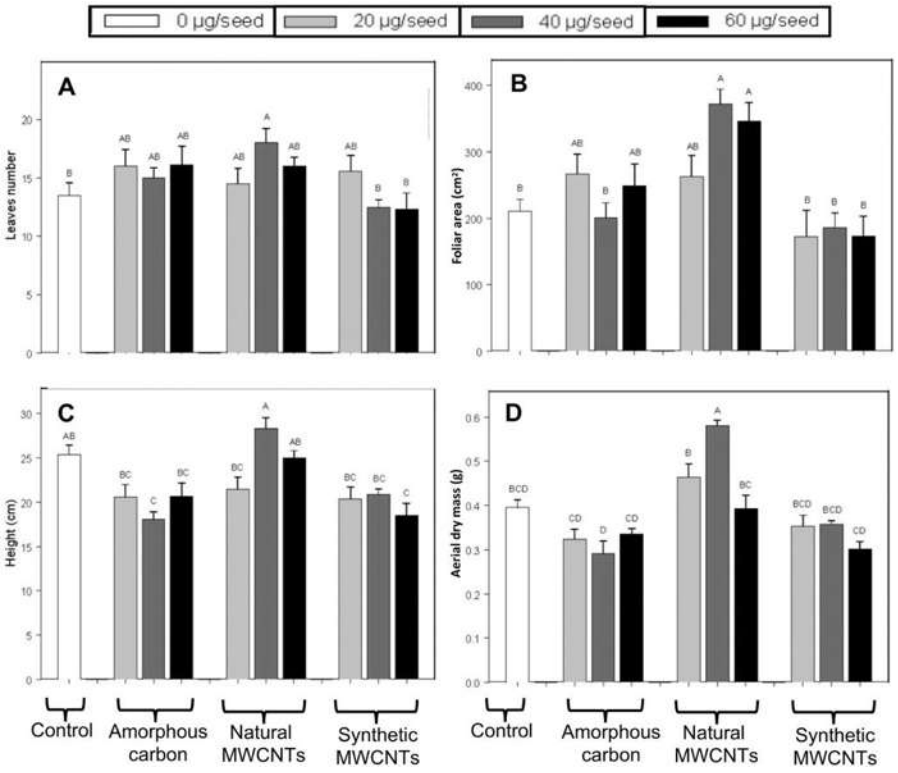


Figure 2 Effect of synthetic MWCNTs, amorphous carbon and natural MWCNTs on aerial biometric parameters of *Eysenhardtia polystachya* plants. Seeds of *E. polystachya* were supplemented with 1.0 mL suspension containing either 0 (control), 20, 40, or 60 µg/mL of the different carbon materials. After 60 days of planting the plants were harvested and biometric variables were recorded. (A) Leaves number, (B) foliar area, (C) height, (D) aerial dry weight. Bars represent mean ± SE of three independent assays. n = 8. One-way analysis of variance (ANOVA) was carried out with Tukey's post hoc test; statistical significance ($P < 0.05$) between treatments with respect to control is indicated with different lowercase letters.
DOI: 10.7717/peerj.8634/fig-2

The responses of this legume to the MWCNTs treatments were contrasting, depending on the origin of the nanomaterial, i.e., MWCNTs of natural origin collected from forest fires events promoted early emergence and increased the germination percentage of the seeds, while synthetic MWCNTs negatively affected seed germination (Table 1). It has been previously reported that the effects of MWCNTs in plants and other organisms depend on their physicochemical properties, such as surface area, length, and diameter, the presence of functional groups, load, shape, and solubility.

In this study, the MWCNTs formed naturally after forest fires lead to better tested plant growth and development than MWCNTs obtained from chemical synthesis. It has been shown that MWCNTs with different characteristics affect seed germination. Early germination induced by synthetic MWCNTs has been reported in tomato seeds, soybean, barley, corn (Lahiani et al., 2013), oat (Joshi et al., 2018b), wheat (Wang et al., 2012; Joshi et al., 2018a), and *Lupinus elegans* (Lara-Romero et al., 2017). Increased seed germination has been associated with increased water uptake during seed imbibition, facilitated by the formation of new pores during penetration of seed coat and cell walls by the MWCNTs.

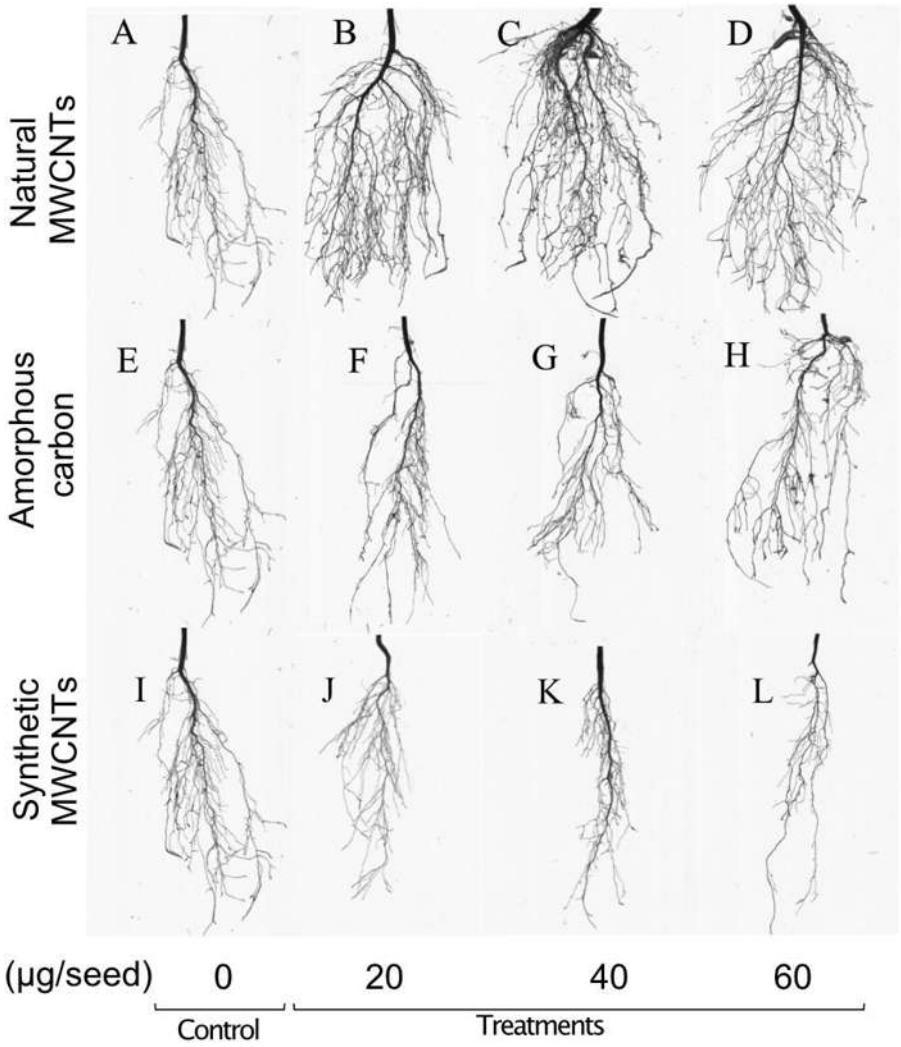


Figure 3 Effect of natural MWCNTs, amorphous carbon and synthetic MWCNTs on root development of *Eysenhardtia polystachya*. The images show root architecture changes in response to different carbon materials in *E. polystachya* roots harvested 60 days after planting. (A–D) Natural MWCNTs. (E–H) Amorphous carbon. (I–L) Synthetic MWCNTs. DOI: 10.7717/peerj.8634/fig-3

however, the action mechanism of these structures on seed germination is not completely clear. In that context, it has been documented that several chemical and physical factors can influence the biochemical and physiological events that control the germination in seeds (Nelson et al., 2012; Asghar et al., 2017).

The effect of MWCNTs has also been documented in other physiological stages of plant development. It has been suggested that a plant response to these nanomaterials depends on their intrinsic chemical characteristics, concentration (Lahiani et al., 2013; Lara-Romero et al., 2017), dispersion method (Joshi et al., 2018a; Joshi et al., 2018b), and also on the plant species (Zhai et al., 2015; Zaytseva, 2016) and the experimental conditions in which it develops (Tiwari et al., 2014).

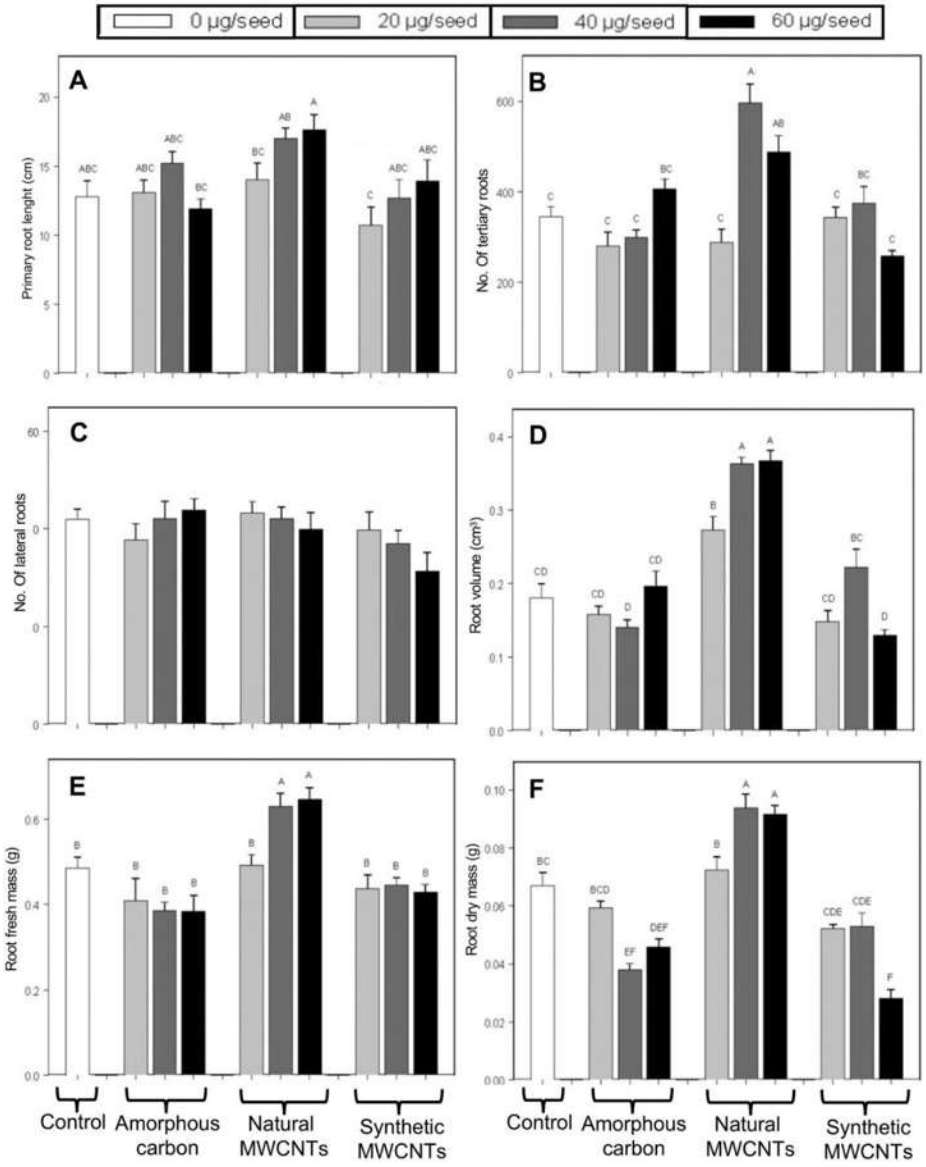


Figure 4 Effect of synthetic MWCNTs, amorphous carbon and natural MWCNTs on root architecture of *Eysenhardtia polystachya* plants. Seeds of *E. polystachya* were supplemented with 1.0 mL suspension containing either 0 (control), 20, 40, or 60 µg/mL of the different carbon materials. After 60 days of planting the plants were harvested and root architecture variables were recorded. (A) Primary root length, (B) lateral roots number, (C) tertiary roots number, (D) root volume, (E) root fresh weight, and (F) root dry weight. Bars represent mean ± SE of three independent assays. n = 8. One-way analysis of variance (ANOVA) was carried out with Tukey's post hoc test; statistical significance ($P < 0.05$) between treatments with respect to control is indicated with different lowercase letters-DOI: 10.7717/peerj.8634/fig-4

Thus, the effects of MWCNTs can be positive, as observed in the *E. polystachya* plants cultivated with 40 µg/mL of natural MWCNTs, where the plants showed greater vegetative area, more abundant foliage, and more aerial area. Our results evidenced that natural MWCNTs modified the root architecture of this legume, as a higher number of

tertiary roots and higher root volume were observed, which is beneficial for its establishment, allowing for greater gaseous exchange and absorption of water and minerals (Lynch, 1995). In addition, plants treated with natural MWCNTs showed a significant increase in dry weights of both shoot and root. Similar effects have been documented for synthetic MWCNTs in oat (Joshi et al., 2018b), wheat (Joshi et al.,2018a), corn (Tiwari et al., 2014; Zhai et al., 2015), and *Lupinus elegans* (Lara-Romero et al., 2017). However, the mechanisms by which MWCNTs promote plant growth and development are not clear. Some reports suggest that MWCNTs activate mechanisms of cell division (Khodakovskaya et al., 2012) and promote elongation of xylem and phloem cells, which consequently influence the uptake of water and nutrients (Joshi et al., 2018a; Joshi et al., 2018b).

It must be noted that toxic effects of synthetic MWCNTs on species of agronomic interest have also been previously reported, such as in *Lactuca sativa* (Ikhtiari et al., 2013), *Amaranthus tricolor* L., and *Cucumis sativus* (Begum, Ikhtiari & Fugetsu, 2014). In this study, we found that synthetic MWCNTs, at the concentrations tested, negatively affected the physiological development of *E. polystachya*, by altering germination, morphometric variables aerial plant parts, and root architecture. The mechanisms associated with MWCNT toxicity have not been elucidated in detail; however, they are associated with cell death in roots and leaves, caused by an increase in the generation of reactive oxygen species (Ikhtiari et al., 2013) and rupture of cell membranes (Begum, Ikhtiari & Fugetsu, 2014).

CONCLUSIONS

In this work, for the first time, we report the effects of natural MWCNTs collected from burned trees after a forest fire. We observed that these MWCNTs improved and accelerated germination in *E. polystachya* seeds and promoted growth, in both aerial and underground parts. We also observed that amorphous carbon did not significantly affect the development of this plant. In contrast, MWCNTs from synthetic origins were observed to negatively affect plant development. These results suggest that natural nanoparticles produced after forest fires may positively affect the growth and development of plants in these ecosystems.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This research was funded by C.I.C./UMSNH grant and a doctoral fellowship grant by CONACYT to Gladys Juárez-Cisneros. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

C.I.C./UMSNH. CONACYT.

Competing Interests

Jesús Campos-García is an Academic Editor for PeerJ.

Author Contributions

Gladys Juárez-Cisneros performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, obtained the suspensions with the different carbon materials, and approved the final draft.

Mariela Gómez-Romero performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, developed the statistical analysis, and approved the final draft.

Homero Reyes de la Cruz analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Jesús Campos-García conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Javier Villegas conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability: The raw measurements are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.8634#supplemental-information.

REFERENCES

Asghar T, Iqbal M, Jamil Y, Zia ul H, Nisar J, Shahid M. 2017. Comparison of He-Ne LASER and sinusoidal non-uniform magnetic field seed pre-sowing treatment effect on Glycine max (Var 90-I) germination, growth and yield. Journal of Photochemistry and Photobiology B 166:212–219 DOI 10.1016/j.jphotobiol.2016.11.018.

Begum P, Ikhtiari R, Fugetsu B. 2014. Potential impact of multi-walled carbon nan- otubes exposure to the seedling stage of selected plant species. Nanomaterials 4:203–221 DOI 10.3390/nano4020203.

Cañas JE, Long M, Nations S, Vadan R, Dai L, Luo M, Ambikapathi R, Lee EH, Olszyk D. 2008. Effects of functionalized and nonfunctionalized single-walled carbon nanotubes on root elongation of select crop species. Environmental Toxicology and Chemistry 27:1922–1931 DOI 10.1897/08-117.1.

De Volder MFL, Tawfick SH, Baughman RH, Hart AJ. 2013. Carbon nanotubes: present and future commercial applications. Science 339:535–539 DOI 10.1126/science.1222453.

Gómez-Romero M, Villegas J, Sáenz-Romero C, Lindig-Cisneros R. 2013. Efecto de la micorrización en el establecimiento de Pinus pseudostrobus en cárcavas. Madera Bosques 19:51–63.

Hatami M. 2017. Toxicity assessment of multi-walled carbon nanotubes on Cucurbita pepo L. under well-watered and water-stressed conditions. Ecotoxicology and Environmental Safety 142:274–283 DOI 10.1016/j.ecoenv.2017.04.018.

Ikhtiari R, Begum P, Watari F, Fugetsu B. 2013. Toxic effect of multiwalled carbon nanotubes on lettuce (Lactuca Sativa). Nano Biomedicine 5:18–24.

Jackson P, Jacobsen NR, Baun A, Birkedal R, Kühnel D, Jensen KA, Vogel U, Wallin H. 2013. Bioaccumulation and ecotoxicity of carbon nanotubes. Chemistry Central Journal 7:1–21 DOI 10.1186/1752-153X-7-154.

Joshi A, Kaur S, Dharamvir K, Nayyar H, Verma G. 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):3148–3160 DOI 10.1002/jsfa.8818.

Joshi A, Kaur S, Singh P, Dharamvir K, Nayyar H, Verma G. 2018b. Tracking multi-walled carbon nanotubes inside oat (Avena sativa L.) plants and assessing their effect on growth, yield, and mammalian (human) cell viability. Applied Nanoscience 8:1399–1414 DOI 10.1007/s13204-018-0801-1.

Khodakovskaya MV, De Silva K, Biris AS, Dervishi E, Villagarcia H. 2012. Carbon nanotubes induce growth enhancement of tobacco cells. ACS Nano 6:2128–2135 DOI 10.1021/nn204643g.

Khodakovskaya MV, Kim B-S, Kim JN, Alimohammadi M, Dervishi E, Mustafa T, Cernigla CE. 2013. Carbon nanotubes as plant growth regulators: effects on tomato growth, reproductive system, and soil microbial community. Small 9:115–123 DOI 10.1002/smll.201201225.

Lahiani MH, Chen J, Irin F, Puretzky AA, Green MJ, Khodakovskaya MV. 2015. Interaction of carbon nanohorns with plants: uptake and biological effects. Carbon 81:607–619 DOI 10.1016/j.carbon.2014.09.095.

Lahiani MH, Dervishi E, Chen J, Nima Z, Gaume A, Biris AS, Khodakovskaya MV. 2013. Impact of carbon nanotube exposure to seeds of valuable crops. ACS Applied Materials and Interfaces 5:7965–7973 DOI 10.1021/am402052x.

Lara-Romero J, Campos-García J, Dasgupta-Schubert N, Borjas-García S, Tiwari DK, Paraguay-Delgado F, Jiménez-Sandoval S, Alonso-Nuñez G, Gómez-Romero M, Lindig-Cisneros R, De la Cruz HR, Villegas JA. 2017. Biological effects of carbon nanotubes generated in forest wildfire ecosystems rich in resinous trees on native plants. PeerJ 5:e3658 DOI 10.7717/peerj.3658.

Liu W-W, Chai S-P, Mohamed AR, Hashim U. 2014. Synthesis and characteriza- tion of graphene and carbon nanotubes: a review on the past and recent de- velopments. Journal of Industrial and Engineering Chemistry 20:1171–1185
DOI 10.1016/j.jiec.2013.08.028.

Lynch J. 1995. Root architecture and plant productivity. Plant Physiology 109:7–13. DOI 10.1104/pp.109.1.7.

McGehee DL, Lahiani MH, Irin F, Green MJ, Khodakovskaya MV. 2017. Mul- tiwalled carbon nanotubes dramatically affect the fruit metabolome of ex- posed tomato plants. ACS Applied Materials and Interfaces 9:32430–32435 DOI 10.1021/acsami.7b10511.

Nelson DC, Flematti GR, Ghisalberti EL, Dixon KW, Smith SM. 2012. Regulation of seed germination and seedling growth by chemical signals from burning vegetation. Annual Review of Plant Biology 63:107–130 DOI 10.1146/annurev-arplant-042811-105545.

Scown TM, Van Aerle R, Tyler CR. 2010. Review: do engineered nanoparticles pose a significant threat to the aquatic environment. Critical Reviews in Toxicology 40:653–670 DOI 10.3109/10408444.2010.494174.

Tiwari DK, Dasgupta-Schubert N, Villaseñor Cendejas LM, Villegas J, Carreto Montoya L, Borjas García SE. 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 4:577–591 DOI 10.1007/s13204-013-0236-7.

Tripathi S, Sonkar SK, Sarkar S. 2011. Growth stimulation of gram (Cicer ari- etinum) plant by water soluble carbon nanotubes. Nanoscale 3:1176–1181 DOI 10.1039/c0nr00722f.

Vithanage M, Seneviratne M, Ahmad M, Sarkar B, Ok YS. 2017. Contrasting effects of engineered carbon nanotubes on plants: a review. Environmental Geochemistry and Health 39:1421–1439 DOI 10.1007/s10653-017-9957-y.

Wang X, Han H, Liu X, Gu X, Chen K, Lu D. 2012. Multi-walled carbon nanotubes can enhance root elongation of wheat (Triticum aestivum) plants. Journal of Nanoparticle Research 14:841–851 DOI 10.1007/s11051-012-0841-5.

Zaytseva O. 2016. Differential impact of multi-walled carbon nanotubes on germination and seedling development of Glycine Max, Phaseolus Vulgaris. European Chemical Bulletin 5:202–210 DOI 10.17628/ECB.2016.5.202.

Zaytseva O, Neumann G. 2016. Carbon nanomaterials: production, impact on plant development, agricultural and environmental applications. Chemical and Biological Technologies in Agriculture 3:1–26 DOI 10.1186/s40538-016-0070-8.

Zhai G, Gutowski SM, Walters KS, Yan B, Schnoor JL. 2015. Charge, size, and cellular selectivity for multiwall carbon nanotubes by maize and soybean. Environmental Science and Technology 49:7380–7390 DOI 10.1021/acs.est.5b01145.

Zhu M, Nie G, Meng H, Xia T, Nel A, Zhao Y. 2013. Physicochemical properties determine nanomaterial cellular uptake, transport, and fate. Accounts of Chemical Research 46:622–631 DOI 10.1021/ar300031y.

CAPÍTULO 2.

Natural multi-walled carbon nanotubes produced after forest fires improved seed germination and plant growth in *Arabidopsis thaliana* by activating the TOR signaling pathway and increasing the amounts of phytoregulators.

Natural multi-walled carbon nanotubes produced after forest fires improved seed germination and plant growth in *Arabidopsis thaliana* by activating the TOR signaling pathway and increasing the amounts of phytoregulators

Gladys Juárez-Cisneros[1]; Jesús Campos-García[1]*, Dhirendra K. Tiwari[2]; Homero Reyes-De la Cruz[1], and Javier Villegas[1]*.

[1]Instituto de Investigaciones Químico Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México.
[2]CONACYT- El Colegio de Michoacán/Ladipa, La Piedad, Michoacán, México.
*** Correspondence:** Javier Villegas, vilj4455@yahoo.com.mx. Tel. 4432271087
Jesús Campos-García, jcgarcia@umich.mx; Tel 4431892100

Abstract

Background: The positives effects of natural multi-walled carbon nanotubes (MWCNTs) produced after forest fires on plant growth promotion have been documented; however, the physiological mechanisms behind of these nanomaterials are unknown. The activity of the target of rapamycin (TOR) signaling pathway and phytoregulator balance play a key role in plant growth. Hence, we evaluated the effect of natural MWCNTs on germination and the development of *Arabidopsis thaliana* plant model and its involvement in the TOR signaling pathway activation. To evaluate the TOR pathway, the *AtTOR/tor1::uidA* activity and the S6K phosphorylation were determined.

Results: The results showed that the natural MWCNTs in the 15-25 µg/mL concentration range promoted seed germination, aerial growth, and biomass, and produced changes in root architecture in *Arabidopsis* compared to amorphous carbon. The activity of the *AtTOR/tor1::uidA* marker was induced by MWCNTs in a doses -dependent manner, and the effects were coincidental with the increased S6K phosphorylation. The MWCNTs also induced the expression in plant roots of the *CycB1;1::uidA* cell division marker in the 10-25 µg/mL concentration range; by contrast, the addition of the AZD8055 TOR-inhibitor repressed the *CycB1;1::uidA* activity. These results confirmed that natural MWCNTs promote *Arabidopsis* plant growth by inducing cell division through the TOR signaling pathway. Additionally, the natural MWCNTs significantly increased the activity of the auxinic marker *DR5::uidA* in the 10-25 µg/mL concentration range; however, the addition of AZD8055 did not inhibits the *DR5::uidA* activity. Natural MWCNTs did not modify the abundance of abscisic acid (ABA); however, the amount of indoleacetic acid (IAA), indole-3-butyric acid (IBA), gibberellic acid (GA), jasmonic acid (JA), and salicylic acid (SA), were increased in a concentration-dependent manner, but contrarily, the kinetin (KT) content was decreased. These findings indicated that the auxin content was increased by natural MWCNTs in *A. thaliana*, which confirms its involvement in the TOR pathway activation, suggesting that the expression of auxin responsive genes activation by the MWCNTs is TOR-independent.

Conclusions: Consequently, the natural MWCNTs promoted the germination and plant growth of *A. thaliana* through a mechanism that involves the TOR signaling pathway, positively impacting the synthesis of the IAA, IBA, GA, JA, and SA phytoregulators.

Keywords: Forest fire; Natural multi-walled carbon nanotubes; TOR signaling; S6K protein; phytoregulators; plant growth-promotion.

Background

Nanotechnology is an emerging science with promising multidisciplinary interests, and it has revolutionized industrial, technological, and scientific development (1). The use of nanomaterials also converges widely in the study of environmental conditions, renewable energies, nanomedicines used for the development of new drugs, the food industry, and certain farm crops in the development of agriculture (2,3). In the last decade, the concept of nano-agriculture appeared (4,5); thus, the study of nanomaterials has focused on innovating strategies to develop more efficient agricultural inputs like the generation of soil-plant nutritional quality monitoring systems, environmental stress, and pest control. Nanomaterials are used as a carrier in the development of slow-release agricultural inputs (6).

Multi-walled carbon nanotubes (MWCNTs) are nanoparticles made up of layers of graphene superimposed and folded into a cylindrical shape (7), which exhibit their own characteristic physicochemical properties (8,9) that promote plant growth (10,11). MWCNTs have been considered nanoparticles of synthetic origin; however, recently, MWCNTs have also been found to originate in nature (12). The natural origin of MWCNTs raised questions about the impact they may have on plants, and there is currently little of no documented information on the subject. Attention has also been focused on explaining the effects of synthetic MWCNTs on the promotion of plant growth. In several plant species, it has been reported that synthetic MWCNTs promote the early emergence of seeds and improves their germination (13); likewise, it has been reported that these nanoparticles increase the chlorophyll content and make photosynthetic activity more efficient (14). Findings so far have shown that these nanoparticles induce the expression of regulatory genes of aquaporin, generating greater water uptake by the plant (10); a higher expression of key *CYCB1* was also reported in the progression of mitosis (15). In addition, MWCNTs reportedly modified the secondary metabolism in tomato (16) and *Satureja khuzestanica* plants (17). Although the study of the effects of MWCNTs physiological mechanisms on plant development is not clear, it is widely documented that plant development is regulated by the availability of nutrients, energy status, environmental signals, and the interaction of plant regulators (18).

In plants, the target of rapamycin (TOR) protein kinase signaling pathway plays a key role in the integration of nutrient availability, energy status, and stress-related cues with growth and metabolic outputs (19). The TOR pathway is also crucial in the regulation of plant growth and development (20). The TORC1 complex is formed by the TOR protein, a regulatory-associated protein of TOR (RAPTOR), and lethal with SEC13 protein 8

(LST8) (21). The TOR protein is a highly conserved serine/threonine kinase (280 kDa) from the family of phosphatidylinositol-3 kinases, and it is an evolutionary structural and functionally conserved protein in eukaryotes, and constitutes the component TOR complex core. In plants, the TORC1 complex participates in diverse processes, such as the progression of the cell cycle, embryogenesis, transcriptional reprogramming of genes that regulate central and secondary metabolism, growth of stems, leaves and root hairs, plant stress-responses, plant senescence and defense against pathogens (22). The TOR complex also integrates the activity of phytoregulators, such as auxins and cytokinins, which acts through cell cycle regulation, meristematic activity, organization of root architecture, and apical dominance, as well as plant organogenesis. Recently, it has been reported that the TOR pathway antagonizes the action of plant defense of phytoregulators, such as jasmonic and salicylic acids. The auxin-TOR interaction regulates plant growth through the phosphorylation of proteins, such as the S6 ribosomal protein kinase (S6K) of the 70 kDa serine/threonine kinase family (23), which is activated in response to growth factors, cytokinins balance, and nutrients. The TOR also participates in the synthesis of other proteins and improves cell survival (24).

Currently, there is a lack of information regarding the molecular and physiological mechanisms of the plant in response to nanoparticles, such as MWCNTs. In addition, the findings related to the synthesis of natural MWCNTs (12) and the limited knowledge of these nanoparticles and their effect on biological systems raises many pertinent questions. Thus, in this work, we evaluated the effect of natural MWCNTs produced after forest fires on the development of the *A. thaliana* plant model and their involvement in the TOR signaling pathway regulation and phytoregulators balance.

Results

Natural MWCNTs and amorphous carbon characterization

MWCNTs characterization was performed by Raman spectroscopy, where two characteristic bands of the MWCNTs were observed in the *P. oocarpa* sample (Figure 1A). The G band was observed at 1575 cm^{-1}, corresponding to the tangential vibrations of the carbon atoms, which arises from the E2g mode of the graphite plane, showing the presence of sp^2 electron hybridization of the carbon (25). Meanwhile, the D band at 1344 cm^{-1} generated by structural disorders in the graphitized materials, corresponding to the sp^3 bonds of the distortions of the network in the curved graphene sheets and ends of the carbon nanotubes (26), rendering 0.88 of ID/IG ratio in the natural MWCNTs. Any bands mentioned were not detected in the *P. montezumae* sample (amorphous carbon), confirming the absence of crystalline materials, such as MWCNTs (Figure 1A). Additionally, the MWCNTs content in the carbonized samples were quantified with TGA, where weight loss at a temperature of ~150°C was associated with the loss of water in the sample, while amorphous carbon compounds, such as cellulose and lignin, were mineralized at 300-550°C (25). The weight loss detected at 550-735ºC corresponded to the combustion of MWCNTs (12). Therefore, in carbonized samples of *P. oocarpa*, ~10% (w/w) of MWCNTs was determined, while that TGA of amorphous carbon confirmed the

absences of crystalline material (MWCNTs) when the total loss of the sample was observed at ~520°C (Figure 1B).

Effect of natural MWCNTs on *A. thaliana* seed germination

Natural MWCNTs accelerated the germination of *A. thaliana* after Col-0 sowing on MS medium supplemented with this nanomaterial. The seeds exposed to different doses of natural MWCNTs showed different germination rates, specifically, at 12-48 h, the germination was promoted at the 1-25 μg/mL doses compared with the control seed (Figure 2). At 48-72 h, the seed germination percentage was increased in seeds treated with MWCNTs, especially at concentrations of 20 and 25 μg/mL, compared with the seed control, while the maximum seed germination was 80% at 72 h. Interestingly, MWCNTs diminished seed germination at 50-100 μg/mL (Figure 2).

Effect of natural MWCNTs on *A. thaliana* plant growth

Natural MWCNTs in *A. thaliana* improved the leaf area and the root architecture at doses ranging between 5 to 25 μg/mL; by contrast, higher doses, such 50-100 μg/mL, of this nanomaterial negatively affected the leaf area and root architecture, but significant differences were not observed when the amorphous carbon sample was used (Figure 3). MWCNTs significantly improved the total height of these plants when cultivated with 15, 20, and 25 μg/mL of natural MWCNTs (Figure 3B), while the leaf area was higher in the treatments with 20 and 25 μg/mL (Figure 3C). The MWCNTs showed a clear effect on root architecture, where concentrations ranged from 15 to 25 μg/mL also increased the formation of lateral roots (Figure 3D). This result was consistent with the total root volume, where the seedings treated with natural MWCNTs showed a higher volume compared to the control seedings (Figure 3E). The effect of natural MWCNTs was also evaluated in biomass, showing an increase in the fresh weight at 15 μg/mL, reaching a maximum effect at 25 μg/mL (Figure 3F). These results coincided with the increases in dry weight, where MWCNTs-treated *A. thaliana* seedings in the range of 15-25 μg/mL, which showed significant increases compared to seedings controls (Figure 3G. By contrast, natural MWCNTs negatively affected plant growth at doses higher than 50 μg/mL, where plants recorded significant decreases in the leaf area, variables of root architecture, and dry and wet biomass.

Effect of natural MWCNTs on the TOR kinase pathway and auxin signaling

To determine the effect of natural MWCNTs on the *TOR* expression, we first studied the *AtTOR/tor1::uidA* expression level in response to the addition of natural MWCNTs nine days after sowing. When the staining *GUS* in the roots was analyzed, we observed that *A. thaliana* roots treated with natural MWCNTs at concentrations between 15-25 μg/mL exhibited a stain intensity significantly higher than the control and similar to the IAA-treated seedlings (Figure 4). By contrast, doses of MWCNTs below 10 μg/mL did not show a significant difference with respect to the control, but at concentrations higher than 50 μg/mL, *GUS* activity significantly decreased (Figure 4B).

The effects of natural MWCNTs in *Arabidopsis* seedings were also evaluated in the downstream TOR target, the S6K protein kinase, measuring the total S6K protein content and its phosphorylated status (p-p70S6-Thr389), which is widely used as an indicator of TOR pathway activity. Similar levels of total S6K protein was detected in both the controls and seedings treated at different concentrations of natural MWCNTs (Figure 5).

Interestingly, the MWCNTs promoted the activation of the S6K protein, showing a notable increase in its phosphorylated isoform as a function of the MWCNTs concentration, obtaining the highest phosphorylation level at 20 μg/mL of this nanomaterial (Figure 5).

Plant growth is the result of a combinatory effect of cell proliferation (increase in cell number) and cell elongation (increase in cell size), and both processes are regulated by the TOR pathway. To determine whether plant growth and development promotion in the *A. thaliana* seedlings induced by MWCNTs was the result of cell elongation or proliferation and whether these processes could be associated and correlates with the higher S6K protein activity and TOR expression induction, we analyzed the cyclin-B expression on plant roots through the transgenic *CycB1;1::uidA* line, which is indicative of cell proliferation. In addition, transgenic plant growth and cyclin-B expression were analyzed in seedings sowed on MS medium supplemented with the TOR-inhibitor AZD8055. As in wild types of seedings, MWCNTs treatments produced an increased germination and development of the seedlings until 25 μg/mL, and a clear inhibition was observed in concentrations of natural MWCNTs above 50-100 μg/mL (Figure 6). These findings were consistent with the effect on cyclin-B expression in the roots, where an increase on *GUS* activity was observed until 25 μg/mL, which is indicative of an induction of cell proliferation (Figure 7). Interestingly, the addition of natural MWCNTs in the presence of the TOR inhibitor AZD8055 caused a strong inhibition of germination and development of the *A. thaliana* seedlings (Figure 6), and a notable reduction of *GUS* activity was observed for all the concentrations of nanomaterials used, which is indicative of a reduction on cyclin-B expression caused by the AZD8055-inhibitor of the TOR pathway (Figure 7).

To determine whether the effects on *Arabidopsis* plant growth promotion and TOR pathway activation by the natural MWCNTs involves auxin signaling, analysis using the *DR5::uidA* transgenic line, which is widely used for visualization of phytohormone localization, was performed. The results showed that natural MWCNTs significantly increased the *GUS* activity at 25 μg/mL, but it was inhibited at levels above 50 μg/mL (Figure 8). Furthermore, the effect of natural MWCNTs on *GUS* activity at concentrations of 25 μg/mL was comparable to the effect of AIA at 1.0 μM. Interestingly, although the AZD8055 strongly diminished *GUS* staining, the auxinic marker was induced by 20-25 μg/mL of MWCNTs even in the presence of the TOR inhibitor (Figure 8). These results indicated that MWCNTs positively modulates auxin content on roots tips, and that under our experimental conditions, this modulation is independent of the TOR pathway activity.

Effect of natural MWCNTs on the *A. thaliana* phytoregulators balance

Given that the auxin content on root tips was increase by MWCNTs, the phytoregulators balance in *A. thaliana* seedlings exposed to MWCNTs was determined by LC-MS/MS analysis. The results indicated that the natural MWCNTs modified the balance of the phytoregulators in the *Arabidopsis* seedlings. The abscisic acid (ABA) phytoregulator concentration was not modified by the addition of natural MWCNTs (Table 1). Importantly, the amount of the phytoregulators indoleacetic acid (IAA), 3-butyric acid (IBA), gibberellic acid (GA), jasmonic acid (JA), and salicylic acid (SA) increased significantly in the *Arabidopsis* seedlings treated with MWCNTs in a concentration-

dependent manner; by contrast, the kinetin (KT) was decreased by the effect of this nanomaterial (Table 1). These findings confirm that the MWCNTs modified the balance of phytoregulators in *A. thaliana*.

Discussion

The effects of synthetic MWCNTs in the plants have been widely explored, being considered nanomaterials with the potential to improve the plant growth; however, recent natural MWCNTs discovery generates a discussion about the possible beneficial or toxic effects of these nanoparticles on the environment. In this work, we provide evidence regarding the beneficial effects of natural MWCNTs on *A. thaliana* growth and development by exploring the molecular mechanism involved. Results showed that the nature of the carbon structures in carbon samples of *P. oocarpa* trees collected after forest fire events confirmed the crystalline structure of MWCNTs (Figure 1A). Raman spectra analysis confirmed the characteristic bands of MWCNTs structures in the *P. oocarpa* trees carbon samples, but not in the amorphous carbon sample obtained from *P. montezumae* trees (Figure 1B). This finding further confirmed that the MWCNTs nanomaterials were contained in the carbon samples obtained from *P. oocarpa* pine trees after forest fire events, as previously described (12).

The plant growth promotion effects of synthetic MWCNTs has been documented for various plant species (27). However, the effects of natural MWCNTs on biological systems have only recently been explored in *Eysenhardtia polystachya*, a leguminous shrub (28). Our results showed that the promotional effects of natural MWCNTs on *A. thaliana* plant-growth and development were similar to those reported for *E. polystachya* dosed with natural MWCNTs, which promoted germination as well as the aerial and root growth. Our findings showed that the natural MWCNTs induced the germination of *A. thaliana* seeds in a concentration-dependent manner, with the germination percentage increasing at MWCNTs doses of 10 to 25 μg/mL, but were reduced at higher doses. Similar behavior was reported in the development of both plant species in the forest ecosystem *Lupinus elegans* and *E. polystachya* treated with synthetic MWCNTs (12). In this sense, the application of nanotubes improved the germination in tomato (29), oat (14), wheat (30), soybean hybrid, barley hybrid, and corn hybrid (27) seeds.

Different plant-promotion mechanisms have been proposed to explain the positive effects of nanoparticles on seed germination. Lahiani *et al*. (2013) suggested that the absorption and promotion effects of MWCNTs on the seeds depend on its interaction with organic materials, while colloidal and heterogeneous nature permits its smooth flow into the plant system (31). Also, it has been documented that several chemical and physical factors can influence the biochemical and physiological events that control the germination of seeds (32). In this context, the effects of nanomaterials on seed germination are caused when the nanotubes penetrate the thick seed coat and support water uptake inside the seed (29). Furthermore, the effect of MWCNTs on the seed germination may be associated with

induced changes at the genomic level, such as the expression of genes that encode water channels (27).

Positive or negative effects of synthetic MWCNTs have also been reported in other developmental stages of plant growth. In our study, seed germination and plant growth promotion effects of natural MWCNTs on *A. thaliana* (15-25 µg/mL) were observed in aerial and root biomass and leaf area (Figure 2). It has been suggested that synthetic MWCNTs in plants facilitate the transport of water and nutrients through a pore formation mechanism in the plant cells. Thus, plants growing under the effect of these nanoparticles promote the formation of extensive root system and larger diameters in the xylem, improving growth and biomass in plants (14,30). However, negative effects in plants have also been observed, which have been associated with root and leaf cell death caused by oxidative stress generation as well as cell membrane rupture (33). If the plant promotion effect of the natural MWCNTs is associated with differential microelements contained in the carbon samples, elemental analysis was carried out using energy dispersive microwaves (EDS). Our findings did not show differences among the elemental composition of natural MWCNTs or amorphous carbon (Figure 9). Thus, the effect of natural MWCNTs observed on *A. thaliana* may be associated with the crystalline structure rather than by a nutritional effect of microelement content in carbonized samples.

Different physiological and molecular mechanisms have been proposed as a way to explain how MWCNTs and others nanoparticles modulate plant growth and development; however, among the mechanisms that have not been explored is the TOR signaling pathway. The TOR signaling complex is essential in cellular differentiation and plant development. It acts as a "switch" during the progression of the cell cycle; the TOR multiprotein complex modulates plant development, senses and integrates biotic and abiotic factors, such as the availability of nutrients and energy, stress, or the balance of phytoregulators, such as auxins and cytokines (20,23), jasmonic acid, and salicylic acid.

In this study, the results of two assays provided evidence of the contribution of TOR signaling pathway on the promotion effect of natural MWCNTs on *A. thaliana* seedlings. The results of the first assay showed an increase of the *GUS* expression in the *AtTOR/tor1::uidA* reporter plant line in response to the addition of natural MWCNTs; where the expression of the *GUS* reporter was significantly higher at MWCNTs ranges of 15 to 25 µg/mL than the staining recorded in control seedlings, though it was equivalent to the intensity obtained with IAA, indicating an increase in *TOR* gene expression (Figure 4). We also analyzed the effect of natural MWCNTs over a target element downstream of TOR protein, the S6 protein kinase, which is the best characterized target of TORC1 complex as the link between TOR pathway with translational control (34). Also, the S6K is involved in the regulation of cell size and glucose homeostasis in plants (35). Our results showed that natural MWCNTs increased the phosphorylation level of the p-S6K-Thr389 protein, while levels of phosphorylation were also in function on the added doses of these nanoparticles; but the total S6K protein expression was unchanged (Figure 5). These results strongly suggest that the plant-growth promotion effects produced by the natural MWCNTs on *A. thaliana* seedlings were associated with the activation of the TOR/S6K-signaling

pathway. This finding is consistent with the essential role of the TOR/S6K pathway, which has been widely reported as a central regulator in the control of cell proliferation and growth (36).

The relationship among the TOR complex and phytoregulators and their effects in plant growth and development regulation have been well documented. The action of auxin or cytokinin signaling events in plants is linked to the increase of the TOR and S6K phosphorylation trough the ROP2 small GTP-proteins (37,38). We also explored the effects of natural MWCNTs on TOR interactions as a possible mechanism that promotes and regulates plant growth in *A. thaliana*. We initially observed that natural MWCNTs significantly increased the *GUS* activity in the roots of the *CycB1;1::uidA* reporter plant line (Figure 7). The induction of the *CycB1;1::uidA* marker in the *A. thaliana* roots by natural MWCNTs and its inhibition by the addition of AZD8055 suggest that cyclin B1 expression is modulated though MWCNTs by a signaling mechanism dependent on the TOR pathway. This result is in agreement with Hartig *et al*. (2006), whose finding that cyclin genes such as *CYCB1* are partially regulated by TOR (39). In addition, our results also support the findings of Khodakovskaya *et al.* (2012), who found that the synthetic MWCNTs promoted the *CycB* in the tobacco cell (15), and showed the analogue role of natural MWCNTs in the promotion of cyclin expression in *Arabidopsis*, such as synthetic MWCNTs. Interestingly, although AZD8055 strongly decreased the induction of *CycB1;1::uidA*, when natural WMCNTs were added in the presence of the TOR inhibitor, a significative increment of *GUS* activity was observed at 20-25 μg/mL of natural MWCNTs (Figure 7). This result suggests that in addition to the TOR signaling pathway, other mechanisms are involved in cell proliferation induction.

Auxins have the ability to induce growth responses throughout the plant life cycle and is one of the most important growth regulators in plants. The auxin signaling pathway modulates diverse aspects of plant growth and development, such as responses to light and gravity, organ patterning, general root and shoot architecture, and vascular development. Auxin elicits responses—cell division and expansion—depending on the cellular and developmental context in which it is perceived (40). Evidence demonstrating that TOR acts as an essential factor for auxin signal transduction in *Arabidopsis* has been reported (23,41,42). Then, auxins were identified as the cellular candidate for a role as upstream TOR effectors (41). The TOR pathway is activated in response to auxin signaling. Glucose and light signals, as well as exogenously applied auxin, were shown to activate S6K1 in shoot meristems as an indicator of TOR pathway activity (18).

The control of cellular proliferation and differentiation in plants is modulated by auxins; in this sense, the promotion effect of natural MWCNTs was evaluated using the transgenic *A. thaliana* plant responsible to auxins, *DR5::uidA*. Results showed that the natural MWCNTs induced the auxin-dependent marker (*DR5::uidA*) in a dose-dependent manner, and the addition of AZD8055 did not totally cause the inhibition of its GUS activity (Figure 8). These findings indicate that the natural MWCNTs stimulate plant growth through induction of auxin synthesis or mobilization, which is modulated by the TOR signaling pathway; however, an additional mechanism independent of TOR could be also involved. Similar results have been reported by Xiong *et al.* (2013), who demonstrated that auxin is decoupled from TOR signaling activation, and thus auxin signaling activity is not altered

when the TOR is suppressed (43). Moreover, the key role that phytoregulators play throughout different stages of plant development has been widely documented (44). However, the effect of synthetic MWCNTs on signaling of phytoregulator synthesis has yet to be fully explored (45), and the effects of natural MWCNTs have not been studied at all. Our findings showed a clear effect of the natural MWCNTs on the amount of several phytoregulators (Table 1). The use of natural MWCNTs no modified the concentration of abscisic acid (ABA) in total *Arabidopsis* seedlings, but the concentration of the indole indoleacetic acid (IAA), butyric acid (IBA), gibberellic acid (GAs), jasmonic acid (JA), and salicylic acid (SA) increased significantly in concentration in the *Arabidopsis* seedlings treated with MWCNTs in a concentration-dependent manner; though by contrast, the kinetin (KT) was diminished. This result is in agreement with prior reports that auxins-cytokinins play an antagonistic role among them to regulate the meristematic activity and development of the root architecture (36). By contrast, it has been reported that synthetic MWCNTs negatively affected growth *Oryza sativa* L. by decreasing the concentrations of IBA, IPA, ABA, JA, and endogenous brassinosteroids (45).

The balance among phytoregulators modulates cell proliferation and the size of plant meristems, and participates in root initiation, formation, and development of lateral and adventitious roots. The role of auxins on the TOR pathway is still poorly understood, it has been documented that GTPase ROP2 activated by auxins promotes the activation of TOR. Meanwhile, the effect of gibberellins on the TOR pathway is not known, though it is possible that, like auxins, their mechanism of signal transduction depends on the TOR pathway, because this phytoregulator participates in different stages of plant development, such as germination, dormancy, regulation of cell elongation, root growth, leaves, stems and fruits, as well as in the flowering. In addition, recent findings have provided further evidence of the correlation between the signaling pathway of TOR and JA as a strategy to counter plant stress (46). Our findings indicated that the promotion effect on *Arabidopsis* seedling by the natural MWCNTs involves a molecular mechanism that modulates phytoregulators content, suggesting an essential participation of the TOR signaling pathway, among others.

Conclusion

The findings obtained in this work provide evidence about the positive effect of natural MWCNTs generated after forest fires on the plant-growth model of *A. thaliana*. The plant-promotor effect on *Arabidopsis* showed significant differences in germination, root architecture modification, and biomass. This research also provided evidence that the plant-growth promotor effect of the natural MWCNTs involves mechanisms dependent on the TOR signaling pathway by cell proliferation induction and modifying the phytoregulators balance.

Methods

Natural MWCNTs and amorphous carbon obtainment and characterization

P. oocarpa and *Pinus montezumae* samples of pyrogenic carbon were obtained twelve weeks after a fire in the community of Nuevo San Juan Parangaricutiro Michoacan,

Mexico. A burned *P. montezumae* sample was collected in the same place as *P. oocarpa*, and it was used as amorphous carbon material by plant bioassays. *P. oocarpa* and *P. montezumae* carbonized samples were calcined at 620 °C for two hours to mineralize ~98% of the non-crystalline organic matter and analyzed with Raman spectroscopy (Lab Ram HR Evolution, Horiba), at a spectral resolution of 5 cm^{-1} using a 20 mW He-Ne laser at 632.8 nm visual excitation. The spectral range was scanned from 110 to 3690 cm^{-1} with an integration time of 5 s (12). MWCNTs quantification was performed by thermogravimetric analysis (TGA), whereby 10 mg of each sample was analyzed in SDT Q600-TA Instruments with progressive temperature intervals ranging from 10 to 800 °C.

Plant growth conditions

Arabidopsis thaliana Col-0 wild type was used in this study and transgenic lines *CycB1;1:uidA* (Colon-Carmona et al., 1999), *DR5::uidA* (Ulmasov et al., 1997), and *AtTOR/tor1::uidA* (Menand et al., 2002). All plants were grown in a growth chamber at 23ºC/photoperiod (16-h light: 8-h darkness) with a light intensity of 100 µE s^{-1}. Seeds of *A. thaliana* wild-type (WT) or were plated with MS 0.5x (Murashige and Skoog) according to (47), supplemented with 0, 5, 10,12.5, 15, 20, 25, 50 and 100 µg/mL of natural MWCNTs carbonized samples with or without the repressor of TOR activity AZD8055 1.0 mM (LC Laboratories) (18). *Arabidopsis* seeds (10 seeds per plate) were grown on MS plates. The seedlings were grown for a further 9-15-days period by placing the plates in the growth chamber in a completely randomized design. All experiments were replicated at least three times. The seed germination was measured at 0, 6, 12, 24, 48, and 72 hours. Plant growth was analyzed 15 days after sowing. The analyzed morphometric variables included height (cm), leaf area (cm^2), as well as fresh and dry weight (g). Variables of the radical architecture were also measured, including root volume (cm^3) and the number of lateral roots with WinRHIZO-software ® Regent Instruments Inc**.**

Natural carbon nanotubes effect on the TOR signaling pathway

To evaluated the effect of natural MWCNTs on the expression of *TOR1*, *CYCB1,* and *DR5*, we evaluated the *GUS* staining on transgenic plants *AtTOR/tor::uidA, CycB1;1::uidA*, and *DR5::uidA*. The *GUS* staining quantification activity was realized according to the protocol described (48). Representative images were captured with a Leica DM750 optical microscope, and the activity measurement of the *GUS* reporter gene was determined using the ImageJ software (NIH Image). Phenotypic analysis of wild type Col-0 and transgenic *AtTOR/tor1:uidA, CycB1;1::uidA*, and *DR5::uidA* plants were used in this study. All experiments were replicated at least three times.

Western blot assay

Arabidopsis plants cultivated in MS supplemented with natural MWCNTs were used nine days after sowing to obtain protein crude extracts (49), and Bradford reagent (Bio-Rad) was used to quantify total protein concentrations. Cellular lysis was carried out in phosphorylation buffer (PB) 300 µL composed of [Hepes 50 mM pH 7.6, sodium-pyrophosphate 50 mM, sodium ortovanadate 1 mM, sodium molybdate 1 mM, EDTA, EGTA 20 mM, benzamidine 1 mM, NaF 20 mM, PMSF 0.2 mM, ß-glycerophosphate 80 mM, mannitol 200 mM, protease inhibitor cocktails 1 µL/mL (all reagents from Sigma-Aldrich Co.)], and 30 µg of protein was mixed with 10 µL of denaturing buffer (Tris-HCl 0.06M, pH6.8, 5% de glycerol, 4% SDS, 4% β-mercaptoethanol and 0.0025%

bromophenol blue) for 5 min at 95 °C in a boiling water bath. Samples were run in a denaturing 12% polyacrylamide gel electrophoresis (SDS-PAGE) in duplicate. One gel was Coomassie blue stained and the other gel was transferred to polyvinylidene difluoride (PVDF, Millipore) membranes for western blot procedure. The membranes were probed with anti-AtS6K polyclonal antibody (1:1000 dilutions, Agrisera AS12 1855), and Goat-anti-rabbit IgG-HRP conjugated (1:3000, BioRad) was used as the secondary antibody. To detect the phosphorylated form of the AtS6K kinase, the anti-AtS6K phosphorylated-Thr(P)-389 polyclonal antibody (1:1000, Agrisera AS13 2664), and secondary antibody Goat-anti-rabbit IgG-HRP conjugated (1:10000, BioRad) were used. The membranes were washed twice with TBS-T buffer and developed using hydrogen peroxide and Supersignal West Pico Luminol (Pierce, Thermo Fisher Scientific). The images were captured in ChemiDoc™ MP System (Bio-Rad). Assays were conducted three times, and representative images are shown. Band intensities in images were quantified using Image J software (NIH Image).

Effect of natural MWCNTs on phytoregulators

Arabidopsis plants were harvested after nine days of growth. The fresh plant material (3 g) was frozen with liquid N_2. The phytoregulators extraction was obtained with methanol-trifluoroacetic acid (TFA) 100:1, vortexed 30 min at room temperature, and centrifuged at (1300 x g, 5 min at 4 °C). The solvents were evaporated in a speed vac (Centrivap Concentrator, Labconco) at 60 °C; the supernatant was transferred to a tube and adjusted to 1.5 mL with methanol. The samples were placed in 2 mL-Bond Eluent sample prep solutions tubes of QuEChERS Dispersive SPE 2 mL, fruits and vegetables with pigments, AOAC (Agilent Technologies), and shaken for 5 min using a vortex. After that, the sample was centrifuged for 5 min at 4000 rpm. Finally, the samples were placed in vials and frozen until analysis. Phytoregulators were quantified by the liquid chromatography-tandem mass spectrometry technique (LC-MS/MS), which consisted of an ACQUITY UPLC and Xevo TQ-S (Waters). The LC-MS/MS measurement was carried out as follows: 1 mL of standard solution or samples previously filtrated were injected into a reverse phase LC column (ACQUITY UPLC BEH C18 1.7 mm, 2.1mm, 50 mm). The phytoregulators were eluted from the column using a gradient of (A) water containing 4 mM of ammonium-formate and 0.1% of formic acid and (B) acetonitrile 100%. A linear gradient was observed from 90% A: 10% B to 60% A: 40% B at 4.0 min, and finally 100% A: 0% B after 1 additional min for equilibration. A flow rate of 0.4 mL/min was used. The total run time (including injection) was 6 min. The column temperature was kept at 40 °C, while the samples in the autosampler were kept at 10 °C. For detection, the Xevo TQ-S was used in MRM mode. The MS/MS transition specific parameters were determined for each phytoregulator. The standards used included Kinetin (KT), indole 3-acetic acid (IAA), indole 3-butyric acid (IBA), gibberellic acid (GA), Jasmonic acid (JA), abscisic acid (ABA), and salicylic acid (SA) all acquired from Sigma-Aldrich. All compounds were measured using a capillary voltage (kV) of 2.20, a de-solvation temperature of 500 °C; a de-solvation gas flow (N_2) (L/h) of 800; a cone gas flow (L/h) of 50; and nebulizer gas flow (Bar) of 7.0. Calculations

were performed using the peak areas of the quantifier transition of each phytoregulator. Assays were conducted two times with two technical determinations for each sample.

List of abbreviations

Abscisic acid (ABA); indoleacetic acid (IAA); indole-3-butyric acid (IBA), gibberellic acid (GA), jasmonic acid (JA), salicylic acid (SA), kinetin (KT); Multi-walled carbon nanotubes (MWCNTs); Target of Rapamycine; liquid chromatography-tandem mass spectrometry technique (LC-MS/MS); thermogravimetric analysis (TGA).

Declarations

Ethics approval and consent to participate: "Not applicable" Consent for publication: "Not applicable" Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Competing interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding: This research was funded by CONACYT (256119 and 222405) grants.

Authors' contributions: G. J-C and D. K-T, performed the experiments, analyzed the data and prepared the figures and/or tables. J. C-G, H. R-C, and J. V. analyzed the data and funding project. G. J-C, J. C-G and J.V. wrote the manuscript. J. C-G and J.V. conceived and designed the experiments, analyzed the data and reviewed drafts of the manuscript, and approved the final draft. All authors read and approved the final manuscript.

Acknowledgements: We thanks to Dr. Benoît Menand (Aix Marseille Université, France) by the *A. thaliana TOR/tor1::uidA* transgenic line which was kindly donated. G. Juárez-Cisneros received a scholarship from CONACYT.

Reference

1. Vithanage M, Seneviratne M, Ahmad M, Sarkar B, Ok YS. Correction to: Contrasting effects of engineered carbon nanotubes on plants: a review. Environ Geochem Health. 2018;39(6):1.
2. Rai PK, Kumar V, Lee SS, Raza N, Kim KH, Ok YS, et al. Nanoparticle-plant interaction: Implications in energy, environment, and agriculture. Environ Int. 2018;119(June):1–19.
3. Verma SK, Das AK, Patel MK, Shah A, Kumar V, Gantait S. Engineered nanomaterials for plant growth and development: A perspective analysis. Sci Total Environ [Internet]. 2018;630:1413–35. Available from: https://doi.org/10.1016/j.scitotenv.2018.02.313

4. Mukhopadhyay SS. Nanotechnology in agriculture: Prospects and constraints. Nanotechnol Sci Appl. 2014;7(2):63–71.
5. Kim DY, Kadam A, Shinde S, Saratale RG, Patra J, Ghodake G. Recent developments in nanotechnology transforming the agricultural sector: a transition replete with opportunities. J Sci Food Agric. 2018;98(3):849–64.
6. Khot LR, Sankaran S, Maja JM, Ehsani R, Schuster EW. Applications of nanomaterials in agricultural production and crop protection: A review. Crop Prot [Internet]. 2012;35:64–70. Available from: http://dx.doi.org/10.1016/j.cropro.2012.01.007
7. Saifuddin N, Raziah AZ, Junizah AR. Carbon nanotubes: A review on structure and their interaction with proteins. J Chem. 2013;2013.
8. Zaytseva O, Neumann G. Carbon nanomaterials: Production, impact on plant development, agricultural and environmental applications. Chem Biol Technol Agric. 2016;3(1):1–26.
9. Scown TM, Van Aerle R, Tyler CR. Review: Do engineered nanoparticles pose a significant threat to the aquatic environment. Crit Rev Toxicol. 2010;40(7):653–70.
10. Lahiani MH, Nima ZA, Villagarcia H, Biris AS, Khodakovskaya M V. Assessment of Effects of the Long-Term Exposure of Agricultural Crops to Carbon Nanotubes. J Agric Food Chem [Internet]. 2017;acs.jafc.7b01863. Available from: http://pubs.acs.org/doi/abs/10.1021/acs.jafc.7b01863
11. Yuan Z, Zhang Z, Wang X, Li L, Cai K, Han H. Novel impacts of functionalized multi-walled carbon nanotubes in plants: promotion of nodulation and nitrogenase activity in the rhizobium-legume system. Nanoscale [Internet]. 2017;9(28):9921–37. Available from: http://xlink.rsc.org/?DOI=C7NR01948C
12. Lara-Romero J, Campos-García J, Dasgupta-Schubert N, Borjas-García S, Tiwari D, Paraguay-Delgado F, et al. Biological effects of carbon nanotubes generated in forest wildfire ecosystems rich in resinous trees on native plants. PeerJ [Internet]. 2017;5:e3658. Available from: https://peerj.com/articles/3658
13. Zaytseva O. Differential Impact of Multi-Walled Carbon Nanotubes on Germination and Seedling Development of Glycine Max , Phaseolus Vulgaris. 2016;202–10.
14. Joshi A, Kaur S, Singh P, Dharamvir K, Nayyar H, Verma G. Tracking multi-walled carbon nanotubes inside oat (Avena sativa L.) plants and assessing their effect on growth, yield, and mammalian (human) cell viability. Appl Nanosci [Internet]. 2018;0(0):0. Available from: http://link.springer.com/10.1007/s13204-018-0801-1
15. Khodakovskaya M V., De Silva K, Biris AS, Dervishi E, Villagarcia H. Carbon nanotubes induce growth enhancement of tobacco cells. ACS Nano. 2012;6(3):2128–35.
16. McGehee DL, Lahiani MH, Irin F, Green MJ, Khodakovskaya M V. Multiwalled Carbon Nanotubes Dramatically Affect the Fruit Metabolome of Exposed Tomato Plants. ACS Appl Mater Interfaces. 2017;9(38):32430–5.
17. Ghorbanpour M, Hadian J. Multi-walled carbon nanotubes stimulate callus induction, secondary metabolites biosynthesis and antioxidant capacity in medicinal plant Satureja khuzestanica grown in vitro. Carbon N Y [Internet]. 2015;94:749–59. Available

from: http://www.sciencedirect.com/science/article/pii/S0008622315300798

18. Li X, Cai W, Liu Y, Li H, Fu L, Liu Z, et al. Differential TOR activation and cell proliferation in Arabidopsis root and shoot apexes. Proc Natl Acad Sci U S A [Internet]. 2017 Mar 7 [cited 2018 Jun 21];114(10):2765–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28223530

19. Rexin D, Meyer C, Robaglia C, Veit B. TOR signalling in plants. Biochem J [Internet]. 2015;470(1):1–14. Available from: http://biochemj.org/lookup/doi/10.1042/BJ20150505

20. Xiong Y, Sheen J. Novel links in the plant TOR kinase signaling network. Curr Opin Plant Biol [Internet]. 2015 Dec [cited 2018 Jun 21];28:83–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26476687

21. Barrada A, Montané MHM, Robaglia C, Menand B. Spatial regulation of root growth: Placing the plant TOR pathway in a developmental perspective. Int J Mol Sci. 2015;16(8):19671–97.

22. De Vleesschauwer D, Filipe O, Hoffman G, Seifi HS, Haeck A, Canlas P, et al. Target of rapamycin signaling orchestrates growth–defense trade-offs in plants. New Phytol. 2018;217(1):305–19.

23. Deng K, Yu L, Zheng X, Zhang K, Wang W, Dong P, et al. Target of Rapamycin Is a Key Player for Auxin Signaling Transduction in Arabidopsis. Front Plant Sci [Internet]. 2016;7(March):291. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27014314%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4786968

24. Fenton TR, Gout IT. Functions and regulation of the 70 kDa ribosomal S6 kinases. Int J Biochem Cell Biol [Internet]. 2011;43(1):47–59. Available from: http://dx.doi.org/10.1016/j.biocel.2010.09.018

25. Liu W-W, Chai S-P, Mohamed AR, Hashim U. Synthesis and characterization of graphene and carbon nanotubes: A review on the past and recent developments. J Ind Eng Chem [Internet]. 2014;20(4):1171–85. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1226086X13003912

26. Lehman JH, Terrones M, Mansfield E, Hurst KE, Meunier V. Evaluating the characteristics of multiwall carbon nanotubes. Carbon N Y [Internet]. 2011;49(8):2581–602. Available from: http://dx.doi.org/10.1016/j.carbon.2011.03.028

27. Lahiani MH, Dervishi E, Chen J, Nima Z, Gaume A, Biris AS, et al. Impact of carbon nanotube exposure to seeds of valuable crops. ACS Appl Mater Interfaces. 2013;5(16):7965–73.

28. Juárez-Cisneros G, Gómez-Romero M, Reyes de la Cruz H, Campos-García J, Villegas J. Multi-walled carbon nanotubes produced after forest fires improve germination and development of Eysenhardtia polystachya. PeerJ. 2020;8:e8634.

29. Khodakovskaya M V., Kim BS, Kim JN, Alimohammadi M, Dervishi E, Mustafa T, et al. Carbon nanotubes as plant growth regulators: Effects on tomato growth, reproductive system, and soil microbial community. Small. 2013;9(1):115–23.

30. Joshi A, Kaur S, Dharamvir K, Nayyar H, Verma G. Multi-walled carbon nanotubes applied through seed-priming influence early germination, root hair, growth and yield of bread wheat (Triticum aestivum L.). J Sci Food Agric. 2018;98(8).

31. Melorose J, Perroy R, Careas S. Carbon and fullerene nanomaterials in plant system. Statew Agric L Use Baseline 2015. 2015;1.

32. Seabra AB, Paula AJ, Durán N. Redox-enzymes, cells and micro-organisms acting on carbon nanostructures transformation: A mini-review. Biotechnol Prog. 2013;29(1):1–10.

33. Begum P, Ikhtiari R, Fugetsu B. Potential Impact of Multi-Walled Carbon Nanotubes Exposure to the Seedling Stage of Selected Plant Species. Nanomaterials [Internet]. 2014;4(4):203–21. Available from: http://www.mdpi.com/2079-4991/4/2/203

34. Barrada A, Montané MH, Robaglia C, Menand B. Spatial regulation of root growth: Placing the plant TOR pathway in a developmental perspective. Int J Mol Sci. 2015;16(8):19671–97.

35. Fingar DC, Salama S, Tsou C, Harlow E, Blenis J. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev. 2002;1472–87.

36. Moubayidin L, Mambro R Di, Sabatini S. Cytokinin – auxin crosstalk. Trends Plant Sci. 2009;4(September).

37. Turck F, Zilbermann F, Kozma SC, Thomas G, Nagy F, Basel CH, et al. Phytohormones Participate in an S6 Kinase Signal Transduction Pathway in Arabidopsis 1. Plant Physiol. 2004;134(April):1527–35.

38. Schepetilnikov M, Ryabova LA. Recent discoveries on the role of tor (Target of rapamycin) signaling in translation in plants. Plant Physiol. 2018;176(2):1095–105.

39. Hartig K, Beck E. Crosstalk between auxin, cytokinins, and sugars in the plant cell cycle. Plant Biol. 2006;8(3):389–96.

40. Schepetilnikov M, Ryabova LA. Auxin Signaling in Regulation of Plant Translation Reinitiation. Front Plant Sci [Internet]. 2017;8(June):1–15. Available from: http://journal.frontiersin.org/article/10.3389/fpls.2017.01014/full

41. Schepetilnikov M, Dimitrova M, Mancera-Martínez E, Geldreich A, Keller M, Ryabova LA. TOR and S6K1 promote translation reinitiation of uORF-containing mRNAs via phosphorylation of eIF3h. EMBO J. 2013;32(8):1087–102.

42. Dong P, Xiong F, Que Y, Wang K, Yu L, Li Z, et al. Expression profiling and functional analysis reveals that TOR is a key player in regulating photosynthesis and phytohormone signaling pathways in Arabidopsis. Front Plant Sci. 2015;6(september):1–15.

43. Xiong Y, McCormack M, Li L, Hall Q, Xiang C, Sheen J. Glucose-TOR signalling reprograms the transcriptome and activates meristems. Nature [Internet]. 2013 Apr 11 [cited 2018 Jun 21];496(7444):181–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23542588

44. Henriques R, Bögre L, Horváth B, Magyar Z. Balancing act: Matching growth with environment by the TOR signalling pathway. J Exp Bot. 2014;65(10):2691–701.

45. Hao Y, Yu F, Lv R, Ma C, Zhang Z, Rui Y, et al. 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.

2016;11(6):e0157264.

46. Song Y, Zhao G, Zhang X, Li L, Xiong F, Zhuo F, et al. The crosstalk between Target of Rapamycin (TOR) and Jasmonic Acid (JA) signaling existing in Arabidopsis and cotton. Sci Rep [Internet]. 2017;7(April):1–15. Available from: http://dx.doi.org/10.1038/srep45830

47. Pozhvanov GA, Medvedev SS. Auxin quantification based on histochemical staining of GUS under the control of auxin-responsive promoter. Russ J Plant Physiol. 2008;55(5):706–11.

48. Malamy JE, Benfey PN. Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development [Internet]. 1997;124(1):33–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9006065

49. De La Cruz HR, Aguilar R, De Jiménez ES. Functional Characterization of a Maize Ribosomal S6 Protein Kinase (ZmS6K), a Plant Ortholog of Metazoan p70S6K. Biochemistry. 2004;43(2):533–9.

Figure legends

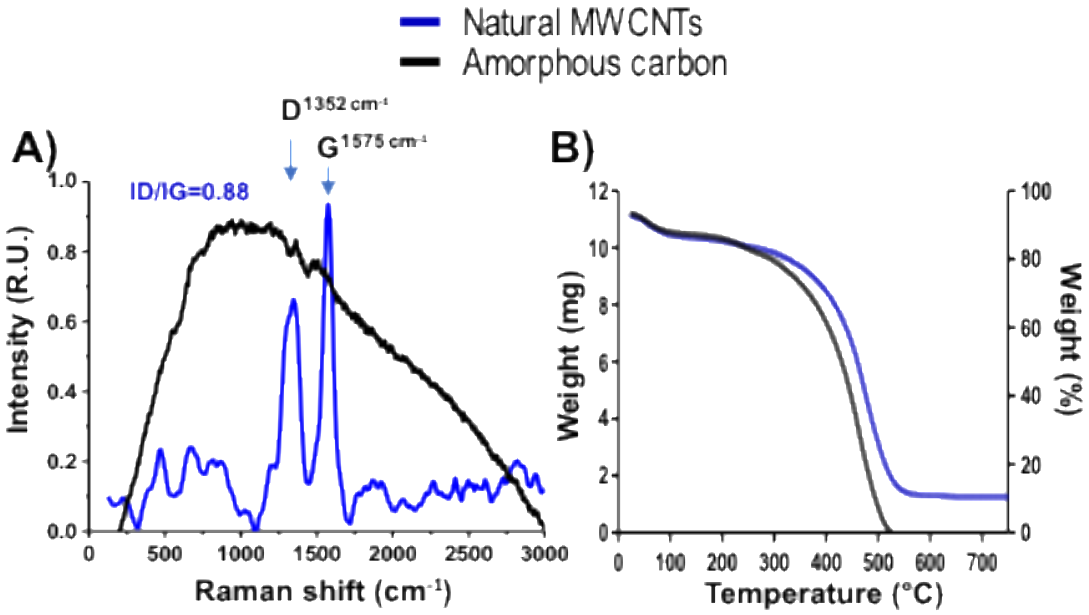


Figure 1. Analysis of natural MWCNTs and amorphous carbon samples obtained after a forest fire. The figure **A**) show Raman spectra scattering spectra (He-Ne laser emitting at 514 nm) of the samples: Characteristic bands of MWCNTs are shown in natural MWCNTs ID band (~1352 cm^{-1}), IG band (~1575 cm^{-1}) (blue line). **B**) TGA analysis shows the presence of material at up 650ºC characteristic of MWCNTs presence. These analyses were repeated three times with similar results, representative graphs are shown.

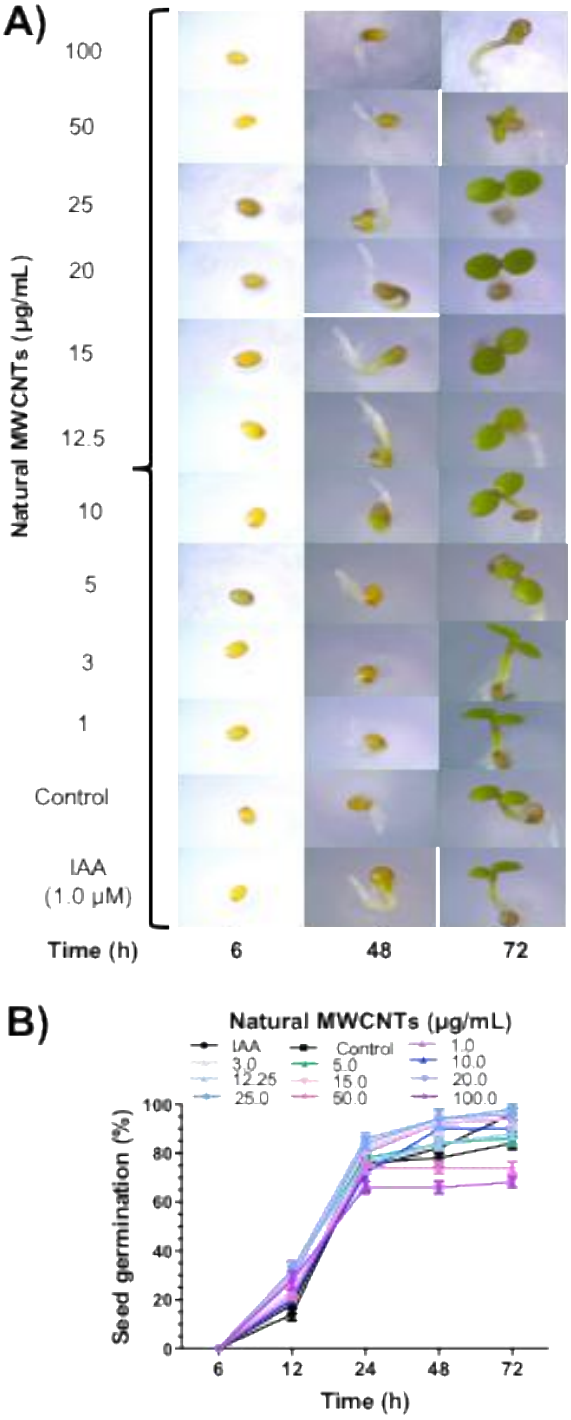


Figure 2. Effect of natural MWCNTs on seed germination of *A. thaliana*. A) Evaluation of seed germination of *A. thaliana* Col-0 in an *in vitro* assay dose-response with natural MWCNTs at 6, 48, and 72 h after seed sowing. This experiment includes different plates per treatment with ten seeds each, which was repeated at least three times with similar results. Photographs of representative seeds and seedlings were taken from plates for each treatment using an optical microscopy LEICA DM750 with 10X objective. The IAA (1.0 µM) was used as positive control. **B**) Seed germination kinetic. Bars represent means ± SE of three independent assays, n= 30 each.

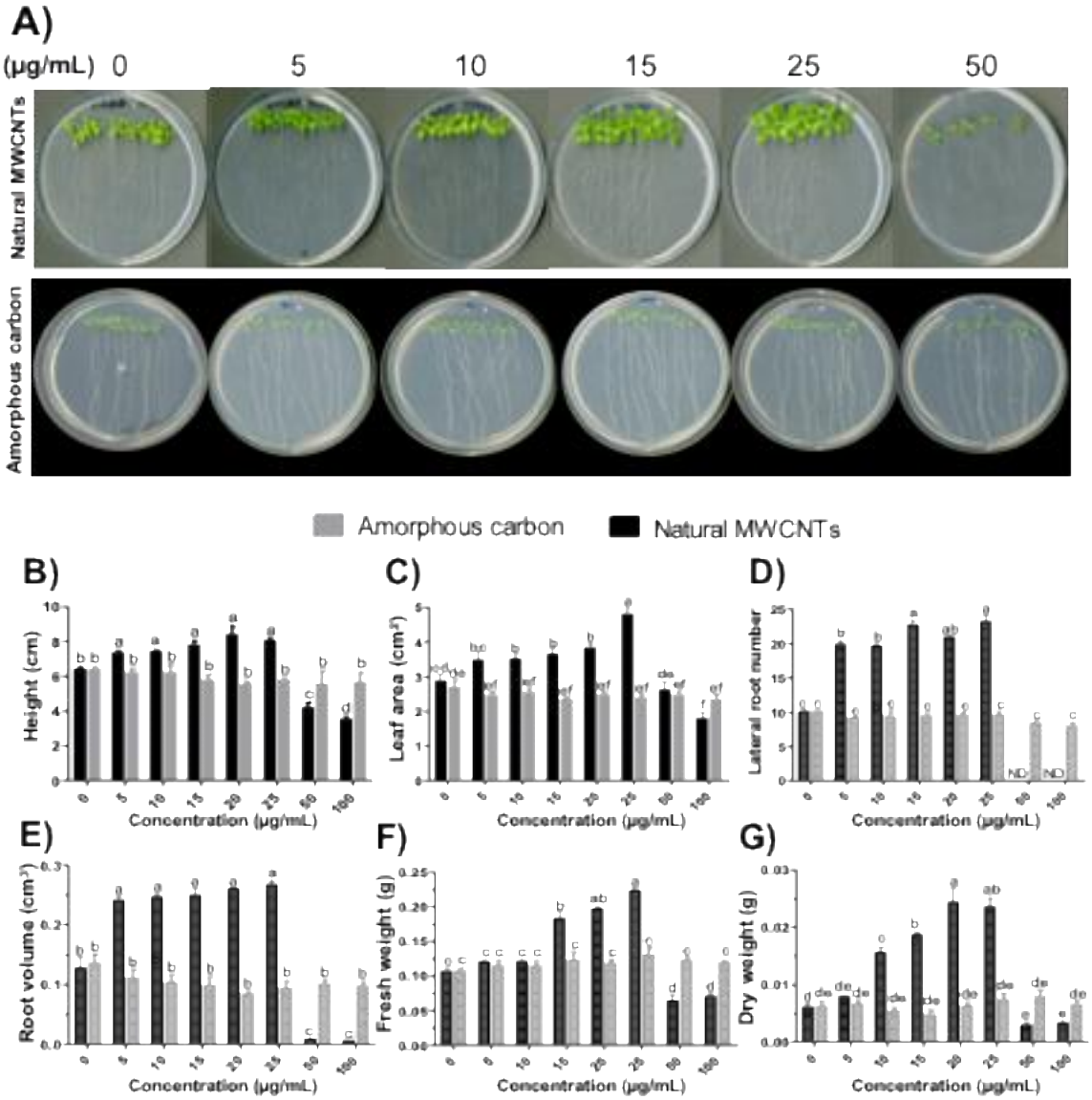


Figure 3. Effect of natural MWCNTs on plant growth and development of *A. thaliana.* **A**) Image of plant growth of *A. thaliana* Col-0 in dose-response assay with natural MWCNTs or amorphous carbon, 15 days after sowing. Representative photographs were taken from plates for each treatment. This experiment includes different plates per treatment with ten seedlings each, which was repeated at least three times with similar results. **B-G)** Morphometric and biomass parameters of *A. thaliana* in the *in vitro* dose-response assay with natural MWCNTs. **B**) Total height, **C**) leaf area, **D**) lateral root number, **E**) root volume, **F**) fresh weight, **G**) dry weight. The analysis was accomplished using the WinRHIZO software, and Instruments Canada Inc., with EPSON Expression 11000XL scan, coupled. Bars represent means ± SE of three independent assays, n= 30 each. One-way analysis of variance (ANOVA) was carried out with Tukey's *post hoc* test; statistical significance ($P < 0.05$) between treatments are indicated with different lowercase letters.

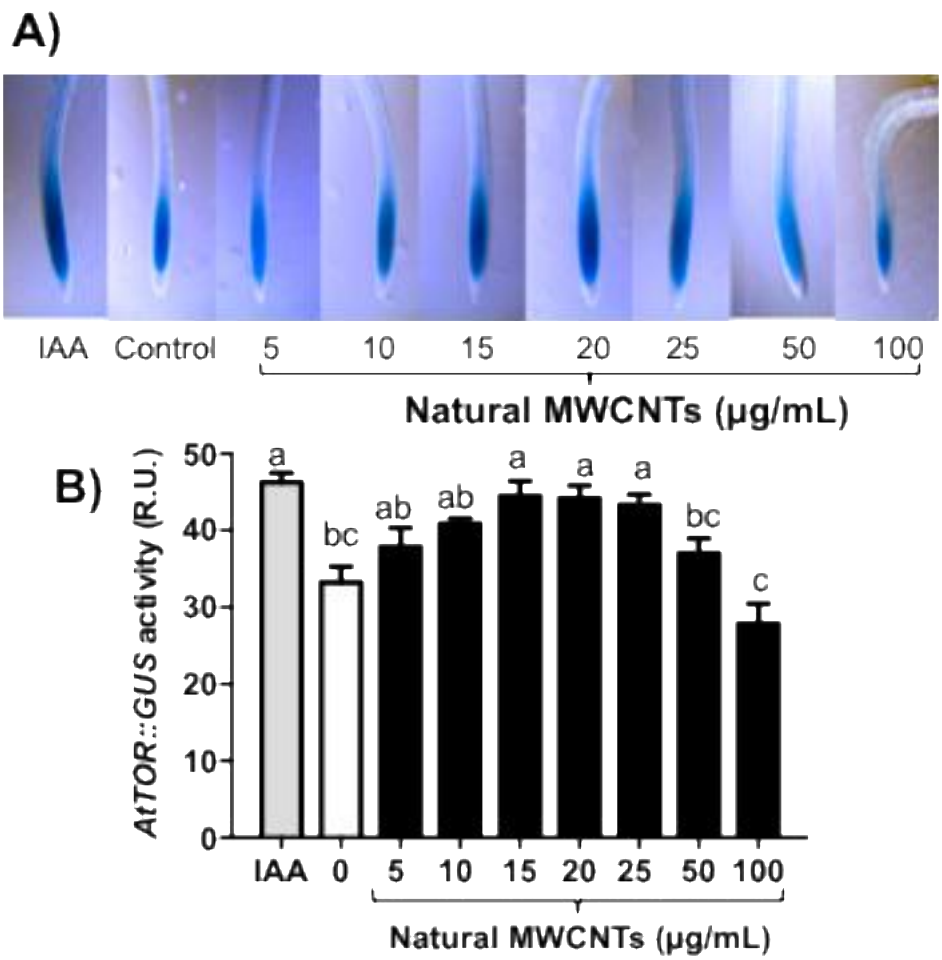


Figure 4. Effect of natural MWCNTs on the *AtTOR/tor1:uidA* expression in *A. thaliana* roots. A) Image of *AtTOR/tor1::uidA* expression in transgenic *A. thaliana* seedlings, β-glucuronidase (GUS) staining in dose-response assay with natural MWCNTs, 15 days after sowing. Representative photographs of individual roots were taken from plates for each treatment GUS-staining at least ten seedlings each, assay was repeated three times with similar results. Photographs of representative roots were obtained with an optical microscopy LEICA DM750 with 10X objective. The IAA (1.0 µM) was used as positive control. **B**) *AtTOR/tor1::uidA* expression kinetic in *Arabidopsis* roots, bars represent means ± SE of three independent assays, n= 10 each. One-way ANOVA was carried out with Tukey's *post hoc* test; statistical significance ($P < 0.05$) between treatments are indicated with different lowercase letters.

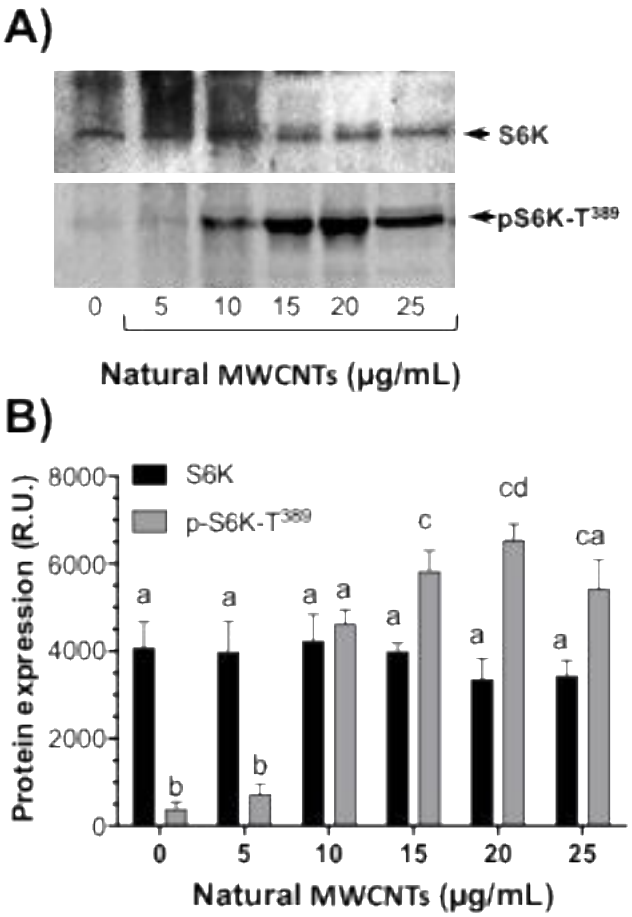


Figure 5. Effect of the natural MWCNTs on expression and phosphorylation of the S6K protein of *A. thaliana* seedlings. Seedlings were homogenized to obtain protein extracts used for Western blot assays as described in Materials and Methods. **A**) Representative images correspond to western blot using protein extracts from each treatment using the anti-AtS6K and anti-AtS6K-phosphorylated Thr(P)-389 antibodies are indicated. **B**) Graph corresponding to determination of the band intensity from the Western blot assays such as in (A), analyzed by densitometry using the Image J software (NIH). Data represent the means ± SE of densitometry determinations, obtained from three independent assays. One-way ANOVA with Tukey's *post-hoc* test was used to compare treatments. Significant differences ($P < 0.05$) between treatments are indicated with different lowercase letters.

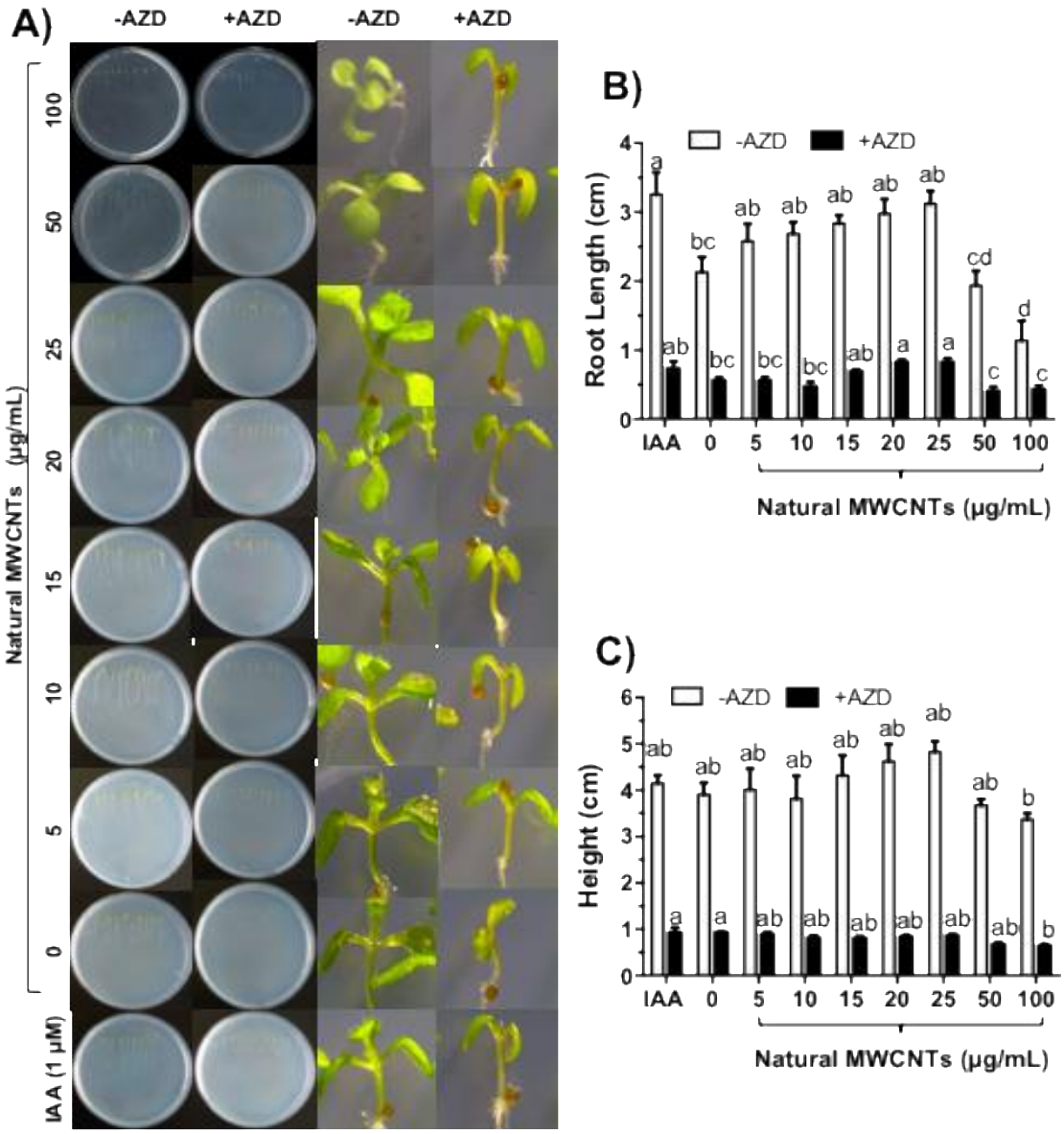


Figure 6. Effect of natural MWCNTs and AZD8055 TOR inhibitor on plant growth and development of *A. thaliana.* A) Image of plant growth of *A. thaliana* Col-0 in dose-response assay with natural MWCNTs and supplemented with AZD8055 (1.0 µM), 15 days after sowing. **B-C)** Morphometric and biomass parameters of *A. thaliana*. **B**) Root length, **C**) total height. The analysis was accomplished using the WinRHIZO software, and Instruments Canada Inc., with EPSON Expression 11000XL scan, coupled. This experiment includes different plates per treatment with ten seedlings each, which was repeated at least three times with similar results. Representative photographs were taken for plates or individual seedlings from each treatment. Bars represent means ± SE of three independent assays, n= 30. One-way ANOVA was carried out with Tukey's *post hoc* test; statistical significance ($P < 0.05$) between treatments are indicated with different lowercase letters.

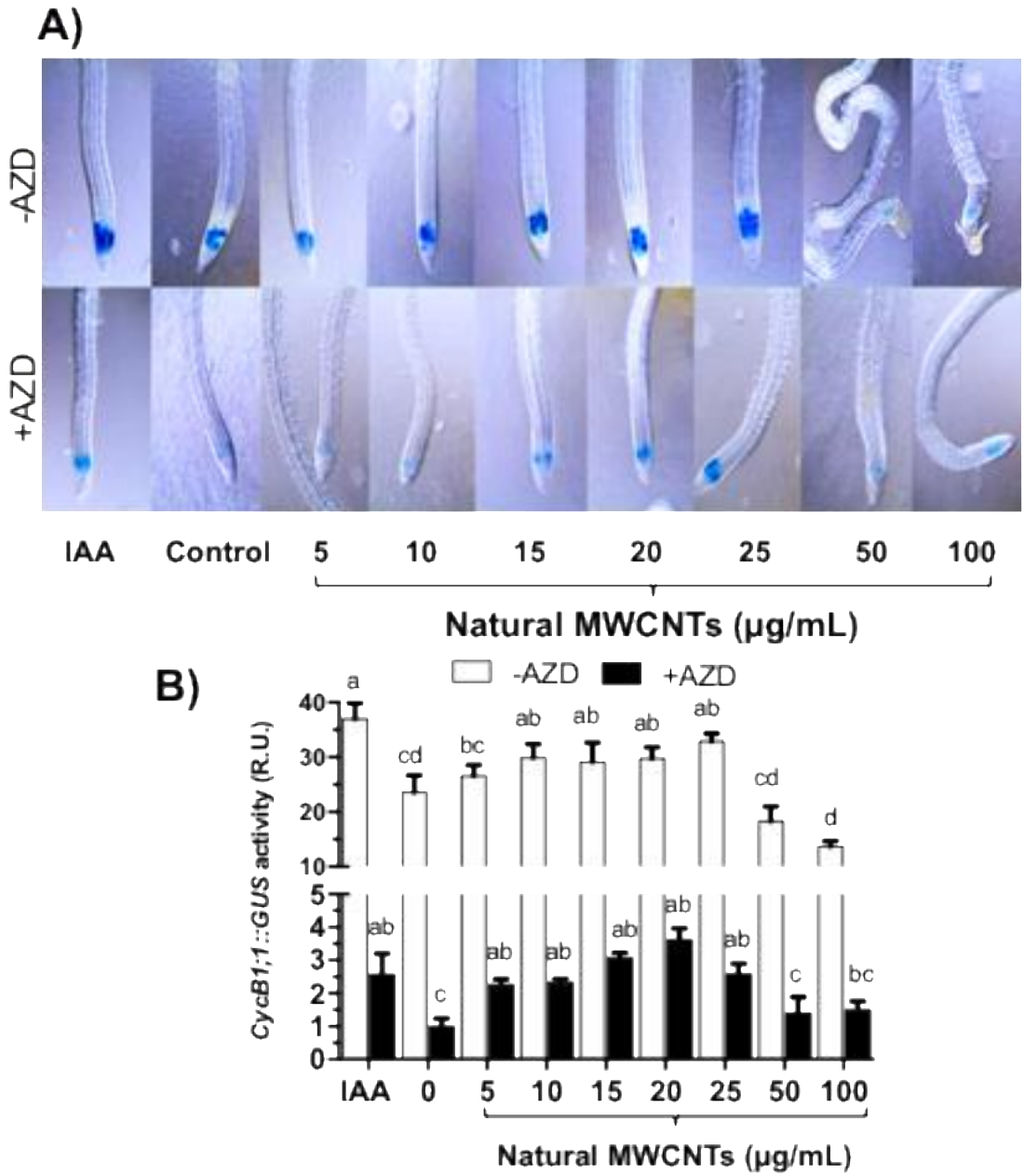


Figure 7. Effect of natural MWCNTs and AZD8055 TOR inhibitor on the *CycB1;1::uidA* expression in *A. thaliana* roots. A) Roots photographs of *CycB1;1::uidA* transgenic Arabidopsis GUS staining supplemented with natural MWCNTs and AZD8055 inhibitor (1.0 µM), 15 days after sowing. Representative photographs of individual roots were taken from plates for each treatment GUS-staining at least ten seedlings each, assay was repeated three times with similar results. Photographs of representative roots were obtained with optical microscopy LEICA DM750 with 10X objective. The IAA (1.0 µM) was used as positive control. **B**) *CycB;1:1:uidA* roots expression kinetic analyzed by densitometry using the Image J software (NIH). Bars represent means ± SE of three independent assays, n= 10 each. One-way ANOVA was carried out with Tukey's *post hoc*

test; statistical significance ($P < 0.05$) between treatments are indicated with different lowercase letters.

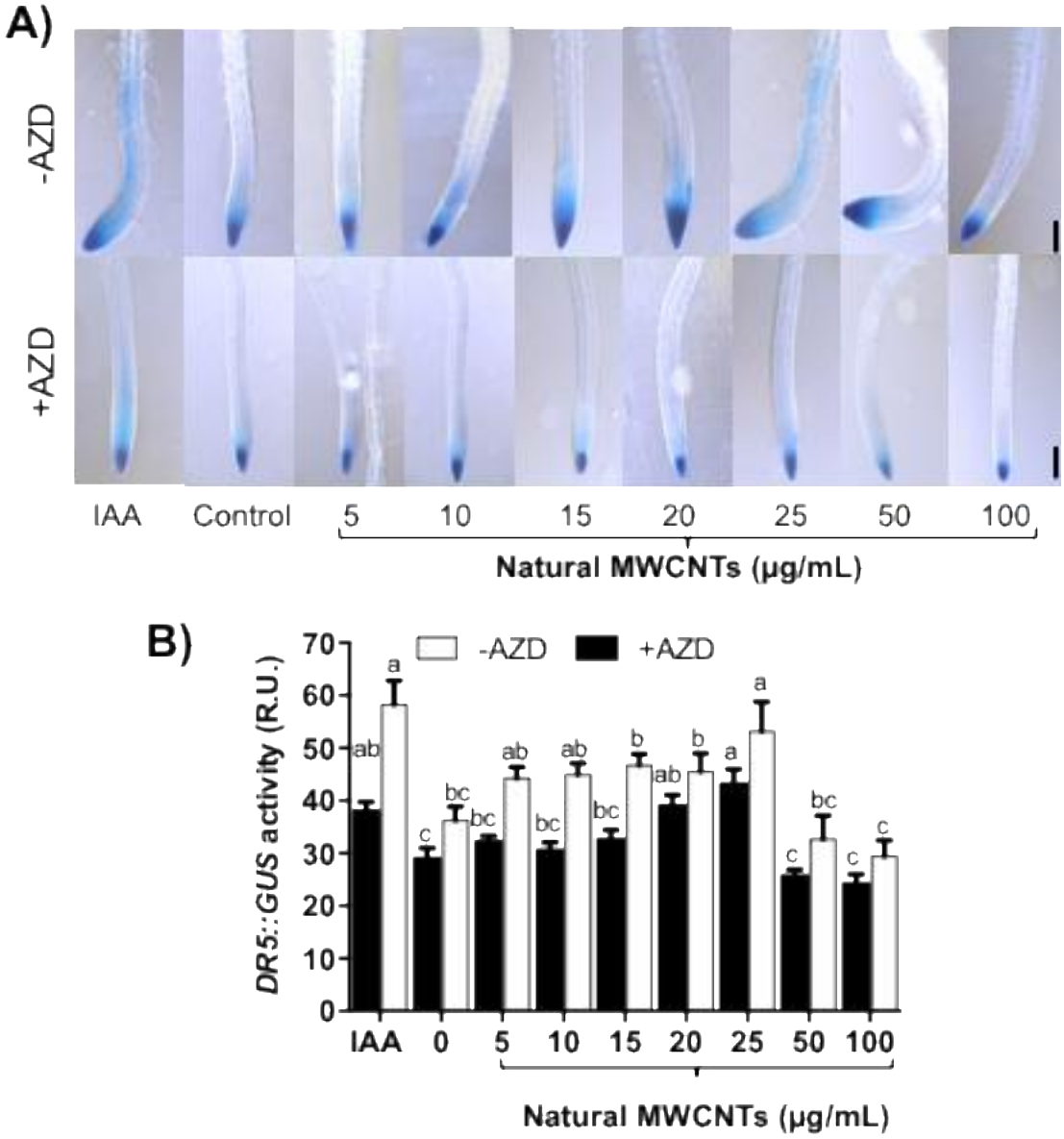


Figure 8. Effect of natural MWCNTs and AZD8055 TOR inhibitor on the *DR5::uidA* expression in *A. thaliana* roots. A) Roots photographs of *DR5::uidA* transgenic *Arabidopsis* GUS-staining supplemented with natural MWCNTs and AZD8055 inhibitor (1.0 µM), 15 days after sowing. Representative photographs of individual roots were taken from plates for each treatment GUS-staining at least ten seedlings each, assay was repeated three times with similar results. Photographs of representative roots were obtained with optical microscopy LEICA DM750 with 10X objective. The IAA (1.0 µM) was used as positive control. **B**) *DR5::uidA* roots expression kinetic analyzed by densitometry using the Image J software (NIH). Bars represent means ± SE of three independent assays, n= 10 each. One-way ANOVA was carried out with Tukey's *post hoc* test; statistical significance ($P < 0.05$) between treatments are indicated with different lowercase letters.

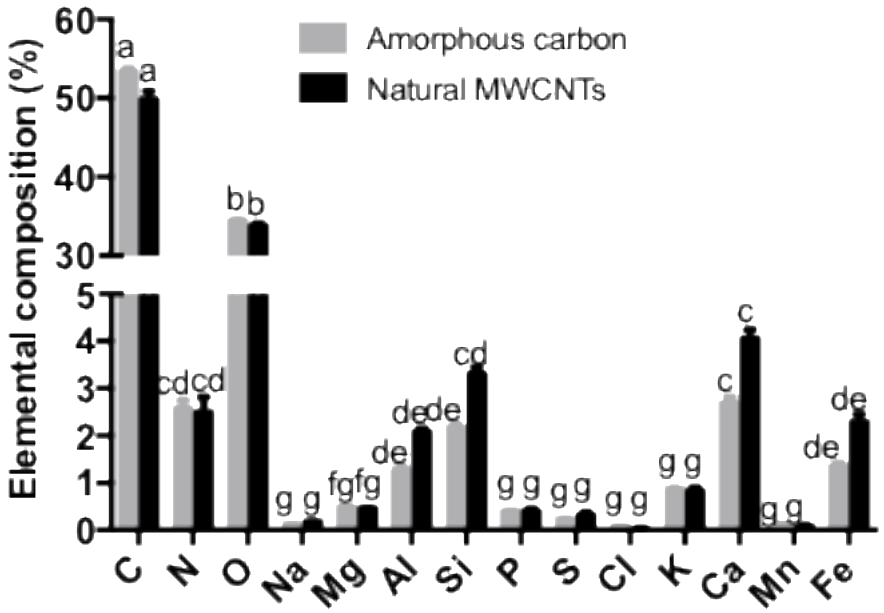


Figure 9. Elemental composition of the natural MWCNTS and amorphous carbon samples obtained after forest fire. Sample obtained after a forest fire (12 weeks later) and utilized for elemental composition by energy dispersive microwaves (EDS) model LK-IE250 OXFORD INCA ENERGY 250. Natural MWCNTs were collected from *P. oocarpa* carbonized tree samples; while as amorphous carbon collected from *P. montezumae* carbonized tree samples. Bars represent means ±SE and n= 3. One-way ANOVA was carried out with Tukey's *post hoc* test; statistical significance (P< 0.05) between samples are indicated with different lowercase letters.

Table 1. Effect of the natural MWCNTs on the phytoregulators balance in *A. thaliana* Col-0 seedlings

	MWCNTs (µg/ml)					
Compound	**0**	**5**	**10**	**15**	**20**	**25**
IAA	11.36	6.18	9.81	**16.53***	15.45*	12.21
IBA	26.71	27.01	34.69*	41.40*	**71.84***	**84.78***
KT	**1.36***	0.14	0.13	0.19	0.16	0.04
ABA	0.22	0.12	0.28	0.13	0.33	0.18
GAs	0.11	0.17	**0.39***	**0.42***	**0.48***	1.00*
JA	2.70	8.70*	4.21	2.70	**10.99***	**11.46***
SA	13.51	14.084375	13.87375	14.155625	18.410625*	**20.10***

Seedling of *Arabidopsis thaliana* Col-0 (3 g) grown in an *in vitro* assay dose-response 15 days after germination supplemented with natural MWCNTs were homogenized, phytoregulators extracted with solvents and analyzed by UPLC-MS/MS as described in Materials and Methods. The standards used were: IAA (indoleacetic acid), abscisic acid (ABA), indole 3-butyric acid (IBA), gibberellic acid (GA), jasmonic acid (JA), kinetin (KT), and salicylic acid (SA). The means of phytoregulator concentrations are expressed in ppb. Calculations were performed using the peak areas of the quantifier transition of each phytoregulator. Quantitation was conducted by two independent assays with two technical determinations for each sample; the coefficients of variance are expressed as percentage (%CV). Multiple Statistic significant differences determined by Student's *t*-test from treatments are represented with asterisks at $P < 0.05$ with respect to the control (without MWCNTs addition).

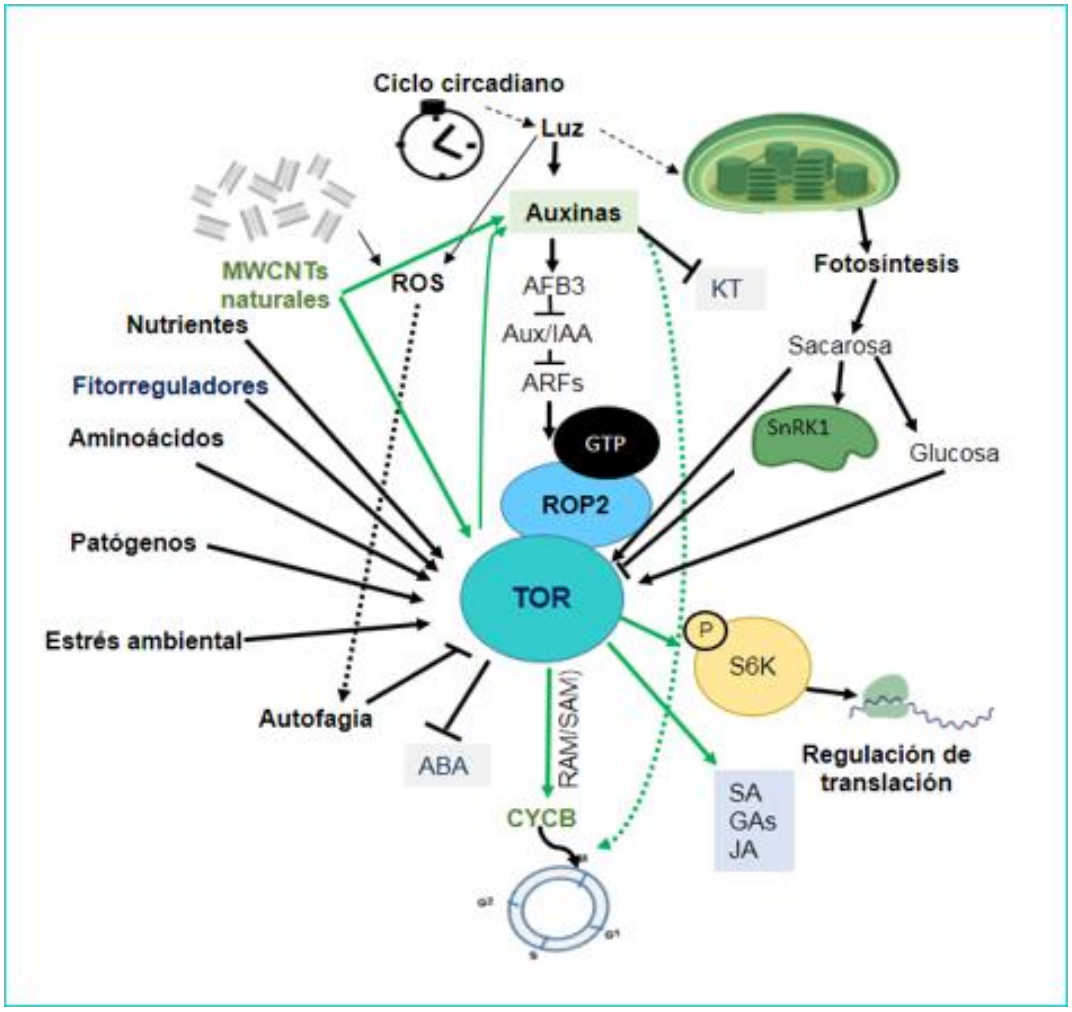


Figure 10. Effect of natural MWCNTs on the TOR pathway and its interaction with phytoregulators as a mechanism involved in promoting plant growth.
*Root and shot apical meristem RAM/SAM

CAPÍTULO 3.

Synthetic multi-walled carbon nanotubes affects *Arabidopsis thaliana* growth through blocking the TOR signaling pathway

Gladys Juárez-Cisneros[1]; Jesús Campos-García[1]*, Dhirendra K. Tiwari[2]; Homero Reyes-De la Cruz[1], and Javier Villegas[1]*.

[1] Instituto de Investigaciones Químico Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México.
[2] CONACYT- El Colegio de Michoacán/Ladipa, La Piedad, Michoacán, México.

Running title: MWCNTs inhibits plant development by TOR blocking

Abstract:

Phytotoxicity and beneficial effects of synthetic multi-walled carbon nanotubes (MWCNTs) on plant growth have been documented. However, the physiological mechanisms behind on plant toxicity of these nanomaterials are unknown. The TOR signaling pathway activity and phytoregulators balance play key roles in plant growth regulation. In this work, we evaluated the effect of synthetic MWCNTs on germination and development in the *Arabidopsis thaliana* plant model and its involvement on the TOR signaling pathway. To evaluate the TOR pathway, the *A. thaliana AtTOR/tor1::uidA* activity and the S6K phosphorylation was determined. Results showed that the synthetic MWCNTs at 10-15 µg/mL promoted seed germination, but in higher concentration the seeds germination was inhibited. Synthetic MWCNTs in a range greater than 10 µg/mL repressed aerial growth, biomass, and produced changes in root architecture. The activity of the *AtTOR/tor1::uidA* marker was repressed at 10 µg/mL of synthetic MWCNTs and correlated with S6K phosphorylation inhibition. Synthetic MWCNTs also affected the expression of the *AtCycB1;1::uidA* cell cycle marker in roots plants at 15 µg/mL; in a similar way, the AZD8055 TOR-inhibitor addition also repressed the *AtCycB1;1::uidA* activity. These results suggest that synthetic MWCNTs affected the *Arabidopsis* plant growth by affecting cell division through an that this effect involves the TOR signaling pathway. Additionally, the synthetic MWCNTs significantly repressed the activity of the auxinic marker *DR5::uidA* at 15 µg/mL in a synergic way to the AZD8055 addition. Synthetic MWCNTs not modified the abscisic acid (ABA) abundance; however, the indole acetic acid (IAA), indole butyric acid (IBA), gibberellic acid (GA) and jasmonic acid (JA) amounts were increased in a concentration-dependent manner, but contrary to, the kinetin content was decreased. In conclusion, the synthetic MWCNTs inhibited the germination and plant growth of *A. thaliana* through modifying the synthesis of phytoregulators and by a mechanism that involves the TOR signaling pathway.

Keywords: Synthetic multi-walled carbon nanotubes; TOR signaling; S6K protein; phytoregulators; plant growth.

1 Introduction

The nanotechnology is an emergent area of multidisciplinary interest due to the nanoparticles have extraordinary physic and chemical properties that make them of growing relevance for industrial, technology, and scientific development [1]. Among nanomaterials, synthetic multi-walled carbon nanotubes (MWCNTs) have been considered nanoparticles with potential nanoagriculture applications by promoting plant growth [2]. This nanomaterials have been associated with the early emergence of seeds and their germination [3]; likewise these nanomaterials improve the chlorophyll content rendering the photosynthetic activity more efficient [4]. Findings so far show that these nanoparticles induce the expression of aquaporin-regulatory genes increasing water uptake by the plant [5]. However, phytotoxic effects of synthetic MWCNTs have also been reported [6]. Those contrasting effects has been associated with the nanoparticles intrinsic properties, especially, their shape, dimensions, electrical conductivity, stability, and their limited solubility [7], as well as the concentration employed and the plant species used as model [8]. In example, in *Cucurbita pepo* (zucchini), the addition of synthetic MWCNTs affected seed germination and plant biomass during a 15-d in a hydroponic assay [9], induced reduction in germination percentage, root and shoot length, biomass, and vigor plant after 14-d of exposition [10]. In *Lactuca sativa* L., MWCNTs inhibited germination and limited growth and plant biomass by inducing cell death [11]. In other agronomic species such as, *Amaranthus tricolor* L. and *Cucumis sativus* [12], phytotoxic effects have also been reported. Nevertheless, the mechanisms involved in the MWCNTs phytotoxicity have not been totally elucidated, however, these effects have been associated with the oxidative stress generated by an increment and accumulation of reactive oxygen species (ROS), membrane damage [10], the reduction of antioxidant enzymes like superoxide dismutase, catalase, and peroxidase [10], or by effecting cell proliferation [13]. The study of beneficial and adverse effects on plants have caused increasing interest worldwide because these nanoparticles have been localized in plant and fruit tissues of food and commercial importance, rendering a critical importance by human health risk.

On the other hand, it has been reported that biotic and abiotic factors such as microbial interactions, temperature, light intensity, water availability, essential minerals, and phytoregulators homeostasis play a critical role on plant growth and development under natural conditions [14]. Moreover, in higher plants the TOR (target of rapamycin) signaling pathway plays a key role on plant development by integrating signals that regulate the assimilation of nutrients, energy, response to biotic or abiotic stress, and phytoregulators biosynthesis [15]. TOR pathway is key player in the cell cycle progression and plant development in response to environmental factors and phytoregulators. This pathway is regulated by the availability of nutrients, amount of light, CO_2 uptake, concentration of photosynthates such as glucose and sucrose, or the presence of NO_3^- [16]. TOR pathway regulates cell proliferation and elongation, autophagy [17], ribosomal embryogenesis, transcriptional reprogramming of genes that regulate central and secondary metabolism, the growth of stems, leaves and root hairs, stress plant responses, senescence [18], and defense against pathogens [16].

TOR regulates plant growth through the phosphorylation of a downstream canonical target, the S6 ribosomal protein kinase (S6K) [19]. In mammals, S6K is presents as the cytosolic isoform p70 S6K1 and nuclear p85 S6K1 [70] whose activity is modulated in response to growth factors, homeostasis of cytokines, or nutrients [20]. As in mammals, overexpression of S6K protein has

been reported to increase cell size in plants [21] and stimulate protein synthesis [22]; this kinase in turn phosphorylates the factor eIF3h to maintain translation through uORF in polysomes, increasing protein syntesis [23]. Thus, the TOR pathway represents a point of convergence of the nutrient, energy, and auxin pathways to regulate plant growth [24]. Auxins are positive regulators of cell division that activate and promote TOR loading in polysomes, activate S6K1, and initiate a signaling cascade that facilitate protein translation [23]. The TOR complex is also dependent on the homeostasis of phytoregulators such as auxins and cytokinins [16,19] mainly, key phytoregulators in cell cycle progression, meristematic activity, root architecture organization and apical dominance, as well as plant organogenesis [25–27]. Auxins have been documented to induce TOR activity and induce restart of mRNA translation through S6K1, whereas deficiency in TOR signaling impaired auxin-mediated root gravitropism [23]. Furthermore, Yunting *et al.*, (2017) suggested that auxins interact with stress response molecules in plants through the regulation of TOR activity [17]. Recently, TOR has been reported to play an antagonistic role with phytoregulators such as jasmonic acid and salicylic acid, which are regulated in response to stress caused by biotic and abiotic factors [18,28]. In this work, we evaluate the effect of synthetic MWCNTs on the development of *A. thaliana* plant model to deepening in the mechanism involved in the beneficial or adverse effects on plants, which represent a scientific interest at worldwide because the potential human health risk of the utilization of nanoparticles in agriculture. In this sense, the effect of synthetic MWCNTs on germination, auxins and TOR pathway signaling were evaluated in Arabidopsis plants.

2 Materials and methods:

2.1 MWCNTs specifications and characterization

The synthetic MWCNTs present a purity >98% (Aldrich, Cat 698849). The MWCNTs sample were analyzed with Raman spectroscopy according with Lara Romero *et al.*, (2017)[29]. Functional groups in the MWCNTs was analyzed by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, and spectra were recorded via Nicolet iS10 spectrophotometer (Thermo Scientific) by the attenuated total reflection (ATR) technique [29].

2.2 Plant growth conditions

Arabidopsis thaliana Col-0 was used in this study. All plants were grown in a growth chamber at 23ºC/photoperiod (16-h light: 8-h darkness) with a light intensity of 100 μE.2s1. Seeds of *A. thaliana* wild-type (WT) were plated with MS 0.5x (Murashige and Skoog) according to [30], supplemented with 0, 5, 10, 15, 20 or 25 μg/ml of MWCNTs. The seed germination was measured at 0, 6, 12, 24, 48, and 72 hours. Plant growth was analyzed 15 days after sowing. The analyzed morphometric variables included height (cm), leaf area (cm^2), as well as fresh and dry weight (g). Variables of the radical architecture were also measured, including root volume (cm^3) and the number of lateral roots with WinRHIZO-software ® Regent Instruments Inc**.**

2.3 Effect of synthetic MWCNTs on the TOR and auxins signaling, and cell proliferation.

To evaluated the effect of synthetic MWCNTs on *TOR* and auxins signailing, and cell cycle, we first evaluated the gene expression of the transgenic plants *AtTOR/tor1::uidA*, *DR5::uidA* and *AtCycB1;1::uidA* respectively, through the *GUS* staining. The quantification activity was realized according to the protocol described by Malamy and Benfey (1997) [31]. Representative images were captured with a Leica DM750 optical microscope, and the activity measurement of the *GUS* reporter gene was determined using the ImageJ software (NIH Image).
Phenotypic analysis of transgenic *DR5::uidA* and *AtCycB1;1::uidA* plants were used in this study. *GUS* staining and quantification activity were used to evaluate the effect of the MWCNTs with and without AZD8055 1.0 mM, a repressor of TOR activity [32].

2.4 Western blot assay

Arabidopsis plants cultivated in MS supplemented with synthetic MWCNTs were used nine days after sowing to obtain protein crude extracts [33], and Bradford reagent (Bio-Rad) was used to quantify total protein concentrations. Cellular lysis was carried out in phosphorylation buffer (PB) 300 µL composed of [Hepes 50 mM pH 7.6, sodium-pyrophosphate 50 mM, sodium ortovanadate 1 mM, sodium molybdate 1 mM, EDTA, EGTA 20 mM, benzamidine 1 mM, NaF 20 mM, PMSF 0.2 mM, ß-glycerophosphate 80 mM, mannitol 200 mM, protease inhibitor cocktails 1 µL/mL (all reagents from Sigma-Aldrich Co.)], and 30 µg of protein was mixed with 10 µL of denaturing buffer (Tris-HCl 0.06M, pH6.8, 5% de glycerol, 4% SDS, 4% β-mercaptoethanol and 0.0025% bromophenol blue) for 5 min at 95 °C in a boiling water bath. Samples were run in a denaturing 12% polyacrylamide gel electrophoresis (SDS-PAGE) in duplicate. One gel was Coomassie blue stained and the other gel was transferred to polyvinylidene difluoride (PVDF, Millipore) membranes for western blot procedure. The membranes were probed with rabbit anti-S6K polyclonal antiserum (1:5000 dilutions, Santa Cruz Biotechnologies), and anti-mouse IgG (1:3000) was used as the secondary antibody. To detect the phospho-p70 S6 kinase Thr(P)-389 polyclonal antibody (1:5000, Santa Cruz Biotechnologies), and secondary antibody anti-rabbit IgG (1:10000) were used. The membranes were washed twice with TBS-T buffer and developed using hydrogen peroxide and Supersignal West Pico Luminol (Pierce, Thermo Fisher Scientific). The images were captured in ChemiDoc™ MP System (Bio-Rad). Assays were conducted at least three times, and representative images are shown. Band intensities in images were quantified using Image J software (NIH Image).

2.5 Effect of synthetic MWCNTs on phytoregulators

Arabidopsis plants were harvested after nine days of growth. The fresh plant material (3 g) was frozen with liquid N_2. The phytoregulators extraction was obtained with methanol-trifluoroacetic acid (TFA) 100:1, vortexed 30 min at room temperature, and centrifuged at (1300 x g, 5 min at 4 °C). The solvents were evaporated in a speed vac (Centrivap Concentrator, Labconco) at 60 °C; the supernatant was transferred to a tube and adjusted to 1.5 ml with methanol. The samples were placed in 2 mL-Bond Eluent sample prep solutions tubes, QuEChERS Dispersive SPE 2 mL, fruits and vegetables with pigments, AOAC (Agilent Technologies), and shaken for 5 min using a vortex. After that, the sample was centrifuged for 5 min at 4000 rpm. Finally, the samples were placed in vials and frozen until analysis.

Phytoregulators were quantified by the liquid chromatography-tandem mass spectrometry technique (LC-MS/MS), which consisted of an ACQUITY UPLC and Xevo TQ-S (Waters). The LC-MS/MS measurement was carried out as follows: 1 mL of standard solution or samples previously filtrated were injected into a reverse phase LC column (ACQUITY UPLC BEH C18 1.7 mm, 2.1mm, 50 mm). The phytoregulators were eluted from the column using a gradient of (A) water containing 4 mM of ammonium-formate and 0.1% of formic acid and (B) acetonitrile 100%. A linear gradient was observed from 90% A: 10% B to 60% A: 40% B at 4.0 min, and finally 100% A: 0% B after 1 additional min for equilibration. A flow rate of 0.4 ml/min was used. The total run time (including injection) was 6 min. The column temperature was kept at 40 °C, while the samples in the autosampler were kept at 10 °C. For detection, the Xevo TQ-S was used in MRM mode. The MS/MS transition specific parameters were determined for each phytoregulator. The standards used included Kinetin (Kt), indole 3-acetic acid (IAA), indole 3-butyric acid (IBA), gibberellic acid (GAs), Jasmonic acid (JA), and abscisic acid (ABA), all acquired from Sigma-Aldrich. All compounds were measured using a capillary voltage (kV) of 2.20, a de-solvation temperature of 500 °C; a de-solvation gas flow (N_2) (L/h) of 800; a cone gas flow (L/h) of 50; and nebulizer gas flow (Bar) of 7.0. Calculations were performed using the peak areas of the quantifier transition of each phytoregulator.

2.6 Statistical analysis:

The results were obtained from three independent assays. The media reporter was obtained of 5 petri dishes with 10 plants each, and each treatment was conducted with 5 replicates n=10. One-way analysis of variance (ANOVA) was carried out with Tukey's post hoc test. Statistical significance ($P < 0.05$) between treatments concerning control is indicated with different lowercase letters.

3 Results

3.1 Synthetic MWCNTs characterization

MWCNTs characterization by Raman spectroscopy was performed, where three bands characteristic of the MWCNTs were observed. The G band at 1563 cm^{-1} corresponding to the tangential vibrations of the carbon atoms, characteristic of the E2g mode of the graphite plane by presence of sp2 electron hybridization of the carbon, the D band a1319 cm^{-1} generated the structural disorder in the graphitized materials [34], corresponding to the sp3 bonds of the distortions of the curved graphene sheets in the carbon nanotube [34,35], and finally, the G´ or 2D band at 2654 cm^{-1}, this band provides information on the number of graphene layers that constitute the nanotubes, a monolayer graphene shows lower intensity of G band compared to bi-, tri or multi-layer graphene, the number of graphene layers increases proportionally with an increase in G band intensity [36]. The synthetic MWCNTs ID/IG ratio obtained was 1.16, showing the defect ratio above the density in graphene sheets [36] (Figure 1A). Also, the sample was analyzed by FTIR (Figure 1B) showing a predominant peak at 3300 cm^{-1} associated with the vibrational modes of the O-H y 1635cm-1 C=C functional groups [35,37]. Finally, microscopy image of synthetic MWCNTs sample by HR-TEM showed that the MWCNTs presented outer diameter of 6–13 nm, the internal diameter of ~4.0 nm, length of ~20 μm, average wall thickness 7–13 graphene layers (Figure 1C).

3.2 Effect of synthetic MWCNTs on *A. thaliana* seed germination and plant growth

Synthetic MWCNTs strongly repressed the germination of *A. thaliana* seeds at 50-100 µg/mL, where only 20 and 10 % of germination was observed, respectively. Although, the seeds exposed to synthetic MWCNTs showed slightly increased germination percentages at 12 h of treatment in all doses evaluated in comparison with the control seeds, at 24-72 h of treatment, the percentage of germinated seeds was similar at concentrations of MWCNTs below of 25 µg/mL (Figures 2A-2B).
In respect to *A. thaliana* growth, synthetic MWCNTs negatively affected the foliar area and the root development at all doses of MWCNTs tested compared with the control plants (Figures 3). The results showed significant reduction in the total height of plants cultivated with 15, 20, and 25 µg/mL of synthetic MWCNTs (Figure 3B), while the leaf area was decreasing in function of the concentration of MWCNTs added (Figure 3C). The radical architecture shows strong growth inhibition with MWCNTs, concentrations above to 10 µg/mL diminished the root volume, inhibiting total growth and development of primary and lateral roots at 20-25 µg/mL (Figure 3D). Biomass was also evaluated in *A. thaliana* seedlings, fresh weight and dry weight were negatively affected at doses above to 15 µg/mL MWCNTs (Figure 3E-F), showing a total plant growth inhibition at 25 µg/mL (Figure 3A).

3.4 Effect of synthetic MWCNTs on the TOR kinase pathway

To determine whether the effects on plant growth involve to the TOR kinase pathway, the effect of synthetic MWCNTs on the *TOR* expression were first analyzed. The level of TOR expression were determined using the transgenic line *AtTOR::uidA* in response to the addition of synthetic MWCNTs nine days after sowing. *GUS* staining in the roots showed that *A. thaliana* seedlings treated with synthetic MWCNTs above to 10 µg/mL, decreased the expression levels of GUS marker, compared with the control treatments without MWCNTs or plants supplemented with IAA 1.0 µM (Figure 4), indicating these nanomaterials reduce the expression of *TOR*. Further, the effects of synthetic MWCNTs in *Arabidopsis* was also evaluated in the downstream TOR target, the S6K protein kinase by measuring the total S6K protein content and its phosphorylated status (p-S6K-Thr389), which is widely used as an indicator of TOR pathway activity. Similar levels of total S6K protein were detected in both the control and protein extracts from Arabidopsis Col-0 seedlings treated with 5 and 10 µg/mL of synthetic MWCNTs; but at 15 µg/mL of synthetic MWCNTs, the S6K protein expression was totally inhibited (Figure 5). Similar effect was detected in its phosphorylated isoform; at 5 and 10 µg/ml of MWCNTs, an increment of S6K protein phosphorylation was observed, but inhibited at 15 µg/mL MWCNTs (Figure 5B).

3.5 Effect of synthetic MWCNTs on cell proliferation and auxin signaling

The Plant growth its regulated by the balance of cell proliferation (increase in cell number) and cell elongation (increase in cell size), both processes are modulated by the TOR signaling pathway. To determine whether negative effect on *A. thaliana* plant growth and development induced by synthetic MWCNTs was the result of repression of cell elongation or proliferation and the correlation with the repression of the TOR and S6K proteins, we analyzed the *cyclin-B* expression on plant roots using the transgenic *AtCycB1;1::uidA* line. Synthetic MWCNTs

treatments on the transgenic plant, showed similar effects as in wild type plants, producing development repression of the seedlings at all the concentrations used (Figure S1). In a similar way, the addition of the TOR inhibitor AZD8055 also affected the plant growth, suggesting the involvement of the TOR pathway. Moreover, we observed that synthetic MWCNTs negative affected *cyclin-B* expression in the roots, compared with the control treatments at concentrations above to 10 μg/mL (Figure 6). A synergic effect was observed when plants were grown on both, synthetic MWCNTs and AZD5055, further suggesting the involvement of TOR signaling (Figure 6).

To determine whether the synthetic MWCNTs effects on *Arabidopsis* plants involves auxin signaling, we tested the *GUS* activity in the *DR5::uidA* transgenic line. The results showed that the synthetic MWCNTs at concentrations of 15 μg/mL significantly decreased the *GUS* activity (Figure 7). Additionally, the supplementation of the AZD8055 TOR-inhibitor caused decreased levels of *GUS* marker in a synergic way with the synthetic MWCNTs (Figure 7), further suggesting the participation of the TOR pathway.

3.6 Effect of synthetic MWCNTs on the *A. thaliana* phytoregulators content

Given that the auxin content on root tips was modulated by synthetic MWCNTs *in A. thaliana* seedlings, the phytoregulators content on seedlings exposed to MWCNTs (10 μg/mL) was measured by LC-MS/MS. The results showed that the synthetic MWCNTs changed the balance of the phytoregulators in the *Arabidopsis* seedlings. The indole acetic acid (IAA) and abscisic acid (ABA) phytoregulators concentration were not modified by the addition of synthetic MWCNTs (Table 1). However, auxins such as the 3-butyric acid (IBA), and other phytoregulators such as gibberellic acid (GAs) and jasmonic acid (JA) increased significantly their amounts; in contrast, the kinetin (Kt) was decreased by the synthetic MWCNTs (Table 1).

4 Discussion

The findings showed that the synthetic MWCNTs inhibits the germination of *A. thaliana* seeds at doses higher than 15 μg/mL (Figure 2). Similar effects for MWCNTs have been reported for *Cucurbita pepo* (zucchini) [9,10] and in *Lactuca sativa* L [11]. According to Lahiani *et al*., (2013) the effects of MWCNTs on germination depend on their translocation inside the seed, influencing the interaction of suspended organic materials, their colloidal nature and the vehicles that allow their flow [38]. We also detected negative effects of synthetic MWCNTs on *A. thaliana* at doses higher than 15 μg/ml in the foliage area, root architecture, and biomass (Figure 3). Previous studies have documented negative effects of synthetic MWCNTs, where the phytotoxicity caused by this nanomaterials had been attributed to the cell death in roots and leaves due to an increase in the generation and accumulation of ROS [11] and the breakdown of cell membranes [12].

To explore the effect of synthetic MWCNTs on the TOR pathway, we first studied the GUS activity in the transgenic *AtTOR::uidA* line in response to the addition of MWCNTs. In the root of *A. thaliana*, it was observed that at10 μg/mL of MWCNTs, the GUS activity was decreased, compared to the control plants without MWCNTs or supplemented with IAA (Figure 4). Likewise, we analyzed the effect of synthetic MWCNTs on S6 kinase, a target protein downstream of TOR, considered as the main target of TOR pathway in plants. In plants, the S6K is also involved in the regulation of the cell size and homeostasis of glucose [21]. Our results showed that the synthetic MWCNTs affected the expression and phosphorylation of the

S6K protein at 15 µg/mL of MWCNTs (Figure 5). Thus, the decrease in TOR expression on *AtTOR/tor1:: uidA* and the S6K inhibition, strongly suggests that the synthetic MWCNTs negatively affect plant growth through repression/activation of TOR/S6K signaling pathway.
Inhibition of the TOR pathway has been reported to generate a short root system as a consequence of an early exit from proliferation and entry into the differentiation program [24,39]. Furthermore, this pathway plays a key role in thr translation and biogenesis of ribosomes, regulating the composition and structure of the cell wall [40] suggesting that repression of the TOR pathway collaterally inhibits the expression of other genes that regulate cell expansion and therefore plant growth [24].

Due to the critical role of the TOR/S6K pathway, as a central regulator in the control of cell growth and proliferation [25], we initially evaluated the effect of MWCNTs on the *AtCycB1;1::uidA* transgenic line, widely used as cell division marker. The results obtained with AZD8055 addition, showed, that the addition of this TOR-inhibitor generated small plants with roots and foliage with limited growth, a typic phenotype on plants with the addition of competitive ATP inhibitors of TOR [41](Figures S1 and 6). It was also observed that the synthetic MWCNTs significantly affected the GUS activity in the roots in *AtCycB1;1::uidA* at 15 µg/mL of these nanomaterials; however, at lower doses no significant differences were detected compared to the control plants.
The inhibition of TOR by the action of AZD8055 significantly suppressed the induction of the *AtCycB1;1::uidA* marker in the roots of *A. thaliana* in all the tested concentrations of synthetic MWCNTs; this inhibition suggests that the expression *Cyclin B1* is modulated through synthetic MWCNTs by a signaling mechanism dependent on the TOR pathway. Our results are in agreement with Hartig et al. (2006), who reported that Cyclin genes as *CYCB1* are partially regulated by these TOR-S6K kinases [42].
The influence of phytoregulators on the TOR/S6K pathway has also been documented, for example, signaling of auxins in plants through ROP2, induce the downstream signaling and phosphorylation cascade of TOR and S6K [43,44]. For this reason, the effect of synthetic MWCNTs was evaluated using the *A. thaliana DR5::uidA* transgenic plants to evaluate the activity of auxins (Figure 7). The results showed that synthetic MWCNTs at a concentration above 10 µg/mL affected the GUS activity, suggesting that MWCNTs interferes with the auxins effects. Furthermore, the inhibitory effect of AZD8055 on the GUS activity of this transgenic line together with MWCNTs, indicates that synthetic MWCNTs affects plant growth by regulating synthesis or mobilization of auxin in a TOR-dependent pathway. The relationship between auxins and TOR signaling pathway and the influence of TOR in the auxin-mediated plant development has been documented [23]. Our results strongly support this interconnection. According to Yokawa and Balusˇka (2016), in plant roots the TOR pathway can be activated by the accumulation of ROS to control its downstream processes, including the regulation of auxins, and negatively the autophagy [45]. In other hand, the auxin participates in the regulation of plant growth throughout the plant life cycle and is one of the most important growth regulators in plants. Previous reports demonstrate that TOR acts as an essential effector for auxin signal transduction *in Arabidopsis* [19, 23, 46]. Then, auxins can be participating as upstream TOR effectors [47], and regulates the cell cycle, according with the cellular cycle phase [23]. Auxins have been documented to induce TOR activity and induce restart of mRNA translation through S6K1, whereas deficiency in TOR signaling impaired auxin-mediated root gravitropism [23].

The key role played by plant regulators during plant development has been extensively documented [24]. However, the effect of synthetic MWCNTs on modulating the synthesis of phytoregulators has not yet been explored [48]. Our findings showed a clear effect of MWCNTs on phytoregulators amounts. Synthetic MWCNTs slightly modified the abundance of salicylic acid (SA), jasmonic Acid (JA), and abscisic acid (ABA) in *Arabidopsis* seedlings, we also detected increases in the abundance of gibberellic acid (GAs) and a strong increase in indole butyric acid (IBA), a precursor of IAA; in contrast, there was a reduction of kinetin (Kt). This result is consistent with Moubayidin et al., 2009), they reported that auxins-cytokinins play an antagonistic role among each other to regulate meristematic activity and the development of root architecture [25], participating in root initiation, formation and development of lateral, and adventitious roots [49]. Likewise, it has been documented that synthetic MWCNTs negatively affect the growth of *Oryza sativa* L. by decreasing the concentrations of phytoregulators such as IBA, IPA, ABA, JA and endogenous brassiosteroids [48].

5 Conclusion

Our results provide evidence about the negative effect of synthetic MWCNTs on the plant-growth model of *A. thaliana*. The negative effect on *Arabidopsis* showed significant differences in germination, aereal growth, root architecture modification, and biomass. This research also provided evidences relating the negative effects of the synthetic MWCNTs to a mechanisms involving the TOR/S6K signaling pathway and modifying the phytoregulators balance.

6 Conflict of Interest

The authors declare that no exist competing interest.

7 Acknowledgments

This research was funded by CONACYT (256119) and C.I.C. 2.14/UMSNH grants. G. Juárez-Cisneros received a scholar-ship of CONACYT.

8 References

[1] X. Qu, P.J.J. Alvarez, Q. Li, Applications of nanotechnology in water and wastewater treatment, Water Res. 47 (2013) 3931–3946. doi:10.1016/j.watres.2012.09.058.

[2] P. Wang, E. Lombi, F. Zhao, P.M. Kopittke, Nanotechnology : A New Opportunity in Plant Sciences, Trends Plant Sci. xx (2016) 1–14. doi:10.1016/j.tplants.2016.04.005.

[3] M.H. Lahiani, E. Dervishi, J. Chen, Z. Nima, A. Gaume, A.S. Biris, M. V. Khodakovskaya, Impact of carbon nanotube exposure to seeds of valuable crops, ACS Appl. Mater. Interfaces. 5 (2013) 7965–7973. doi:10.1021/am402052x.

[4] A. Joshi, S. Kaur, P. Singh, K. Dharamvir, H. Nayyar, G. Verma, Tracking multi-walled carbon nanotubes inside oat (Avena sativa L.) plants and assessing their effect on growth, yield, and mammalian (human) cell viability, Appl. Nanosci. 0 (2018) 0. doi:10.1007/s13204-018-0801-1.

[5] M. V. Khodakovskaya, K. De Silva, A.S. Biris, E. Dervishi, H. Villagarcia, Carbon nanotubes induce growth enhancement of tobacco cells, ACS Nano. 6 (2012) 2128–2135. doi:10.1021/nn204643g.

[6] M. Vithanage, M. Seneviratne, M. Ahmad, B. Sarkar, Y.S. Ok, Contrasting effects of

engineered carbon nanotubes on plants: a review, Environ. Geochem. Health. 39 (2017) 1421–1439. doi:10.1007/s10653-017-9957-y.

[7] T.M. Scown, R. Van Aerle, C.R. Tyler, Review: Do engineered nanoparticles pose a significant threat to the aquatic environment, Crit. Rev. Toxicol. 40 (2010) 653–670. doi:10.3109/10408444.2010.494174.

[8] P. Jackson, N.R. Jacobsen, A. Baun, R. Birkedal, D. Kühnel, K.A. Jensen, U. Vogel, H. Wallin, Bioaccumulation and ecotoxicity of carbon nanotubes, Chem. Cent. J. 7 (2013) 1–21. doi:10.1186/1752-153X-7-154.

[9] D. Stampoulis, S.K. Sinha, J.C. White, Assay-dependent phytotoxicity of nanoparticles to plants, Environ. Sci. Technol. 43 (2009) 9473–9479. doi:10.1021/es901695c.

[10] M. Hatami, Toxicity assessment of multi-walled carbon nanotubes on Cucurbita pepo L. under well-watered and water-stressed conditions, Ecotoxicol. Environ. Saf. 142 (2017) 274–283. doi:10.1016/j.ecoenv.2017.04.018.

[11] R. Ikhtiari, P. Begum, F. Watari, B. Fugetsu, Toxic Effect of Multiwalled Carbon Nanotubes on Lettuce (Lactuca Sativa), 5 (2013) 18–24.

[12] P. Begum, R. Ikhtiari, B. Fugetsu, Potential Impact of Multi-Walled Carbon Nanotubes Exposure to the Seedling Stage of Selected Plant Species, Nanomaterials. 4 (2014) 203–221. doi:10.3390/nano4020203.

[13] X. ming Tan, C. Lin, B. Fugetsu, Studies on toxicity of multi-walled carbon nanotubes on suspension rice cells, Carbon N. Y. 47 (2009) 3479–3487. doi:10.1016/j.carbon.2009.08.018.

[14] U. Bechtold, B. Field, Molecular mechanisms controlling plant growth during abiotic stress, J. Exp. Bot. 69 (2018) 2753–2758. doi:10.1093/jxb/ery157.

[15] D. Rexin, C. Meyer, C. Robaglia, B. Veit, TOR signalling in plants, Biochem. J. 470 (2015) 1–14. doi:10.1042/BJ20150505.

[16] Y. Xiong, J. Sheen, Novel links in the plant TOR kinase signaling network, Curr. Opin. Plant Biol. 28 (2015) 83–91. doi:10.1016/j.pbi.2015.09.006.

[17] Y. Pu, X. Luo, D.C. Bassham, Tor-dependent and -independent pathways regulate autophagy in arabidopsis thaliana, Front. Plant Sci. 8 (2017) 1–13. doi:10.3389/fpls.2017.01204.

[18] D. De Vleesschauwer, O. Filipe, G. Hoffman, H.S. Seifi, A. Haeck, P. Canlas, J. Van Bockhaven, E. De Waele, K. Demeestere, P. Ronald, M. Hofte, Target of rapamycin signaling orchestrates growth–defense trade-offs in plants, New Phytol. 217 (2018) 305–319. doi:10.1111/nph.14785.

[19] K. Deng, L. Yu, X. Zheng, K. Zhang, W. Wang, P. Dong, J. Zhang, M. Ren, Target of Rapamycin Is a Key Player for Auxin Signaling Transduction in Arabidopsis., Front. Plant Sci. 7 (2016) 291. doi:10.3389/fpls.2016.00291.

[20] T.R. Fenton, I.T. Gout, Functions and regulation of the 70 kDa ribosomal S6 kinases, Int. J. Biochem. Cell Biol. 43 (2011) 47–59. doi:10.1016/j.biocel.2010.09.018.

[21] D.C. Fingar, S. Salama, C. Tsou, E. Harlow, J. Blenis, Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E, Genes Dev. (2002) 1472–1487. doi:10.1101/gad.995802.

[22] J. Van Leene, C. Han, A. Gadeyne, D. Eeckhout, C. Matthijs, B. Cannoot, N. De Winne, G. Persiau, E. Van De Slijke, B. Van de Cotte, E. Stes, M. Van Bel, V. Storme, F. Impens, K. Gevaert, K. Vandepoele, I. De Smet, G. De Jaeger, Capturing the phosphorylation and protein interaction landscape of the plant TOR kinase, Nat. Plants. 5 (2019) 316–

327. doi:10.1038/s41477-019-0378-z.
[23] M. Schepetilnikov, M. Dimitrova, E. Mancera-Martínez, A. Geldreich, M. Keller, L.A. Ryabova, TOR and S6K1 promote translation reinitiation of uORF-containing mRNAs via phosphorylation of eIF3h, EMBO J. 32 (2013) 1087–1102. doi:10.1038/emboj.2013.61.
[24] R. Henriques, L. Bögre, B. Horváth, Z. Magyar, Balancing act: Matching growth with environment by the TOR signalling pathway, J. Exp. Bot. 65 (2014) 2691–2701. doi:10.1093/jxb/eru049.
[25] L. Moubayidin, R. Di Mambro, S. Sabatini, Cytokinin – auxin crosstalk, Trends Plant Sci. 4 (2009). doi:10.1016/j.tplants.2009.06.010.
[26] J. Kyozuka, Control of shoot and root meristem function by cytokinin, Plan Biol. 1 (2007) 442–446. doi:10.1016/j.pbi.2007.08.010.
[27] A. Sofo, A. Vitti, M. Nuzzaci, G. Tataranni, A. Scopa, Correlation between hormonal homeostasis and morphogenic responses in Arabidopsis thaliana seedlings growing in a Cd / Cu / Zn multi-pollution context, Physiol. Plant. (2013) 487–498. doi:10.1111/ppl.12050.
[28] M.A. Salem, Y. Li, K. Bajdzienko, J. Fisahn, M. Watanabe, R. Hoefgen, M.A. Schöttler, P. Giavalisco, RAPTOR Controls Developmental Growth Transitions by Altering the Hormonal and Metabolic Balance, Plant Physiol. 177 (2018) 565–593. doi:10.1104/pp.17.01711.
[29] J. Lara-Romero, J. Campos-García, N. Dasgupta-Schubert, S. Borjas-García, D.K. Tiwari, F. Paraguay-Delgado, S. Jiménez-Sandoval, G. Alonso-Nuñez, M. Gómez-Romero, R. Lindig-Cisneros, H.R. De la Cruz, J.A. Villegas, Biological effects of carbon nanotubes generated in forest wildfire ecosystems rich in resinous trees on native plants, PeerJ. 2017 (2017) 1–15. doi:10.7717/peerj.3658.
[30] G.A. Pozhvanov, S.S. Medvedev, Auxin quantification based on histochemical staining of GUS under the control of auxin-responsive promoter, Russ. J. Plant Physiol. 55 (2008) 706–711. doi:10.1134/S1021443708050166.
[31] J.E. Malamy, P.N. Benfey, Organization and cell differentiation in lateral roots of Arabidopsis thaliana., Development. 124 (1997) 33–44. doi:VL - 124.
[32] X. Li, W. Cai, Y. Liu, H. Li, L. Fu, Z. Liu, L. Xu, H. Liu, T. Xu, Y. Xiong, Differential TOR activation and cell proliferation in Arabidopsis root and shoot apexes., Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 2765–2770. doi:10.1073/pnas.1618782114.
[33] H.R. De La Cruz, R. Aguilar, E.S. De Jiménez, Functional Characterization of a Maize Ribosomal S6 Protein Kinase (ZmS6K), a Plant Ortholog of Metazoan p70S6K, Biochemistry. 43 (2004) 533–539. doi:10.1021/bi035222z.
[34] W.-W. Liu, S.-P. Chai, A.R. Mohamed, U. Hashim, Synthesis and characterization of graphene and carbon nanotubes: A review on the past and recent developments, J. Ind. Eng. Chem. 20 (2014) 1171–1185. doi:10.1016/j.jiec.2013.08.028.
[35] J.H. Lehman, M. Terrones, E. Mansfield, K.E. Hurst, V. Meunier, Evaluating the characteristics of multiwall carbon nanotubes, Carbon N. Y. 49 (2011) 2581–2602. doi:10.1016/j.carbon.2011.03.028.
[36] W.W. Liu, S.P. Chai, A.R. Mohamed, U. Hashim, Synthesis and characterization of graphene and carbon nanotubes: A review on the past and recent developments, J. Ind. Eng. Chem. 20 (2014) 1171–1185. doi:10.1016/j.jiec.2013.08.028.
[37] A.N. Mohan, M. B, S. Panicker, Facile synthesis of graphene-tin oxide nanocomposite derived from agricultural waste for enhanced antibacterial activity against Pseudomonas

aeruginosa, Sci. Rep. 9 (2019) 4170. doi:10.1038/s41598-019-40916-9.
[38] J. Melorose, R. Perroy, S. Careas, Carbon and fullerene nanomaterials in plant system, Statew. Agric. L. Use Baseline 2015. 1 (2015). doi:10.1017/CBO9781107415324.004.
[39] B. Menand, T. Desnos, L. Nussaume, F. Berger, D. Bouchez, C. Meyer, C. Robaglia, Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene., Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 6422–7. doi:10.1073/pnas.092141899.
[40] R.-M. Leiber, F. John, Y. Verhertbruggen, A. Diet, J.P. Knox, C. Ringli, The TOR Pathway Modulates the Structure of Cell Walls in *Arabidopsis*, Plant Cell. 22 (2010) 1898–1908. doi:10.1105/tpc.109.073007.
[41] A. Barrada, M. Djendli, T. Desnos, R. Mercier, C. Robaglia, M.-H. Montané, B. Menand, A TOR-YAK1 signaling axis controls cell cycle, meristem activity and plant growth in Arabidopsis , Development. 146 (2019) dev171298. doi:10.1242/dev.171298.
[42] K. Hartig, E. Beck, Crosstalk between auxin, cytokinins, and sugars in the plant cell cycle, Plant Biol. 8 (2006) 389–396. doi:10.1055/s-2006-923797.
[43] F. Turck, F. Zilbermann, S.C. Kozma, G. Thomas, F. Nagy, C.H. Basel, P. Biology, Phytohormones Participate in an S6 Kinase Signal Transduction Pathway in Arabidopsis 1, Plant Physiol. 134 (2004) 1527–1535. doi:10.1104/pp.103.035873.1.
[44] M. Schepetilnikov, L.A. Ryabova, Recent discoveries on the role of tor (Target of rapamycin) signaling in translation in plants, Plant Physiol. 176 (2018) 1095–1105. doi:10.1104/pp.17.01243.
[45] K. Yokawa, F. Balusˇka, The TOR complex: An emergency switch for root behavior, Plant Cell Physiol. (2016). doi:10.1093/pcp/pcv191.
[46] P. Dong, F. Xiong, Y. Que, K. Wang, L. Yu, Z. Li, M. Ren, Expression profiling and functional analysis reveals that TOR is a key player in regulating photosynthesis and phytohormone signaling pathways in Arabidopsis, Front. Plant Sci. 6 (2015) 1–15. doi:10.3389/fpls.2015.00677.
[47] L. Bögre, R. Henriques, Z. Magyar, TOR tour to auxin., EMBO J. 32 (2013) 1069–71. doi:10.1038/emboj.2013.69.
[48] Y. Hao, F. Yu, R. Lv, C. Ma, Z. Zhang, Y. Rui, L. Liu, W. Cao, B. Xing, 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 (2016) e0157264. doi:10.1371/journal.pone.0157264.

9 Figures and legends

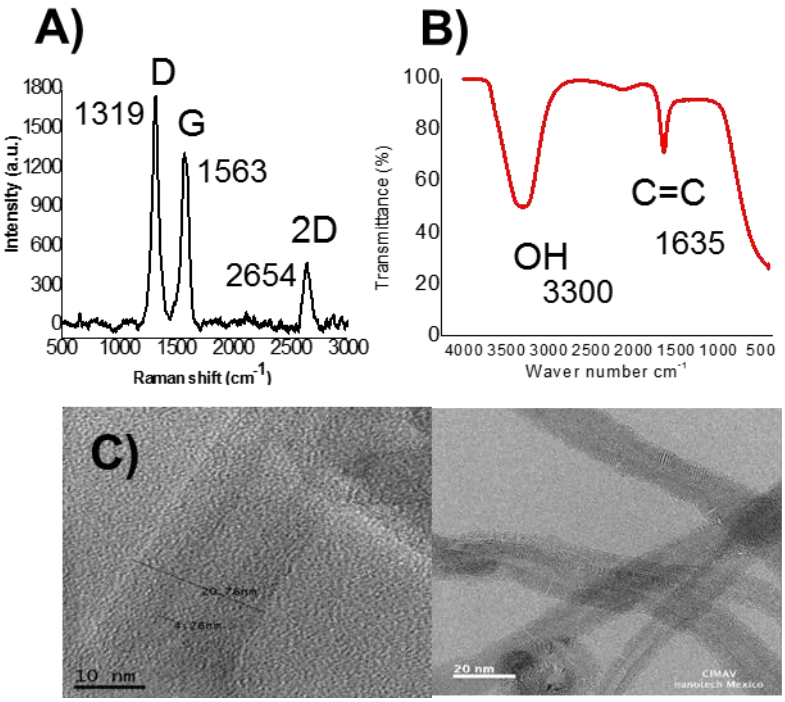


Figure 1. Analysis of synthetic MWCNTs with a purity >98% (Aldrich, Cat 698849). The figure A) show Raman spectra scattering spectra (He-Ne laser emitting at 514 nm) of the sample: Characteristic bands of MWCNTs are shown in synthetic MWCNTs ID band (~1319 cm^{-1}), IG band (~1563 cm^{-1}) and 2D band (1654cm^{-1}). The ID/IG ratio values corresponding are shown. B) FTIR spectra shows functional groups characteristic of MWCNTs; C) HR-TEM images of synthetic MWCNTs. Representative images are shown.

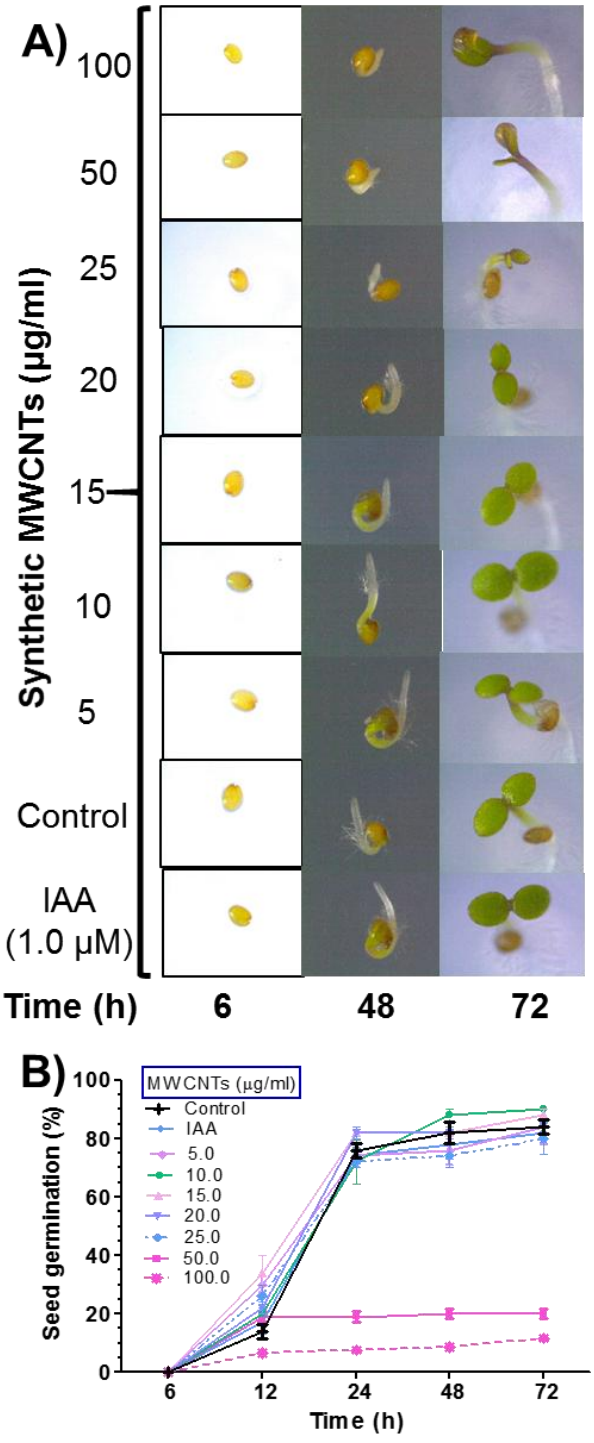


Figure 2. Effect of synthetic MWCNTs on seed germination of *A. thaliana*. A) Evaluation of seed germination of *A. thaliana WT* in an *in vitro* assay dose-response with synthetic MWCNTs at 6, 48, and 72 h after seed sowing. The IAA (1.0 µM) was used as positive control. B) Seed germination kinetic. Bars represent means ± SE of three independent assays. n= 30 each.

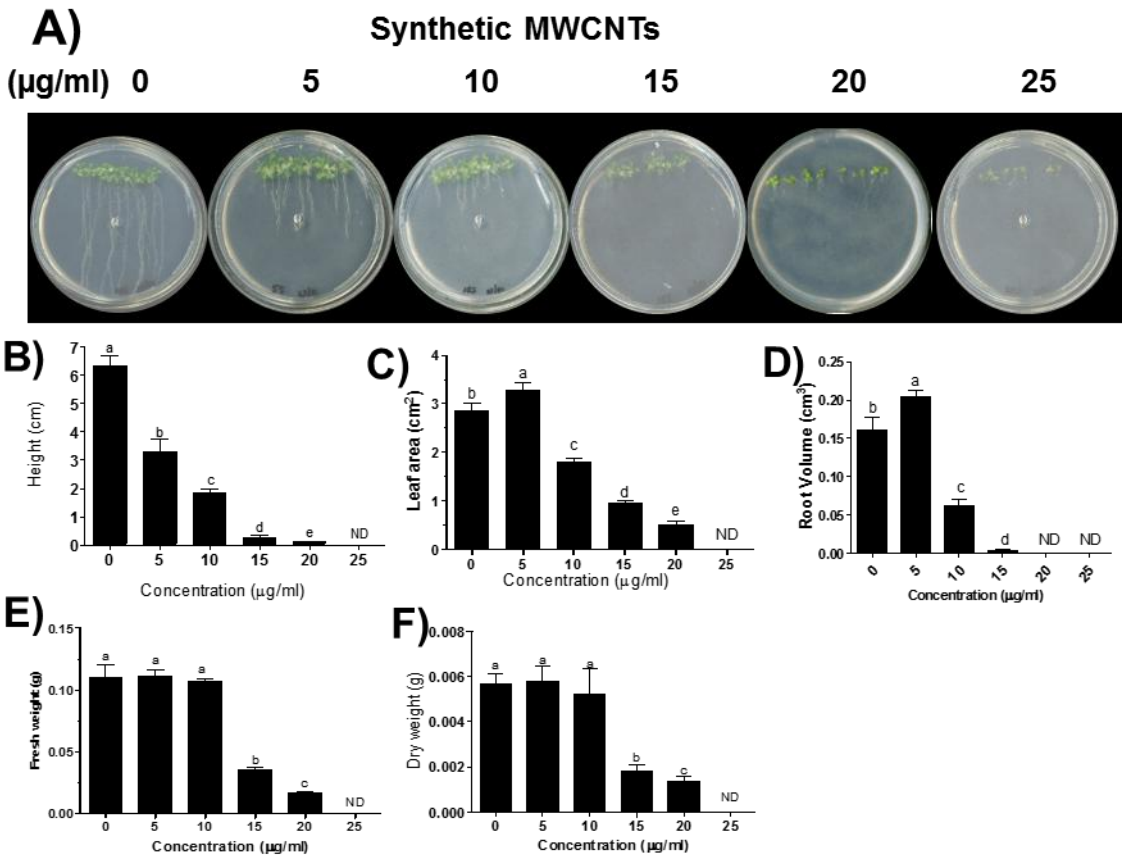


Figure 3. Effect of synthetic MWCNTs on plant growth and development of *A. thaliana*. A) Image of plant growth of *A. thaliana* WT in dose-response assay with synthetic MWCNTs 15 days after sowing. B-G) Morphometric and biomass parameters of *A. thaliana* in the *in vitro* dose-response assay with synthetic MWCNTs. B) Total height, C) leaf area, D) root volume, E) fresh weight, F) dry weight. Bars represent means ± SE of three independent assays. n= 30 each. One-way analysis of variance (ANOVA) was carried out with Tukey's post hoc test; statistical significance ($P < 0.05$) between treatments are indicated with different lowercase letters.

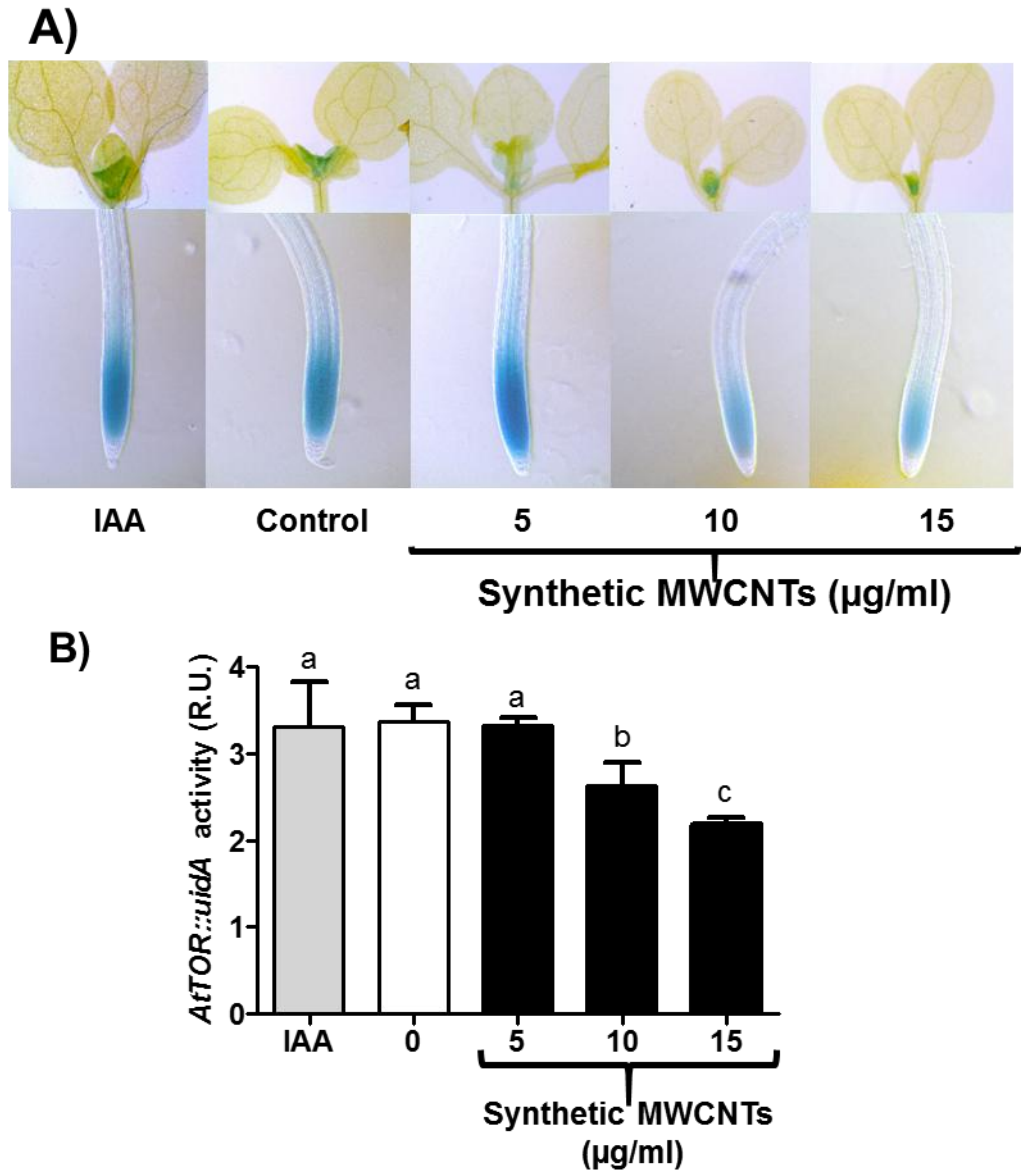


Figure 4. Effect of natural MWCNTs on the *AtTOR/tor1:: uidA* expression in *A. thaliana* roots. A) Image of *AtTOR/tor1:: uidA* expression in transgenic *A. thaliana* seedlings, β-glucuronidase (*GUS*) staining in dose-response assay with synthetic MWCNTs, 9 days after sowing. The IAA (1.0 µM) was used as positive control. B) *AtTOR/tor1:: uidA* roots expression kinetic, Bars represent means ± SE of three independent assays. n= 30 each.

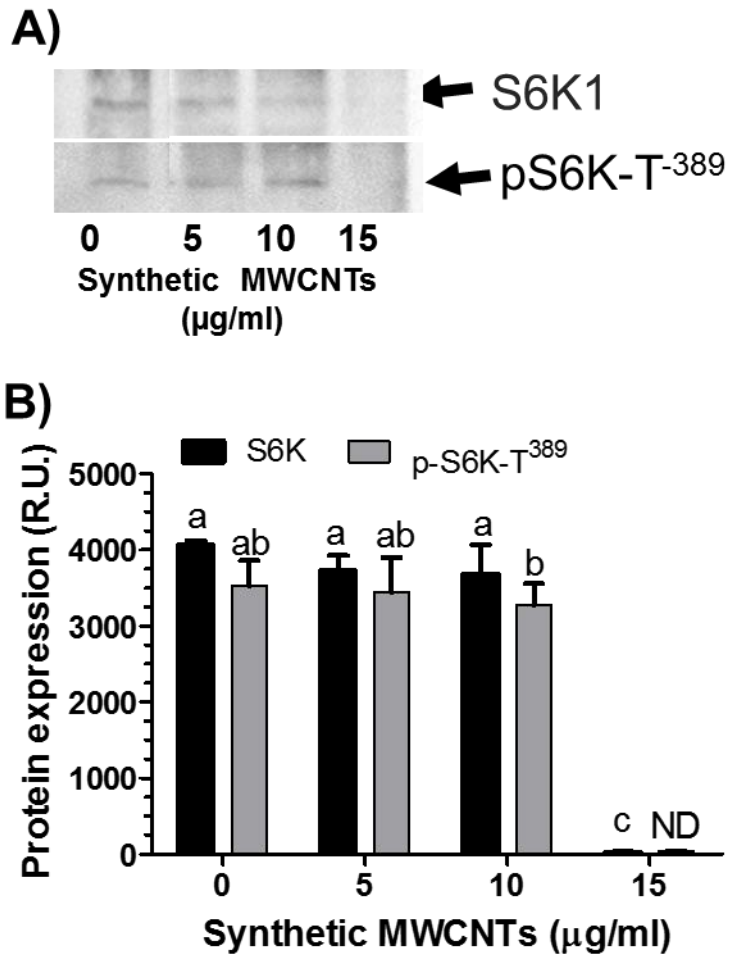


Figure 5. Effect of the synthetic MWCNTs on expression and phosphorylation of the S6K protein of *A. thaliana* seedlings. Seedlings were homogenized to obtain protein extracts used for Western blot assays as described in Materials and Methods. A) Representative images correspond to western blot using protein extracts of at least three independent assays using the indicated antibody. The anti-S6K and anti-S6K-Thr(P)-389 antibodies used are indicated. B) Graph corresponding to determination of the band intensity from the Western blot assay (A), analyzed by densitometry. Data represent the means ± SE of densitometry determinations, using protein extracts obtained from at least three independent assays. One-way ANOVA with Tukey's post-hoc test was used to compare treatment-times with respect to control. Significant differences ($P < 0.05$) between treatments are indicated with different lowercase letters. Bars represent means ± SE of three independent assays. n= 30 each.

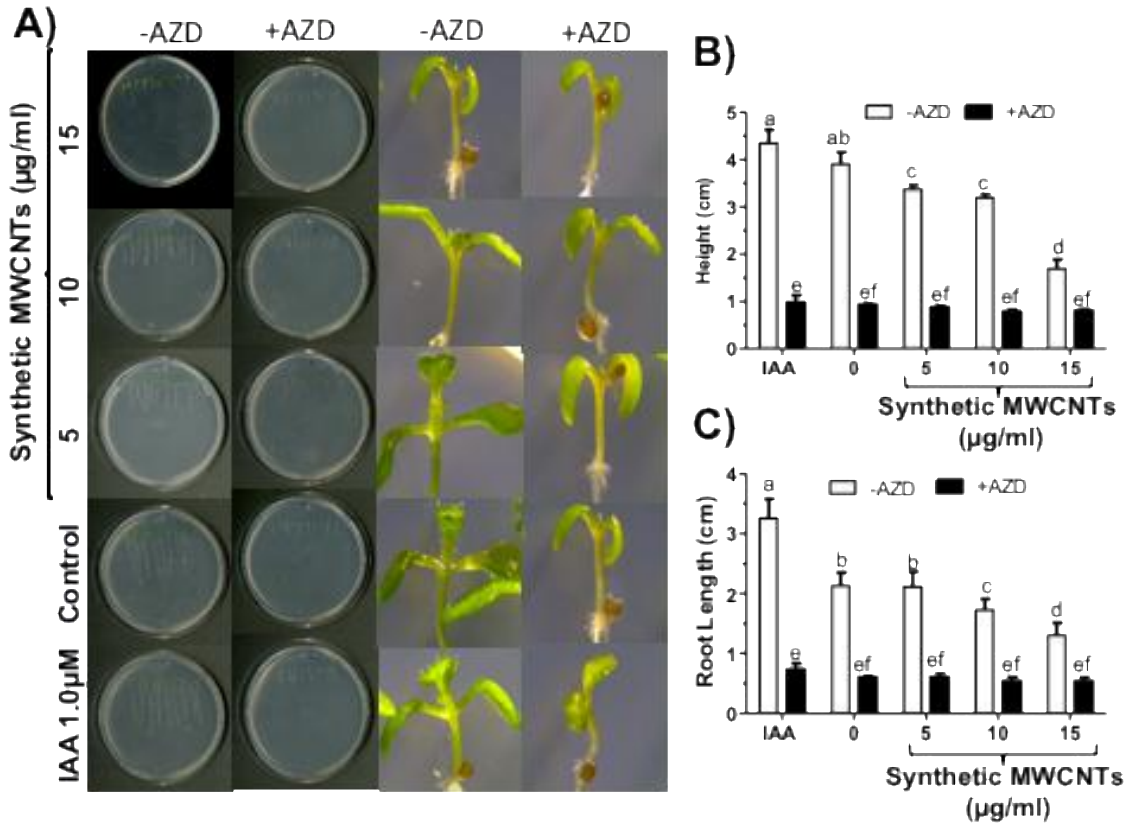


Figure 6. Effect of synthetic MWCNTs and AZD8055 TOR inhibitor on plant growth and development of *A. thaliana*. A) Image of plant growth of *A. thaliana* WT in dose-response assay with synthetic MWCNTs and supplemented with AZD8055 (1.0 µM), 9 days after sowing. B-C) Morphometric and biomass parameters of *A. thaliana*. B) Root length, C) total height. One-way analysis of variance (ANOVA) was carried out with Tukey's post hoc test; statistical significance ($P < 0.05$) between control and treatments are indicated with different lowercase letters. Bars represent means ± SE of three independent assays. n= 30 each.

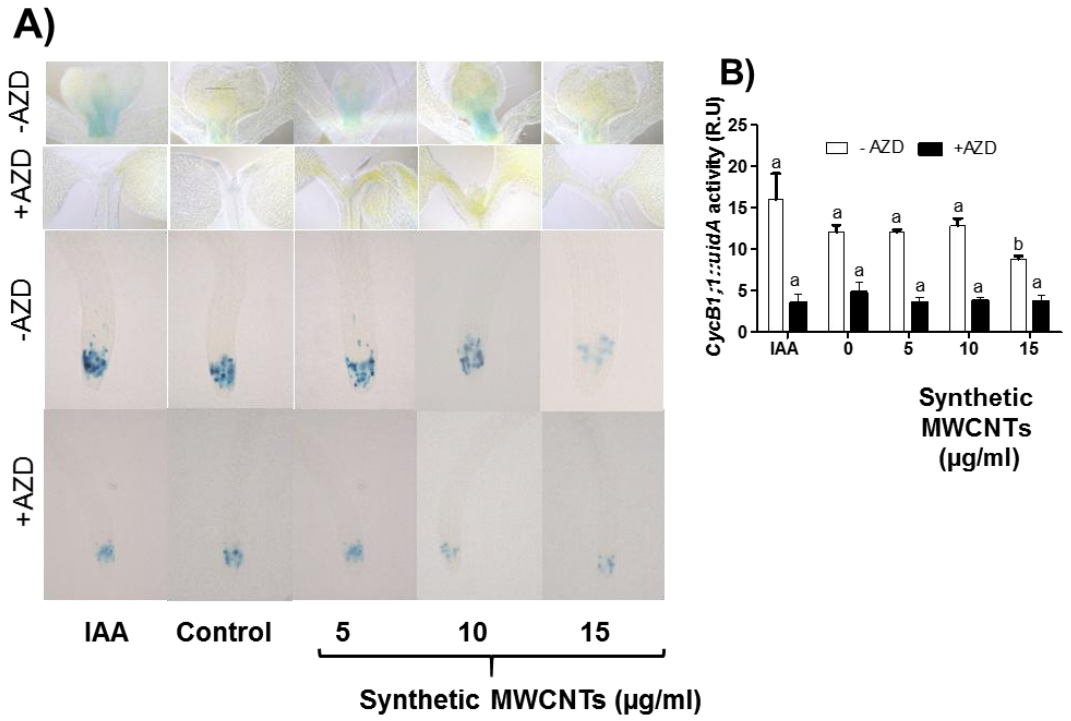


Figure 7. Effect of synthetic MWCNTs and AZD8055 TOR inhibitor on the *CYCB1;1::uidA* expression in *A. thaliana* roots. A) Roots photographs of *CYCB1;1::uidA* transgenic *Arabidopsis GUS* staining supplemented with synthetic MWCNTs and AZD8055 inhibitor (1.0 µM), 9 days after sowing. The IAA (1.0 µM) was used as positive control. B) *CYCB1;1::uidA* roots expression kinetic analyzed by densitometry using the Image J software (NIH). One-way ANOVA was carried out with Tukey's post hoc test; statistical significance ($P < 0.05$) between control and treatments are indicated with different lowercase letters. Bars represent means ± SE of three independent assays. n= 30 each.

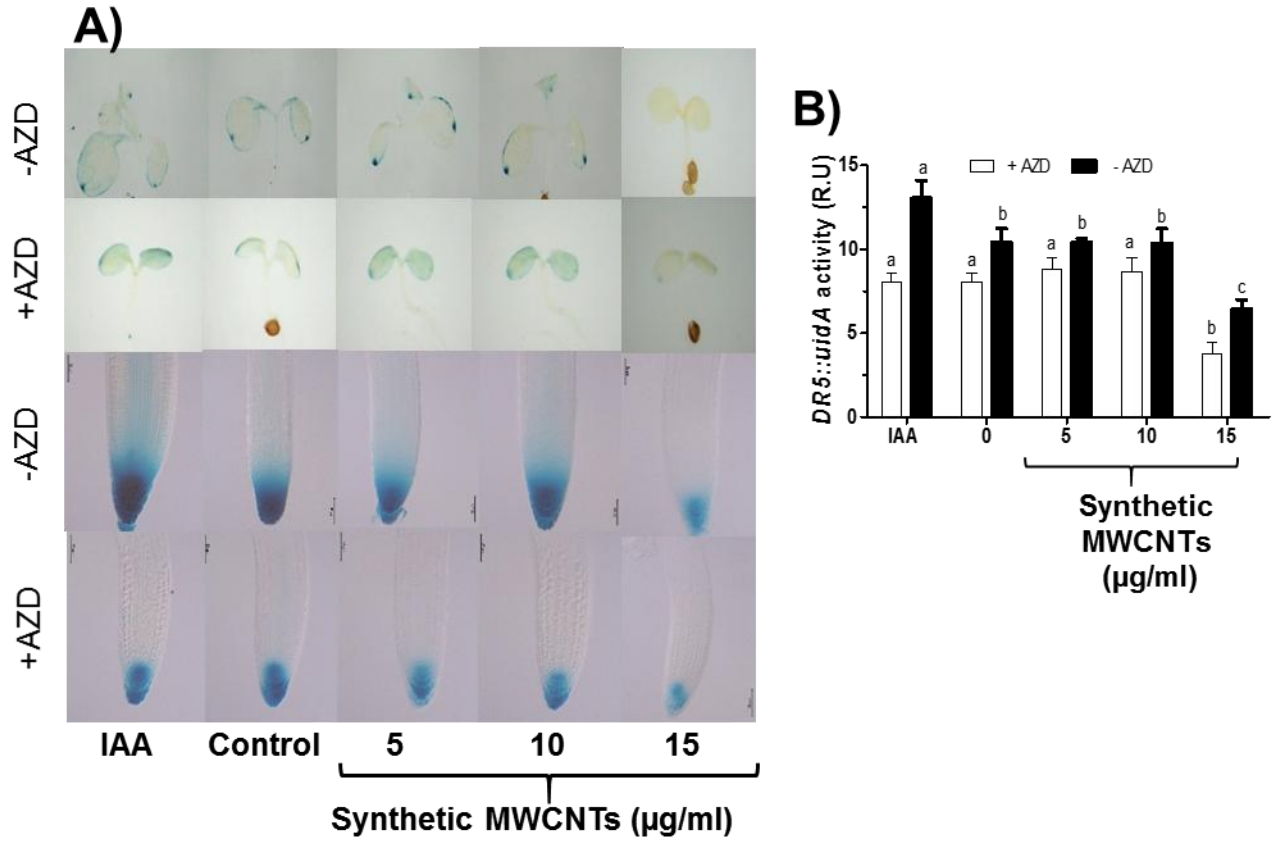


Figure 8. Effect of synthetic MWCNTs and AZD8055 TOR inhibitor on the *DR5::uidA* expression in *A. thaliana* roots. A) Roots photographs of *DR5::uidA* transgenic *Arabidopsis GUS* staining supplemented with synthetic MWCNTs and AZD8055 inhibitor (1.0 µM), 9 days after sowing. The IAA (1.0 µM) was used as positive control. B) *DR5::uidA* roots expression kinetic analyzed by densitometry using the Image J software (NIH). ANOVA was carried out with Tukey's post hoc test; statistical significance ($P < 0.05$) between control and treatments are indicated with different lowercase letters. Bars represent means ± SE of three independent assays. n= 30 each.

Table 1. Effect of the synthetic MWCNTs on the phytoregulators balance in *A. thaliana* WT seedlings.

	Control	Synthetic MWCNTs (10 ug/ml)
ABA	0.22	0.32
GA	0.11	**1.75***
IAA	11.36	13.91
IBA	26.71	**78.46***
JA	2.70	**3.68***
KT	1.36*	0.009
SA	13.51	14.766875

IAA (indoleacetic acid), abscisic acid (ABA), indole 3-butyric acid (IBA), gibberellic acid (GA), jasmonic acid (JA), kinetin (KT), and salicylic acid (SA). Phytoregulators concentration in ppb are shown. Values correspond to means with percentage of variance coefficient less than 10%, three independent assays. T-Students test was carried out, statistical significance ($P < 0.05$) between treatments comparing with the control are indicated with asterisks.

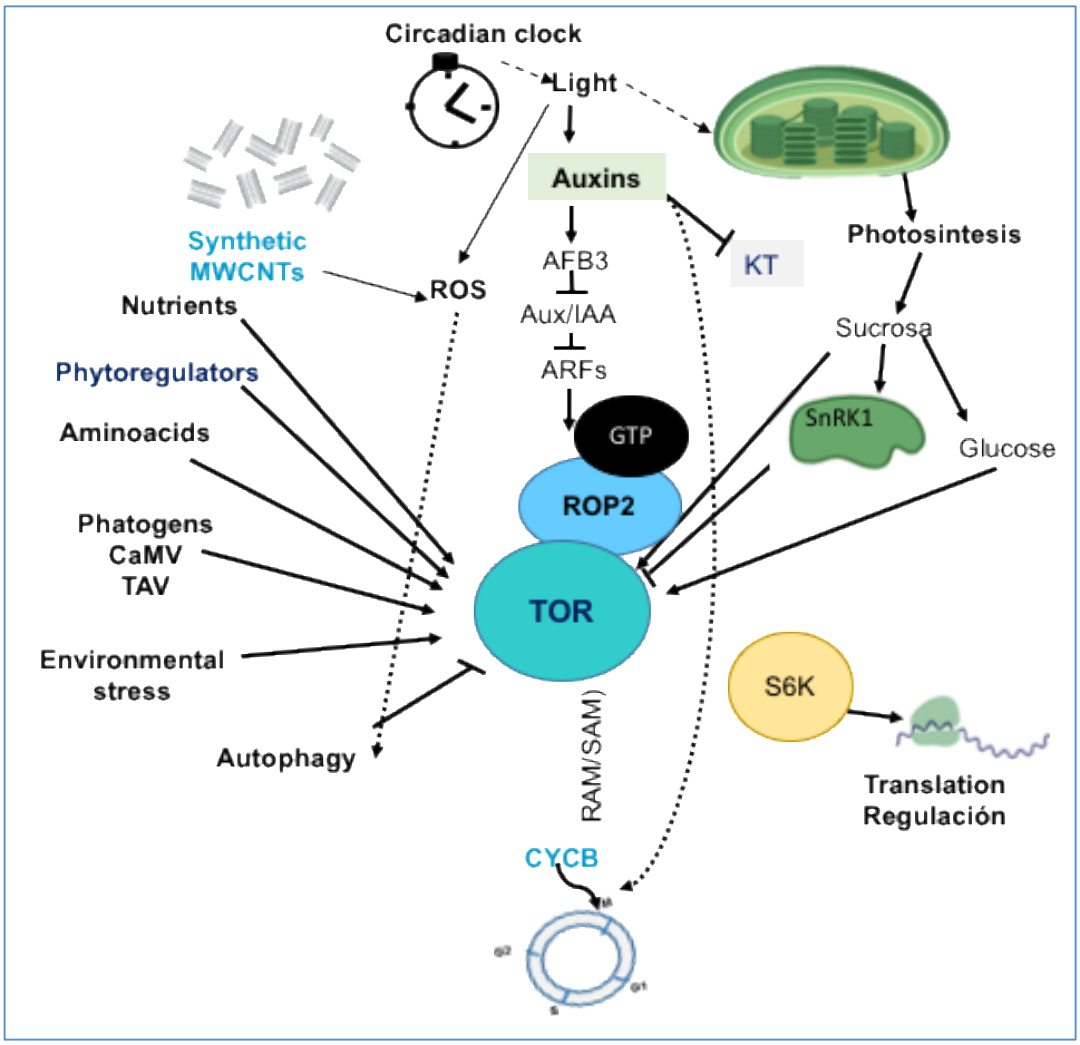


Figure 9. Effect of synthetic MWCNTs on the TOR signaling pathway and its interaction with phytoregulators as an affected mechanism during plant development.

*Root and shot apical meristem RAM/SAM

8. DISCUSIÓN

La nanotecnología es una disciplina emergente de interés multidisciplinario debido a que las nanopartículas tienen propiedades físicas y químicas extraordinarias que las hacen de interés industrial, tecnológico y científico [1,2]. Entre los nanomateriales, los nanotubos de carbono sintéticos de paredes múltiples (MWCNTs) se han considerado de origen sintético, sin embargo, recientemente se demostró que estos nanomateriales pueden generarse también durante los incendios forestales [28] y a la fecha se desconoce totalmente los efectos de estas nanopartículas en los sistemas biológicos.

Por otro lado, los MWCNTs sintéticos se han considerado nanopartículas con aplicaciones potenciales en la agricultura al promover el crecimiento vegetal [3–5]. Sin embargo, también se han documentado sus efectos fitotóxicos [13,14] por lo que se busca elucidar los diferentes mecanismos fisiológicos y moleculares que los MWCNTs afectan positiva o negativamente durante el desarrollo vegetal.

En este trabajo, aportamos el primer reporte sobre los efectos de los MWCNTs de origen natural en el desarrollo vegetal; se realizó un análisis comparativo de los efectos de los MWCNTs de origen natural *vs* MWCNTs de origen sintético a diferentes concentraciones sobre dos diferentes especies vegetales. *E. polystachya* al ser una leguminosa de importancia forestal en México y *A. thaliana*, una planta usada ampliamente como modelo biológico. Los resultados obtenidos mostraron que a dosis equivalentes los MWCNTs de origen natural promovieron el desarrollo vegetal en ambas plantas, mientras que los MWCNTs de origen sintético afectaron negativamente tanto a *E. polystachya* como en *A. thaliana*. Dichos efectos contrastantes del uso de MWCNTs sintéticos en las plantas se han atribuido a las propiedades intrínsecas de las nanopartículas: forma, dimensiones, conductividad eléctrica, estabilidad y su solubilidad limitada [5], así como la concentración de nanopartículas aplicada a las plantas y la especie vegetal utilizada como modelo biológico probado [15]. Por otro lado, la adición de carbono amorfo obtenido del mismo sitio de recolección de los MWCNTs de origen natural no causó diferencias significativas en el crecimiento y desarrollo vegetal en *E. polystachya* y en *A. thaliana*. Por lo que nuestros resultados sugieren que la concentración de nanopartículas adicionadas es un factor determinante en el efecto causado sobre el crecimiento y desarrollo vegetal de *E. polystachya* y *A. thaliana*; sin embargo, fue evidente que las propiedades físico-químicas de los MWCNTs afectó el desarrollo de estas plantas, y la diferencia estructural

entre los MWCNTs naturales y sintéticos puede ser un factor que contribuyó en los efectos contrastantes detectados en ambas plantas.

El desarrollo vegetal en condiciones naturales, involucra factores bióticos como las interacciones microbianas, insectos y nematodos [55], y abióticos, principalmente la temperatura, intensidad de la luz o la disponibilidad de agua, minerales esenciales y la homeostasis de fitorreguladores [56,57]. En la última década, el estudio de MWCNTs ha sido creciente, debido a los beneficios que estos nanomateriales pueden generar en las plantas, especialmente en cultivos de interés agronómico. En *E. polystachya*, así como en *A. thaliana* los MWCNTs naturales favorecieron la emergencia temprana de las semillas e incrementaron el porcentaje de germinación, por el contrario, los MWCNTs de origen sintético a dosis equivalentes afectaron la germinación de semillas. Estos resultados fueron consistentes con lo reportado por Lara et al., (2018) con el uso de MWCNTs de origen sintético, donde estas nanopartículas en *Lupinuss elegans* y *E. polystachya* incrementaron la tasa de germinación de semillas. Otros trabajos de investigación han documentado efectos similares sobre la germinación temprana e incrementos en el porcentaje de germinación generados por MWCNTs sintéticos en semillas de *Solanum lycopersicum* [40], *Avena sativa* [42], *Triticum* spp [41,144], en *Glycine max*, *Hordeum vulgare* y *Zea mays* [31]. El aumento de la germinación de semillas se ha asociado con una mayor absorción de agua en las semillas, facilitado por la formación de nuevos poros de la capa de las semillas y las paredes celulares por los MWCNT; sin embargo, el mecanismo de acción de estas estructuras en la germinación de semillas no es completamente claro. Por otro lado, el uso de MWCNTs sintéticos causó efectos negativos sobre la germinación tanto de *E. polystachya* como de *A. thaliana* a dosis equivalentes a las usadas de MWCNTs naturales. Efectos similares se han reportado en *Cucurbita pepo* (calabacín) [48,49] y en *Lactuca sativa* L [50]. De acuerdo con Lahiani *et al.*, (2013) los efectos de los MWCNTs en la germinación dependen de su translocación al interior de la semilla, influye la interacción de los materiales orgánicos suspendidos, su naturaleza coloidal y los vehículos que permiten su flujo [145]. Además, se ha documentado que varios factores químicos y físicos pueden influir en los eventos bioquímicos y fisiológicos que controlan la germinación en las semillas [146,147], por ejemplo la regulación de acuaporinas durante el proceso de germinación de las semillas [31].

Los resultados anteriores fueron consistentes durante las diferentes etapas fisiológicas de *E. polystachya* y de *A. thaliana*, donde se detectó que los MWCNTs formados naturalmente promovieron el crecimiento y desarrollo de ambas plantas, modificaron la arquitectura de la raíz de esta leguminosa, al generar un mayor número de raíces terciarias y un mayor volumen de raíz, lo que beneficia a la planta para su establecimiento, tener un mayor intercambio gaseoso, absorción de agua y minerales [143]. Además, las plantas tratadas con MWCNTs naturales mostraron un aumento significativo en los pesos secos tanto del follaje como de la raíz. Se ha documentado que los MWCNTs activan mecanismos de división celular [43] y promueven el alargamiento de las células de xilema y floema, que en consecuencia influyen en la absorción de agua y nutrientes [41,42].

En contraste, los MWCNTs sintéticos afectaron negativamente el follaje y desarrollo de la raíz, así como la biomasa y supervivencia de esta leguminosa a las dosis evaluadas. Los efectos tóxicos de los MWCNTs sintéticos en especies de interés agronómico también se han reportado previamente, como en *Lactuca sativa* [50], *Amaranthus tricolor* L. *sativus*.y *Cucumis sativus* [51]. En este estudio, encontramos que los MWCNT sintéticos, a las concentraciones probadas, afectaron negativamente el desarrollo de *E. polystachya,* al alterar la germinación, las variables morfométricas de las partes aéreas de la planta y la arquitectura de la raíz. Los mecanismos asociados con la toxicidad de los MWCNTs no se han dilucidado; sin embargo, están asociados con la inducción de muerte celular en las raíces y las hojas, al aumentar el estrés oxidativo causado por los MWCNTs, por incremento y acumulación de especies reactivas de oxígeno (ROS), daños en las membranas [7,17], la inhibición de enzimas antioxidantes [17], represión de la proliferación celular y muerte celular [22]. Lo anterior es de creciente interés, debido a que estas nanopartículas se han localizado en frutas y tejidos vegetales con importancia alimentaria y comercial.

Diferentes mecanismos fisiológicos y moleculares se han propuesto para intentar explicar cómo los MWCNTs y otras nanopartículas afectan el desarrollo vegetal; sin embargo, el efecto de estos nanomateriales en el complejo de señalización TOR no ha sido explorados, a pesar de que desempeña un papel clave como “interruptor” durante la progresión del ciclo celular [73,120]. La función de TOR implica modular el desarrollo vegetal, censar e integrar factores bióticos y abióticos como la disponibilidad de nutrientes y energía a través de procesos

metabólicos en las plantas [71], está involucrado en la regulación de la respuesta a estrés [27] o la homeostasis de fitorreguladores como las auxinas y citocininas [60,67], el ácido jasmónico y el ácido salicílico [65,77,78]; por lo que es una vía de señalización crucial en la regulación del crecimiento y desarrollo vegetal [60]. En este trabajo aportamos la primera evidencia del efecto de los MWCNTs naturales y sintéticos sobre la vía de señalización TOR como un mecanismo mediante el cual se modula el desarrollo vegetal.

Para determinar el papel de los MWCNTs en la vía de señalización de TOR, primero se estudió la expresión de la ß-glucuronidasa en la línea transgénica *AtTOR/tor::uidA* como respuesta a la adición de MWCNTs naturales. En la raíz de *A. thaliana* a dosis equivalentes, los MWCNTs naturales favorecieron la expresión del reportero *GUS*, equivalente a la obtenida en plantas con IAA, y superior que las plantas control (sin MWCNTs). Por el contrario, con el uso de MWCNTs de origen sintético, la expresión de la ß-glucuronidasa en la línea reportera *AtTOR/tor1::uidA* se observó que la expresión del reportero *GUS* en la raíz fue disminuyendo al incrementar la concentración de MWCNTs sintéticos. Por otro lado, se evaluó el efecto de los MWCNTs en la actividad de la S6K, una cinasa "rio abajo" de la proteína TOR, se cuantificó la proteína S6K y su estado fosforilado (p-p70S6-Thr389), usado ampliamente como indicador de la actividad de la ruta TOR. Se detectaron niveles similares de proteína S6K total tanto en el control como en las plantas tratadas a diferentes concentraciones de MWCNTs naturales. Interesantemente, los MWCNTs promovieron la activación de la proteína S6K, mostrando un aumento en su isoforma fosforilada en función de la concentración de MWCNTs naturales. En contraste, los resultados mostraron que los MWCNTs sintéticos afectaron la fosforilación de la proteína p-S6K-Thr^{389}, dependiente de las dosis agregadas de estas nanopartículas, a concentración de 15 µg/ml de MWCNTs la expresión total de la proteína S6K se inhibió. Se ha documentado que la inhibición de la vía TOR genera un sistema de raíz corto como consecuencia de la salida temprana de la etapa de la división celular e inicio de la fase de diferenciación anticipada [73,151]. Además, esta vía desempeña un papel clave en traducción y biogénesis de ribosomas, la regulación de la composición y estructura de la pared celular [152], lo que sugiere que la represión de la vía TOR podría estar inhibiendo colateralmente la expresión de otros genes que regulan la expansión celular y por tanto el crecimiento vegetal [73].

La proteína S6K desempeña diversas funciones debido a que es una parte integral conservada de la vía de señalización de TOR, es considerada el vínculo directo entre la vía TOR con el control de traducción, además está involucrada en la regulación del tamaño celular y la homeostasis de la glucosa en las plantas [150]. Por lo tanto, la estimulación de la expresión y la actividad de TOR indican, que los MWCNTs están modificando la actividad de la vía de señalización de TOR/S6K durante el desarrollo vegetal.

El crecimiento de las plantas es el resultado de un efecto combinado de proliferación celular (aumento en el número de células) y elongación celular (aumento en el tamaño de las células), ambos procesos están regulados por la vía TOR. Para determinar si el crecimiento y la promoción del desarrollo de las plantas en las plántulas de *A. thaliana* inducidas por MWCNTs son el resultado del alargamiento o proliferación celular y si estos procesos podrían asociarse y correlacionarse con la mayor actividad de la proteína S6K y la inducción de la expresión de TOR, analizamos la expresión de ciclina B1 en raíces de plantas a través de la línea transgénica *AtCycB1;1::uidA*, que es indicativa de proliferación celular [16]. Adicionalmente, se analizó el crecimiento de plantas transgénicas y la expresión de ciclina B1 utilizando el inhibidor de TOR, AZD8055. Sin el inhibidor, al igual que en las plantas de tipo silvestre, los tratamientos con MWCNTs naturales produjeron un mayor desarrollo de las plántulas, mientras que con los MWCNTs sintéticos se disminuyó el tamaño de las plántulas. Estos hallazgos fueron consistentes con el efecto sobre la expresión de ciclina B1 en las raíces, donde los MWCNTs favorecieron la actividad de *GUS*, indicativo de un aumento en la expresión de ciclina B1; pero por el contrario, los MWCNTs sintéticos afectaron la expresión de ciclina B1. Inesperadamente, la adición de MWCNTs naturales en presencia de AZD8055 (inhibidor de TOR) causó una fuerte inhibición en la germinación y el desarrollo de las plántulas de *A. thaliana* con NTC naturales y sintéticos, la adición de AZD8055 generó plantas pequeñas cuyas raíces y follaje mostraron crecimiento limitado, fenotipo característico de la adición de inhibidores ATP competitivos de TOR [153]. Asimismo, se observó una disminución de la actividad del marcador *AtCycB1;1::uidA* en las raíces de *A. thaliana* en todas las concentraciones de NTC aplicadas; esta inhibición causada por la adición de AZD8055 sugieren que la expresión de ciclina B1 modulada por la adición de MWCNTs sintéticos ocurre mediante un mecanismo de señalización dependiente de la ruta TOR. De acuerdo con Hartig et al. (2006), los genes de ciclina como *CYCB1* están parcialmente

regulados por cinasas del tipo TOR-S6K [154], lo que indica que la reducción en la expresión de ciclina B1 fue causada por la presencia de los MWCNTs ocurrió por la inhibición de la vía TOR.

Por otro lado, se ha documentado ampliamente el papel que desempeñan las auxinas sobre la división celular y el desarrollo vegetal, al inducir respuestas de crecimiento en todas las etapas del desarrollo del ciclo de vida de las plantas; por ejemplo, regulan respuestas a la luz y la gravedad, la organogénesis, la arquitectura radical y de brotes y el desarrollo vascular, asimismo, estas inducen respuestas (división y expansión celular) dependiente de la etapa fisiológica de desarrollo de la planta [83]. Además, se ha documentado, que la vía TOR actúa como un factor esencial para la transducción de señales de auxina en *Arabidopsis* [67,72,148] y las auxinas a su vez desencadenan la fosforilación de la proteína TOR y posteriormente la de la S6K a través de la proteina ROP2, "rio abajo" en la cascada de señalización [155,156]. Por lo que, las auxinas se han propuesto como activadores de las reacciones rio arriba de la vía de señalización TOR [72]; involucradas en la señalización de disponibilidad de glucosa y luz, así como en el efecto de auxinas aplicadas exógenamente, estas activaban S6K1 en los meristemos de los brotes como indicador de la actividad de la ruta TOR.

Para determinar el papel de las auxinas en la interacción MWCNTs-TOR durante el desarrollo vegetal, se analizó la expresión de la línea transgénica *DR5::uidA*, usada ampliamente para la visualización de la localización de auxinas. Los resultados mostraron que los MWCNTs naturales aumentaron significativamente la actividad de *GUS*, equivalente al efecto de AIA a 1,0 μM aplicado exógenamente. Mientras que el efecto de los MWCNTs sintéticos afectó negativamente la expresión *DR5::uidA* al ir incrementando la concentración de estas nanopartículas. Interesantemente, en la línea transgénica *DR5::uidA* cultivada en presencia de AZD8055 y de MWCNTs naturales, la actividad del marcador auxínico no fue afectado. En contraste, con el uso de MWCNTs sintéticos se detectó que al inhibir la vía TOR con el AZD8055, también la actividad auxínica se vio afectada. Estos resultados sugieren que tanto los MWCNTs naturales como sintéticos durante el desarrollo vegetal intervienen directamente en la modulación de la síntesis o movilización de auxinas, lo cual está directamente interconectado con la vía de señalización de TOR. Sin embargo, el uso de MWCNTs naturales también puede regular la actividad de auxinas mediante un mecanismo independiente de la vía de TOR.

Por otro lado, la deficiente señalización de TOR afecta el desarrollo de las plantas mediado por auxinas [72]. De acuerdo con Yokawa y Balusˇka1, en raíces la vía de TOR puede ser activada por la acumulación de ROS para controlar esta vía "rio abajo", incluidas la regulación de auxinas, y negativamente el proceso de autofagia [157].

Además de las auxinas, diversos fitorreguladores desempeñan un papel clave durante las diferentes etapas del ciclo biológico vegetal [73]. Por lo que evaluamos los efectos de los MWCNTs naturales y sintéticos en *A. thaliana*. Nuestros hallazgos mostraron un claro efecto de los MWCNTs naturales en la estimulación de la acumulación de diferentes fitorreguladores. Por ejemplo, en las plántulas de manera dependiente de la concentración adicionada de nanopartículas se detectaron incrementos significativos en la abundancia de auxinas como el ácido-indol-3-butírico y el ácido 3-butírico (IBA), también en el ácido giberélico (GA), ácido jasmónico (JA) y ácido salicílico (SA). En contraste, con la adición de MWCNTs naturales se detectó una fuerte disminución de Kt. Con la adición de MWCNTs de origen sintético en plántulas de *Arabidopsis*, se detectó que el fitorregulador más abundante fue el IBA, un precursor del IAA, seguido de GAs, también se detectaron incrementos significativos en la abundancia SA y ABA, mientras que hubo una disminución de Kt. Estos fitorreguladores en sinergia regulan la proliferación celular y tamaño de los meristemos vegetales [61], participan en la iniciación de la raíz, en la formación y desarrollo de raíces laterales y adventicias [49]. Sin embargo, el papel de estos fitorreguladores en la vía TOR aún es desconocido: por ejemplo, se ha reportado que el papel de las auxinas sobre la vía TOR es através de la GTPasa ROP2, la cual al ser activada por las auxinas desencadena una cascada de señalización "rio abajo" hasta la fosforilación de S6K [59,60]. Mientras que el papel de las giberelinas ha sido menos estudiado en esta vía, sin embargo es posible que al igual que las auxinas participe en estimular la vía TOR, ya que este fitorregulador participa en diferentes etapas del desarrollo vegetal, desde la iniciación de la germinación, dormancia, regular la elongación vegetal, crecimiento de la raíz, hojas, tallos y frutos, así como en la floración [56]. Asimismo, se ha documentado que fitorreguladores como el ABA, o el JA y SA juegan un papel antagónico en la vía de TOR [17,28].

A la fecha, el papel de los MWCNTs sintéticos en el balance de fitorreguladores comienza a ser explorado. El primer reporte sobre el efecto de MWCNTs sintéticos de diferentes características estructurales fue documentado por Hao *et al.*, (2016), estos investigadores

demostraron que los MWCNTs afectaron negativamente el crecimiento *Oryza sativa* L. al disminuir las concentraciones de IBA, IPA, ABA, JA y brasioesteroides endógenos [149], por lo que los resultados obtenidos sugieren que los efectos generados en *A. thaliana* son dependiente de esta alteración en el balance de fitorreguladores causado por los MWCNTs.

9. CONCLUSIÓN

Los MWCNTs naturales y sintéticos generaron efectos contrarios en el desarrollo de *E. Polystachya* y *A. thaliana*. Mientras que los MWCNTs naturales promovieron el crecimiento de estas plantas, los MWCNTs sintéticos inhibieron el desarrollo vegetal. Dichos efectos implican la actividad de la vía de señalización de TOR y su correlación con el balance de fitorreguladores. Adicionalmente, los resultados sugieren que los MWCNTs naturales actúan sobre la vía de señalización dependiente de auxinas a través de la vía TOR, pero también mediante algún mecanismo independiente de esta vía de señalización.

10.REFERENCIAS

[1] M. Vithanage, M. Seneviratne, M. Ahmad, B. Sarkar, Y.S. Ok, Correction to: Contrasting effects of engineered carbon nanotubes on plants: a review, Environ. Geochem. Health. 39 (2018) 1. doi:10.1007/s10653-017-0050-3.

[2] I. Khan, K. Saeed, I. Khan, Nanoparticles: Properties, applications and toxicities, Arab. J. Chem. (2017). doi:10.1016/j.arabjc.2017.05.011.

[3] C. Schmidt, J. Storsberg, Nanomaterials—Tools, Technology and Methodology of Nanotechnology Based Biomedical Systems for Diagnostics and Therapy, Biomedicines. 3 (2015) 203–223. doi:10.3390/biomedicines3030203.

[4] M.K. Teli, S. Mutalik, G.K. Rajanikant, Nanotechnology and nanomedicine: going small means aiming big., Curr. Pharm. Des. 16 (2010) 1882–92. http://www.ncbi.nlm.nih.gov/pubmed/20222866.

[5] P.K. Rai, V. Kumar, S.S. Lee, N. Raza, K.H. Kim, Y.S. Ok, D.C.W. Tsang, Nanoparticle-plant interaction: Implications in energy, environment, and agriculture, Environ. Int. 119 (2018) 1–19. doi:10.1016/j.envint.2018.06.012.

[6] S.K. Verma, A.K. Das, M.K. Patel, A. Shah, V. Kumar, S. Gantait, Engineered nanomaterials for plant growth and development: A perspective analysis, Sci. Total Environ. 630 (2018) 1413–1435. doi:10.1016/j.scitotenv.2018.02.313.

[7] Y.Q. Deng, J.C. White, B.S. Xing, Interactions between engineered nanomaterials and agricultural crops: Implications for food safety, J. Zhejiang Univ. Sci. A. 15 (2014) 552–572. doi:10.1631/jzus.A1400165.

[8] Y. Ju-Nam, J.R. Lead, Manufactured nanoparticles: An overview of their chemistry, interactions and potential environmental implications, Sci. Total Environ. 400 (2008) 396–414. doi:10.1016/j.scitotenv.2008.06.042.

[9] S.K. Verma, A.K. Das, S. Gantait, V. Kumar, E. Gurel, Applications of carbon nanomaterials in the plant system: A perspective view on the pros and cons, Sci. Total Environ. 667 (2019) 485–499. doi:10.1016/j.scitotenv.2019.02.409.

[10] M. Sorbiun, E. Shayegan Mehr, A. Ramazani, A. Mashhadi Malekzadeh, Biosynthesis of metallic nanoparticles using plant extracts and evaluation of their antibacterial properties ARTICLE INFO, Nanochem Res. 3 (2018) 1–16. doi:10.22036/ncr.2018.01.001.

[11] M.S. Mauter, M. Elimelech, Environmental applications of carbon-based nanomaterials, Environ. Sci. Technol. 42 (2008) 5843–5859. doi:10.1021/es8006904.

[12] A. Astefanei, O. Núñez, M.T. Galceran, Characterisation and determination of fullerenes: A critical review, Anal. Chim. Acta. 882 (2015) 1–21. doi:10.1016/j.aca.2015.03.025.

[13] W.-W. Liu, S.-P. Chai, A.R. Mohamed, U. Hashim, Synthesis and characterization of graphene and carbon nanotubes: A review on the past and recent developments, J. Ind. Eng. Chem. 20 (2014) 1171–1185. doi:10.1016/j.jiec.2013.08.028.

[14] E.T. Thostenson, Z. Ren, T. Chou, N19-P-AM13.pdf, 61 (2001) 1899–1912. doi:10.1016/S0266-3538(01)00094-X.

[15] N. Saifuddin, A.Z. Raziah, A.R. Junizah, Carbon nanotubes: A review on structure and their interaction with proteins, J. Chem. 2013 (2013). doi:10.1155/2013/676815.

[16] J.N. Coleman, U. Khan, W.J. Blau, Y.K. Gun'ko, Small but strong: A review of the mechanical properties of carbon nanotube-polymer composites, Carbon N. Y. 44

(2006) 1624–1652. doi:10.1016/j.carbon.2006.02.038.
[17] S. Lijima, Helical microtubules of graphitic carbon, Nature. 354 (1991) 56–58.
[18] M.F.L. De Volder, S.H. Tawfick, R.H. Baughman, a J. Hart, Carbon nanotubes: present and future commercial applications., Science. 339 (2013) 535–9. doi:10.1126/science.1222453.
[19] M. Yu, B.S. Files, S. Arepalli, R.S. Ruoff, Tensile Loading of Ropes of Single Wall Carbon Nanotubes and their Mechanical Properties, Phys. Rev. Lett. 1 (2000) 1–4.
[20] H. Yuan, S. Hu, P. Huang, H. Song, K. Wang, J. Ruan, R. He, D. Cui, Single Walled Carbon Nanotubes Exhibit Dual-Phase Regulation to Exposed Arabidopsis Mesophyll Cells, Nanoscale Res. Lett. 6 (2011) 1–9. doi:10.1007/s11671-010-9799-3.
[21] J.E. Cañas, M. Long, S. Nations, R. Vadan, L. Dai, M. Luo, R. Ambikapathi, E.H. Lee, D. Olszyk, Effects of functionalized and nonfunctionalized single-walled carbon nanotubes on root elongation of select crop species., Environ. Toxicol. Chem. 27 (2008) 1922–1931. doi:10.1897/08-117.1.
[22] M. V Khodakovskaya, B.-S. Kim, J.N. Kim, M. Alimohammadi, E. Dervishi, T. Mustafa, C.E. Cernigla, Carbon nanotubes as plant growth regulators: effects on tomato growth, reproductive system, and soil microbial community., Small. 9 (2013) 115–23. doi:10.1002/smll.201201225.
[23] J.A. Elliott, Y. Shibuta, H. Amara, C. Bichara, E.C. Neyts, Atomistic modelling of CVD synthesis of carbon nanotubes and graphene, Nanoscale. 5 (2013) 6662–6676. doi:10.1039/c3nr01925j.
[24] W.W. Liu, S.P. Chai, A.R. Mohamed, U. Hashim, Synthesis and characterization of graphene and carbon nanotubes: A review on the past and recent developments, J. Ind. Eng. Chem. 20 (2014) 1171–1185. doi:10.1016/j.jiec.2013.08.028.
[25] R.A. Afre, T. Soga, T. Jimbo, M. Kumar, Y. Ando, Carbon nanotubes by spray pyrolysis of turpentine oil at different temperatures and their studies, Microporous Mesoporous Mater. 96 (2006) 184–190. doi:10.1016/j.micromeso.2006.06.036.
[26] R.J. Andrews, C.F. Smith, A.J. Alexander, Mechanism of carbon nanotube growth from camphor and camphor analogs by chemical vapor deposition, Carbon N. Y. 44 (2006) 341–347. doi:10.1016/j.carbon.2005.07.025.
[27] J. Zhao, Growth of carbon nanotubes on natural organic precursors by chemical vapor deposition, Carbon N. Y. 49 (2011) 2155–2158. doi:10.1016/j.carbon.2011.01.030.
[28] J. Lara-Romero, J. Campos-García, N. Dasgupta-Schubert, S. Borjas-García, D. Tiwari, F. Paraguay-Delgado, S. Jiménez-Sandoval, G. Alonso-Nuñez, M. Gómez-Romero, R. Lindig-Cisneros, H. Reyes De la Cruz, J.A. Villegas, Biological effects of carbon nanotubes generated in forest wildfire ecosystems rich in resinous trees on native plants, PeerJ. 5 (2017) e3658. doi:10.7717/peerj.3658.
[29] S.S. Mukhopadhyay, Nanotechnology in agriculture: Prospects and constraints, Nanotechnol. Sci. Appl. 7 (2014) 63–71. doi:10.2147/NSA.S39409.
[30] D.Y. Kim, A. Kadam, S. Shinde, R.G. Saratale, J. Patra, G. Ghodake, Recent developments in nanotechnology transforming the agricultural sector: a transition replete with opportunities, J. Sci. Food Agric. 98 (2018) 849–864. doi:10.1002/jsfa.8749.
[31] M.H. Lahiani, E. Dervishi, J. Chen, Z. Nima, A. Gaume, A.S. Biris, M. V. Khodakovskaya, Impact of carbon nanotube exposure to seeds of valuable crops, ACS Appl. Mater. Interfaces. 5 (2013) 7965–7973. doi:10.1021/am402052x.

[32] M.H. Lahiani, Z.A. Nima, H. Villagarcia, A.S. Biris, M. V. Khodakovskaya, Assessment of Effects of the Long-Term Exposure of Agricultural Crops to Carbon Nanotubes, J. Agric. Food Chem. (2017) acs.jafc.7b01863. doi:10.1021/acs.jafc.7b01863.

[33] Z. Yuan, Z. Zhang, X. Wang, L. Li, K. Cai, H. Han, Novel impacts of functionalized multi-walled carbon nanotubes in plants: promotion of nodulation and nitrogenase activity in the rhizobium-legume system, Nanoscale. 9 (2017) 9921–9937. doi:10.1039/C7NR01948C.

[34] H. Chen, R. Yada, Nanotechnologies in agriculture: New tools for sustainable development, Trends Food Sci. Technol. 22 (2011) 585–594. doi:10.1016/j.tifs.2011.09.004.

[35] R. Nair, S.H. Varghese, B.G. Nair, T. Maekawa, Y. Yoshida, D.S. Kumar, Nanoparticulate material delivery to plants, Plant Sci. 179 (2010) 154–163. doi:10.1016/j.plantsci.2010.04.012.

[36] L.R. Khot, S. Sankaran, J.M. Maja, R. Ehsani, E.W. Schuster, Applications of nanomaterials in agricultural production and crop protection: A review, Crop Prot. 35 (2012) 64–70. doi:10.1016/j.cropro.2012.01.007.

[37] M. Rai, Role of nanotechnology in agriculture with special reference to pest control, Appl. Microbiol. Biotechnol. 1 (2012) 287–293. doi:10.1007/s00253-012-3969-4.

[38] D.K. Tiwari, N. Dasgupta-Schubert, L.M.V. Cendejas, J. Villegas, L.C. Montoya, S.E.B. García, Interfacing carbon nanotubes (CNT) with plants: enhancement of growth, water and ionic nutrient uptake in maize (Zea mays) and implications for nanoagriculture, Appl. Nanosci. 4 (2014) 577–591. doi:10.1007/s13204-013-0236-7.

[39] S. Tripathi, S.K. Sonkar, S. Sarkar, Growth stimulation of gram (Cicer arietinum) plant by water soluble carbon nanotubes., Nanoscale. 3 (2011) 1176–1181. doi:10.1039/c0nr00722f.

[40] M. V. Khodakovskaya, B.S. Kim, J.N. Kim, M. Alimohammadi, E. Dervishi, T. Mustafa, C.E. Cernigla, Carbon nanotubes as plant growth regulators: Effects on tomato growth, reproductive system, and soil microbial community, Small. 9 (2013) 115–123. doi:10.1002/smll.201201225.

[41] A. Joshi, S. Kaur, K. Dharamvir, H. Nayyar, G. Verma, Multi-walled carbon nanotubes applied through seed-priming influence early germination, root hair, growth and yield of bread wheat (Triticum aestivum L.), J. Sci. Food Agric. 98 (2018). doi:10.1002/jsfa.8818.

[42] A. Joshi, S. Kaur, P. Singh, K. Dharamvir, H. Nayyar, G. Verma, Tracking multi-walled carbon nanotubes inside oat (Avena sativa L.) plants and assessing their effect on growth, yield, and mammalian (human) cell viability, Appl. Nanosci. 0 (2018) 0. doi:10.1007/s13204-018-0801-1.

[43] M. V. Khodakovskaya, K. De Silva, A.S. Biris, E. Dervishi, H. Villagarcia, Carbon nanotubes induce growth enhancement of tobacco cells, ACS Nano. 6 (2012) 2128–2135. doi:10.1021/nn204643g.

[44] D.L. McGehee, M.H. Lahiani, F. Irin, M.J. Green, M. V. Khodakovskaya, Multiwalled Carbon Nanotubes Dramatically Affect the Fruit Metabolome of Exposed Tomato Plants, ACS Appl. Mater. Interfaces. 9 (2017) 32430–32435. doi:10.1021/acsami.7b10511.

[45] M. Ghorbanpour, J. Hadian, Multi-walled carbon nanotubes stimulate callus induction, secondary metabolites biosynthesis and antioxidant capacity in medicinal plant

Satureja khuzestanica grown in vitro, Carbon N. Y. 94 (2015) 749–759. doi:10.1016/j.carbon.2015.07.056.
[46] T.M. Scown, R. Van Aerle, C.R. Tyler, Review: Do engineered nanoparticles pose a significant threat to the aquatic environment, Crit. Rev. Toxicol. 40 (2010) 653–670. doi:10.3109/10408444.2010.494174.
[47] P. Jackson, N.R. Jacobsen, A. Baun, R. Birkedal, D. Kühnel, K.A. Jensen, U. Vogel, H. Wallin, Bioaccumulation and ecotoxicity of carbon nanotubes, Chem. Cent. J. 7 (2013) 154–165. doi:10.1186/1752-153X-7-154.
[48] D. Stampoulis, S.K. Sinha, J.C. White, Assay-dependent phytotoxicity of nanoparticles to plants, Environ. Sci. Technol. 43 (2009) 9473–9479. doi:10.1021/es901695c.
[49] M. Hatami, Toxicity assessment of multi-walled carbon nanotubes on Cucurbita pepo L. under well-watered and water-stressed conditions, Ecotoxicol. Environ. Saf. 142 (2017) 274–283. doi:10.1016/j.ecoenv.2017.04.018.
[50] R. Ikhtiari, P. Begum, F. Watari, B. Fugetsu, Toxic Effect of Multiwalled Carbon Nanotubes on Lettuce (Lactuca Sativa), 5 (2013) 18–24.
[51] P. Begum, R. Ikhtiari, B. Fugetsu, Potential Impact of Multi-Walled Carbon Nanotubes Exposure to the Seedling Stage of Selected Plant Species, Nanomaterials. 4 (2014) 203–221. doi:10.3390/nano4020203.
[52] O. Zaytseva, Differential Impact of Multi-Walled Carbon Nanotubes on Germination and Seedling Development of Glycine Max , Phaseolus Vulgaris, (2016) 202–210. doi:10.17628/ECB.2016.5.202.
[53] M. Hatami, K. Kariman, M. Ghorbanpour, Engineered nanomaterial-mediated changes in the metabolism of terrestrial plants, Sci. Total Environ. 571 (2016) 275–291. doi:10.1016/j.scitotenv.2016.07.184.
[54] X.M. Tan, C. Lin, B. Fugetsu, Studies on toxicity of multi-walled carbon nanotubes on suspension rice cells, Carbon N. Y. 47 (2009) 3479–3487. doi:10.1016/j.carbon.2009.08.018.
[55] Y. Yang, Y. Dou, S. An, Z. Zhu, Abiotic and biotic factors modulate plant biomass and root/shoot (R/S) ratios in grassland on the Loess Plateau, China, Sci. Total Environ. 636 (2018) 621–631. doi:10.1016/j.scitotenv.2018.04.260.
[56] C. Hermans, J.P. Hammond, P.J. White, N. Verbruggen, How do plants respond to nutrient shortage by biomass allocation?, Trends Plant Sci. 11 (2006) 610–617. doi:10.1016/j.tplants.2006.10.007.
[57] U. Bechtold, B. Field, Molecular mechanisms controlling plant growth during abiotic stress, J. Exp. Bot. 69 (2018) 2753–2758. doi:10.1093/jxb/ery157.
[58] D. Rexin, C. Meyer, C. Robaglia, B. Veit, TOR signalling in plants, Biochem. J. 470 (2015) 1–14. doi:10.1042/BJ20150505.
[59] Y. Xiong, J. Sheen, The role of target of rapamycin signaling networks in plant growth and metabolism., Plant Physiol. 164 (2014) 499–512. doi:10.1104/pp.113.229948.
[60] Y. Xiong, J. Sheen, Novel links in the plant TOR kinase signaling network., Curr. Opin. Plant Biol. 28 (2015) 83–91. doi:10.1016/j.pbi.2015.09.006.
[61] Y. Pu, X. Luo, D.C. Bassham, Tor-dependent and -independent pathways regulate autophagy in arabidopsis thaliana, Front. Plant Sci. 8 (2017) 1–13. doi:10.3389/fpls.2017.01204.
[62] A. Barrada, M.H.M. Montané, C. Robaglia, B. Menand, Spatial regulation of root growth: Placing the plant TOR pathway in a developmental perspective, Int. J. Mol.

Sci. 16 (2015) 19671–19697. doi:10.3390/ijms160819671.
[63] M. Mahfouz, Sunghan Kim, Ashton J. Delauney, Desh Pal S. Verma, Arabidopsis TARGET OF RAPAMYCIN Interacts with RAPTOR, Which Regulates the Activity of S6 Kinase in Response to Osmotic Stress Signals, Plant Cell Online. 18 (2006) 477–490. doi:10.1105/tpc.105.035931.
[64] H. Yang, D.G. Rudge, J.D. Koos, B. Vaidialingam, H.J. Yang, N.P. Pavletich, MTOR kinase structure, mechanism and regulation, Nature. 497 (2013) 217–223. doi:10.1038/nature12122.
[65] D. De Vleesschauwer, O. Filipe, G. Hoffman, H.S. Seifi, A. Haeck, P. Canlas, J. Van Bockhaven, E. De Waele, K. Demeestere, P. Ronald, M. Hofte, Target of rapamycin signaling orchestrates growth–defense trade-offs in plants, New Phytol. 217 (2018) 305–319. doi:10.1111/nph.14785.
[66] L. Shi, Y. Wu, J. Sheen, TOR signaling in plants: conservation and innovation, Development. 145 (2018) dev160887. doi:10.1242/dev.160887.
[67] K. Deng, L. Yu, X. Zheng, K. Zhang, W. Wang, P. Dong, J. Zhang, M. Ren, Target of Rapamycin Is a Key Player for Auxin Signaling Transduction in Arabidopsis., Front. Plant Sci. 7 (2016) 291. doi:10.3389/fpls.2016.00291.
[68] O. Meyuhas, A. Dreazen, Ribosomal Protein S6 Kinase. From TOP mRNAs to Cell Size, in: Prog. Mol. Biol. Transl. Sci., 1st ed., Elsevier Inc., 2009: pp. 109–153. doi:10.1016/S1877-1173(09)90003-5.
[69] T.R. Fenton, I.T. Gout, Functions and regulation of the 70 kDa ribosomal S6 kinases, Int. J. Biochem. Cell Biol. 43 (2011) 47–59. doi:10.1016/j.biocel.2010.09.018.
[70] D.C. Fingar, S. Salama, C. Tsou, E. Harlow, J. Blenis, Fingar et al, 2002, (2002) 1472–1487. doi:10.1101/gad.995802.when.
[71] J. Van Leene, C. Han, A. Gadeyne, D. Eeckhout, C. Matthijs, B. Cannoot, N. De Winne, G. Persiau, E. Van De Slijke, B. Van de Cotte, E. Stes, M. Van Bel, V. Storme, F. Impens, K. Gevaert, K. Vandepoele, I. De Smet, G. De Jaeger, Capturing the phosphorylation and protein interaction landscape of the plant TOR kinase, Nat. Plants. 5 (2019) 316–327. doi:10.1038/s41477-019-0378-z.
[72] M. Schepetilnikov, M. Dimitrova, E. Mancera-Martínez, A. Geldreich, M. Keller, L.A. Ryabova, TOR and S6K1 promote translation reinitiation of uORF-containing mRNAs via phosphorylation of eIF3h, EMBO J. 32 (2013) 1087–1102. doi:10.1038/emboj.2013.61.
[73] R. Henriques, L. Bögre, B. Horváth, Z. Magyar, Balancing act: Matching growth with environment by the TOR signalling pathway, J. Exp. Bot. 65 (2014) 2691–2701. doi:10.1093/jxb/eru049.
[74] L. Moubayidin, R. Di Mambro, S. Sabatini, Cytokinin – auxin crosstalk, Trends Plant Sci. 4 (2009). doi:10.1016/j.tplants.2009.06.010.
[75] J. Kyozuka, Control of shoot and root meristem function by cytokinin, Plan Biol. 1 (2007) 442–446. doi:10.1016/j.pbi.2007.08.010.
[76] A. Sofo, A. Vitti, M. Nuzzaci, G. Tataranni, A. Scopa, Correlation between hormonal homeostasis and morphogenic responses in Arabidopsis thaliana seedlings growing in a Cd / Cu / Zn multi-pollution context, Physiol. Plant. (2013) 487–498. doi:10.1111/ppl.12050.
[77] M.A. Salem, Y. Li, K. Bajdzienko, J. Fisahn, M. Watanabe, R. Hoefgen, M.A. Schöttler, P. Giavalisco, RAPTOR Controls Developmental Growth Transitions by Altering the Hormonal and Metabolic Balance, Plant Physiol. 177 (2018) 565–593.

doi:10.1104/pp.17.01711.
[78] Y. Song, G. Zhao, X. Zhang, L. Li, F. Xiong, F. Zhuo, C. Zhang, Z. Yang, R. Datla, M. Ren, F. Li, The crosstalk between Target of Rapamycin (TOR) and Jasmonic Acid (JA) signaling existing in Arabidopsis and cotton, Sci. Rep. 7 (2017) 1–15. doi:10.1038/srep45830.
[79] C. Delatorre, A. Rodríguez, L. Rodríguez, J.P. Majada, R.J. Ordás, I. Feito, Hormonal profiling: Development of a simple method to extract and quantify phytohormones in complex matrices by UHPLC–MS/MS, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1040 (2017) 239–249. doi:10.1016/j.jchromb.2016.11.007.
[80] M. Vanstraelen, E. Benková, Hormonal interactions in the regulation of plant development., Annu. Rev. Cell Dev. Biol. 28 (2012) 463–87. doi:10.1146/annurev-cellbio-101011-155741.
[81] L. Moubayidin, S. Perilli, R. Dello Ioio, R. Di Mambro, P. Costantino, S. Sabatini, The rate of cell differentiation controls the arabidopsis root meristem growth phase, Curr. Biol. 20 (2010) 1138–1143. doi:10.1016/j.cub.2010.05.035.
[82] X. Li, W. Cai, Y. Liu, H. Li, L. Fu, Z. Liu, L. Xu, H. Liu, T. Xu, Y. Xiong, Differential TOR activation and cell proliferation in Arabidopsis root and shoot apexes., Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 2765–2770. doi:10.1073/pnas.1618782114.
[83] M. Schepetilnikov, L.A. Ryabova, Auxin Signaling in Regulation of Plant Translation Reinitiation, Front. Plant Sci. 8 (2017) 1–15. doi:10.3389/fpls.2017.01014.
[84] E. Sundberg, L. Østergaard, Distinct and dynamic auxin activities during reproductive development., Cold Spring Harb. Perspect. Biol. 1 (2009). doi:10.1101/cshperspect.a001628.
[85] M. Ruiz Rosquete, E. Barbez, J. Kleine-Vehn, Cellular auxin homeostasis: Gatekeeping is housekeeping, Mol. Plant. 5 (2012) 772–786. doi:10.1093/mp/ssr109.
[86] N.D. Tivendale, J.J. Ross, J.D. Cohen, The shifting paradigms of auxin biosynthesis, Trends Plant Sci. 19 (2014) 44–51. doi:10.1016/j.tplants.2013.09.012.
[87] N.D. Tivendale, J.D. Cohen, Analytical History of Auxin, J. Plant Growth Regul. 34 (2015) 708–722. doi:10.1007/s00344-015-9519-4.
[88] L. Irina, a C. Villalobos, S. Lee, C. De Oliveira, A. Ivetac, W. Brandt, L. Armitage, L.B. Sheard, X. Tan, G. Parry, H. Mao, N. Zheng, R. Napier, S. Kepinski, M. Estelle, A combinatorial TIR1/AFB-Aux/IAA co-receptor system for differential sensing of auxin, Nat. Chem. Biol. 8 (2012) 477–485. doi:10.1038/nchembio.926.A.
[89] H. Kasahara, Current aspects of auxin biosynthesis in plants, Biosci. Biotechnol. Biochem. 80 (2016) 34–42. doi:10.1080/09168451.2015.1086259.
[90] W.A. Peer, From perception to attenuation: Auxin signalling and responses, Curr. Opin. Plant Biol. 16 (2013) 561–568. doi:10.1016/j.pbi.2013.08.003.
[91] Y. Zhao, Auxin Biosynthesis : A Simple Two-Step Pathway Converts Tryptophan to Indole-3-Acetic Acid in Plants, Mol. Plant. 5 (2012) 334–338. doi:10.1093/mp/ssr104.
[92] S. Simon, J. Petrášek, Why plants need more than one type of auxin, Plant Sci. 180 (2011) 454–460. doi:10.1016/j.plantsci.2010.12.007.
[93] J. Ludwig-Müller, E. Epstein, Indole-3-butyric acid in Arabidopsis thaliana, Plant Growth Regul. 13 (1993) 189–195. doi:10.1007/bf00024261.
[94] E. Epstein, J. Ludwig-Müller, Indole-3-butyric acid in plants: occurrence, synthesis, metabolism and transport, Physiol. Plant. 88 (1993) 382–389. doi:10.1111/j.1399-3054.1993.tb05513.x.

[95] L.C. Strader, A.H. Culler, J.D. Cohen, B. Bartel, Conversion of endogenous indole-3-butyric acid to indole-3-acetic acid drives cell expansion in arabidopsis seedlings, Plant Physiol. 153 (2010) 1577–1586. doi:10.1104/pp.110.157461.
[96] E.M. Frick, L.C. Strader, Roles for IBA-derived auxin in plant development, J. Exp. Bot. 69 (2018) 169–177. doi:10.1093/jxb/erx298.
[97] T. Kudo, T. Kiba, H. Sakakibara, Metabolism and long-distance translocation of cytokinins, J. Integr. Plant Biol. 52 (2010) 53–60. doi:10.1111/j.1744-7909.2010.00898.x.
[98] R. Koprna, N. De Diego, L. Dundálková, L. Spíchal, Use of cytokinins as agrochemicals, Bioorganic Med. Chem. 24 (2016) 484–492. doi:10.1016/j.bmc.2015.12.022.
[99] B. Zürcher, E. Müller, Cytokinin Synthesis, Signaling, and Function-Advances and New Insights, in: Int. Rev. Cell Mol. Biol., Elsevier Inc., 2016: pp. 1–38. doi:10.1016/bs.ircmb.2016.01.001.
[100] S. In, V. Cellular, D.B. Plant, N.M. Apr, D.B. Plant, Cytokinins : Biosynthesis , Metabolism and Perception Author (s): Machteld C . Mok , Ruth C . Martin , David W . S . Mok Published by : Society for In Vitro Biology Stable URL : http://www.jstor.org/stable/4293319 Your use of the JSTOR archive indicates, In Vitro. 36 (2009) 102–107.
[101] G.E. Schaller, I.H. Street, J.J. Kieber, Cytokinin and the cell cycle, Curr. Opin. Plant Biol. 21 (2014) 7–15. doi:10.1016/j.pbi.2014.05.015.
[102] M. Haruta, M.R. Sussman, Ligand Receptor-Mediated Regulation of Growth in Plants, Curr. Top. Dev. Biol. 123 (2017) 331–363. doi:10.1016/bs.ctdb.2016.11.007.
[103] C.T. Argueso, T. Raines, J.J. Kieber, Cytokinin signaling and transcriptional networks, Curr. Opin. Plant Biol. 13 (2010) 533–539. doi:10.1016/j.pbi.2010.08.006.
[104] H. Sakakibara, K. Takei, N. Hirose, Interactions between nitrogen and cytokinin in the regulation of metabolism and development, Trends Plant Sci. 11 (2006) 440–448. doi:10.1016/j.tplants.2006.07.004.
[105] S. Yamaguchi, Gibberellin Metabolism and its Regulation, Annu. Rev. Plant Biol. 59 (2008) 225–251. doi:10.1146/annurev.arplant.59.032607.092804.
[106] R. Yano, Y. Kanno, Y. Jikumaru, K. Nakabayashi, Y. Kamiya, E. Nambara, CHOTTO1, a putative double APETALA2 repeat transcription factor, is involved in abscisic acid-mediated repression of gibberellin Biosynthesis during seed germination in Arabidopsis, Plant Physiol. 151 (2009) 641–654. doi:10.1104/pp.109.142018.
[107] H. Li, H. Wu, Q. Qi, H. Li, Z. Li, S. Chen, Q. Ding, Q. Wang, Z. Yan, Y. Gai, X. Jiang, J. Ding, T. Gu, X. Hou, M. Richard, Y. Zhao, Y. Li, Gibberellins Play a Role in Regulating Tomato Fruit Ripening, Plant Cell Physiol. 60 (2019) 1619–1629. doi:10.1093/pcp/pcz069.
[108] L. Liu, C. Jia, M. Zhang, D. Chen, S. Chen, R. Guo, D. Guo, Q. Wang, Ectopic expression of a BZR1-1D transcription factor in brassinosteroid signalling enhances carotenoid accumulation and fruit quality attributes in tomato, Plant Biotechnol. J. 12 (2014) 105–115. doi:10.1111/pbi.12121.
[109] H. Cheng, S. Song, L. Xiao, H.M. Soo, Z. Cheng, D. Xie, J. Peng, Gibberellin acts through jasmonate to control the expression of MYB21, MYB24, and MYB57 to promote stamen filament growth in arabidopsis, PLoS Genet. 5 (2009) 20–22. doi:10.1371/journal.pgen.1000440.
[110] K. Marciniak, K. Przedniczek, Comprehensive insight into gibberellin- and jasmonate-

mediated stamen development, Genes (Basel). 10 (2019). doi:10.3390/genes10100811.
[111] P. Achard, P. Genschik, Releasing the brakes of plant growth: How GAs shutdown della proteins, J. Exp. Bot. 60 (2009) 1085–1092. doi:10.1093/jxb/ern301.
[112] P. Hedden, S.G. Thomas, Gibberellin biosynthesis and its regulation, Biochem. J. 444 (2012) 11–25. doi:10.1042/BJ20120245.
[113] J.M. Davière, P. Achard, Gibberellin signaling in plants, Dev. 140 (2013) 1147–1151. doi:10.1242/dev.087650.
[114] S. Chen, X. Wang, L. Zhang, S. Lin, D. Liu, Q. Wang, S. Cai, R. El-Tanbouly, L. Gan, H. Wu, Y. Li, Identification and characterization of tomato gibberellin 2-oxidases (GA2oxs) and effects of fruit-specific SlGA2ox1 overexpression on fruit and seed growth and development, Hortic. Res. 3 (2016) 1–9. doi:10.1038/hortres.2016.59.
[115] V.A. Smith, C.J. Knatt, P. Gaskin, J.B. Reid, The distribution of gibberellins in vegetative tissues of Pisum sativum L. I. Biological and biochemical consequences of the le mutation, Plant Physiol. 99 (1992) 368–371. doi:10.1104/pp.99.2.368.
[116] J. Browse, Jasmonate Passes Muster: A Receptor and Targets for the Defense Hormone, Annu. Rev. Plant Biol. 60 (2009) 183–205. doi:10.1146/annurev.arplant.043008.092007.
[117] A. Robert-Seilaniantz, M. Grant, J.D.G. Jones, Hormone Crosstalk in Plant Disease and Defense: More Than Just JASMONATE-SALICYLATE Antagonism, Annu. Rev. Phytopathol. 49 (2011) 317–343. doi:10.1146/annurev-phyto-073009-114447.
[118] R.A. Creelman, J.E. Mullet, Biosynthesis and Action of Jasmonates in Plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 355–381. doi:10.1146/annurev.arplant.48.1.355.
[119] M. Hamberg, H. Gardner, Oxylipin pathway to jasmonates:biochemestry and biological significance, Biochim. Biophys. Acta. 5 (1992) 1–18.
[120] P. Guan, Dancing with Hormones: A Current Perspective of Nitrate Signaling and Regulation in Arabidopsis, Front. Plant Sci. 8 (2017) 1–20. doi:10.3389/fpls.2017.01697.
[121] R. Finkelstein, Abscisic Acid Synthesis and Response, in: Arab. B., American Society of Plant Biologists, 2013: pp. 1–36. doi:10.1199/tab.0058.
[122] A.R. Kermode, Role of Abscisic Acid in Seed Dormancy, (2005) 319–344. doi:10.1007/s00344-005-0110-2.
[123] B. Ehlting, R. Ha, T. Lange, H. Rennenberg, M. Arend, A. Himmelbach, E. Grill, Expression of the Arabidopsis Mutant abi1 Gene Alters Abscisic Acid Sensitivity , Stomatal Development , and Growth Morphology in Gray Poplars 1 [C], 151 (2009) 2110–2119. doi:10.1104/pp.109.144956.
[124] P. Hsu, Y. Takahashi, S. Munemasa, E. Merilo, K. Laanemets, R. Waadt, D. Pater, Abscisic acid-independent stomatal CO 2 signal transduction pathway and convergence of CO2 and ABA signaling downstream of OST1 kinase, 115 (2018) 9971–9980. doi:10.1073/pnas.1809204115.
[125] C. Zheng, T. Halaly, A.K. Acheampong, Y. Takebayashi, Y. Jikumaru, Abscisic acid (ABA) regulates grape bud dormancy , and dormancy release stimuli may act through modification of ABA metabolism, 66 (2015) 1527–1542. doi:10.1093/jxb/eru519.
[126] C. Lu, M. Chen, R. Liu, L. Zhang, X. Hou, Abscisic Acid Regulates Auxin Distribution to Mediate Maize Lateral Root Development Under Salt Stress, Front. Plant Sci. 10 (2019) 1–16. doi:10.3389/fpls.2019.00716.

[127] C.A. Ondzighi-Assoume, S. Chakraborty, J.M. Harris, Environmental nitrate stimulates abscisic acid accumulation in arabidopsis root tips by releasing it from inactive stores, Plant Cell. 28 (2016) 729–745. doi:10.1105/tpc.15.00946.
[128] A.C. Vlot, D.M.A. Dempsey, D.F. Klessig, Salicylic Acid , a Multifaceted Hormone to Combat Disease, Annu. Rev. Phytopathol. (2009) 177–206. doi:10.1146/annurev.phyto.050908.135202.
[129] D.A. Dempsey, D.F. Klessig, How does the multifaceted plant hormone salicylic acid combat disease in plants and are similar mechanisms utilized in humans?, BMC Biol. 15 (2017) 1–11. doi:10.1186/s12915-017-0364-8.
[130] I. Maruri-López, N.Y. Aviles-Baltazar, A. Buchala, M. Serrano, Intra and extracellular journey of the phytohormone salicylic acid, Front. Plant Sci. 10 (2019) 1–11. doi:10.3389/fpls.2019.00423.
[131] L.S. Weyrich, S. Duchene, J. Soubrier, L. Arriola, B. Llamas, J. Breen, A.G. Morris, K.W. Alt, D. Caramelli, V. Dresely, M. Farrell, A.G. Farrer, M. Francken, N. Gully, W. Haak, K. Hardy, K. Harvati, P. Held, E.C. Holmes, J. Kaidonis, C. Lalueza-Fox, M. De La Rasilla, A. Rosas, P. Semal, A. Soltysiak, G. Townsend, D. Usai, J. Wahl, D.H. Huson, K. Dobney, A. Cooper, Neanderthal behaviour, diet, and disease inferred from ancient DNA in dental calculus, Nature. 544 (2017) 357–361. doi:10.1038/nature21674.
[132] G. Qi, J. Chen, M. Chang, H. Chen, K. Hall, J. Korin, F. Liu, D. Wang, Z.Q. Fu, Pandemonium Breaks Out : Disruption of Salicylic Acid-Mediated Defense by Plant Pathogens, Mol. Plant. 11 (2018) 1427–1439. doi:10.1016/j.molp.2018.10.002.
[133] J. Glazebrook, Contrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens, Annu. Rev. Phytopathol. 43 (2005) 205–227. doi:10.1146/annurev.phyto.43.040204.135923.
[134] A. Metwally, I. Finkemeier, M. Georgi, K. Dietz, Salicylic Acid Alleviates the Cadmium Toxicity in, Plant Physiol. 132 (2003) 272–281. doi:10.1104/pp.102.018457.duced.
[135] L. Rajjou, M. Belghazi, R. Huguet, C. Robin, A. Moreau, C. Job, D. Job, Proteomic investigation of the effect of salicylic acid on arabidopsis seed germination and establishment of early defense mechanisms, Plant Physiol. 141 (2006) 910–923. doi:10.1104/pp.106.082057.
[136] M. Rivas-San Vicente, J. Plasencia, Salicylic acid beyond defence: Its role in plant growth and development, J. Exp. Bot. 62 (2011) 3321–3338. doi:10.1093/jxb/err031.
[137] K. Morris, S.A.H. Mackerness, T. Page, C. Fred John, A.M. Murphy, J.P. Carr, V. Buchanan-Wollaston, Salicylic acid has a role in regulating gene expression during leaf senescence, Plant J. 23 (2000) 677–685. doi:10.1046/j.1365-313X.2000.00836.x.
[138] K. Zhang, R. Halitschke, C. Yin, C.J. Liu, S.S. Gan, Salicylic acid 3-hydroxylase regulates Arabidopsis leaf longevity by mediating salicylic acid catabolism, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 14807–14812. doi:10.1073/pnas.1302702110.
[139] D.F. Klessig, J. Malamy, The salicylic acid signal in plants, Plant Mol. Biol. 26 (1994) 1439–1458. doi:10.1007/BF00016484.
[140] C. Norman, K.A. Howell, A.H. Millar, J.M. Whelan, D.A. Day, Salicylic Acid Is an Uncoupler and Inhibitor of Mitochondrial Electron Transport, Plant Physiol. 134 (2004) 492–501. doi:10.1104/pp.103.031039.
[141] G. Zhai, S.M. Gutowski, K.S. Walters, B. Yan, J.L. Schnoor, Charge, Size and Cellular Selectivity for Multi-wall Carbon Nanotubes by Maize and Soybean, Environ. Sci.

Technol. 49 (2015) 7380−7390. doi:10.1021/acs.est.5b01145.
[142] A. Joshi, S. Kaur, P. Singh, K. Dharamvir, H. Nayyar, G. Verma, Tracking multi-walled carbon nanotubes inside oat (Avena sativa L .) plants and assessing their effect on growth , yield , and mammalian (human) cell viability, (2018) 13204.
[143] J. Lynch, Root Architecture and Plant Productivity, Plant Physiol. 109 (1995) 7–13. doi:10.1104/pp.109.1.7.
[144] X. Wang, H. Han, X. Liu, X. Gu, K. Chen, D. Lu, Multi-walled carbon nanotubes can enhance root elongation of wheat (Triticum aestivum) plants, J. Nanoparticle Res. 14 (2012) 841. doi:10.1007/s11051-012-0841-5.
[145] J. Melorose, R. Perroy, S. Careas, Carbon and fullerene nanomaterials in plant system, Statew. Agric. L. Use Baseline 2015. 1 (2015). doi:10.1017/CBO9781107415324.004.
[146] A.B. Seabra, A.J. Paula, N. Durán, Redox-enzymes, cells and micro-organisms acting on carbon nanostructures transformation: A mini-review, Biotechnol. Prog. 29 (2013) 1–10. doi:10.1002/btpr.1673.
[147] N.K. Moghadam, M. Hatami, S. Rezaei, M. Bayat, B.A. Lajayer, Induction of Plant Defense Machinery Against Nanomaterials Exposure, Adv. Phytonanotechnology. (2019) 241–263. doi:10.1016/B978-0-12-815322-2.00010-9.
[148] P. Dong, F. Xiong, Y. Que, K. Wang, L. Yu, Z. Li, M. Ren, Expression profiling and functional analysis reveals that TOR is a key player in regulating photosynthesis and phytohormone signaling pathways in Arabidopsis, Front. Plant Sci. 6 (2015) 1–15. doi:10.3389/fpls.2015.00677.
[149] Y. Hao, F. Yu, R. Lv, C. Ma, Z. Zhang, Y. Rui, L. Liu, W. Cao, B. Xing, 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 (2016) e0157264. doi:10.1371/journal.pone.0157264.
[150] D.C. Fingar, S. Salama, C. Tsou, E. Harlow, J. Blenis, Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E, Genes Dev. (2002) 1472–1487. doi:10.1101/gad.995802.
[151] B. Menand, T. Desnos, L. Nussaume, F. Berger, D. Bouchez, C. Meyer, C. Robaglia, Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene., Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 6422–7. doi:10.1073/pnas.092141899.
[152] R.-M. Leiber, F. John, Y. Verhertbruggen, A. Diet, J.P. Knox, C. Ringli, The TOR Pathway Modulates the Structure of Cell Walls in *Arabidopsis*, Plant Cell. 22 (2010) 1898–1908. doi:10.1105/tpc.109.073007.
[153] A. Barrada, M. Djendli, T. Desnos, R. Mercier, C. Robaglia, M.-H. Montané, B. Menand, A TOR-YAK1 signaling axis controls cell cycle, meristem activity and plant growth in Arabidopsis , Development. 146 (2019) dev171298. doi:10.1242/dev.171298.
[154] K. Hartig, E. Beck, Crosstalk between auxin, cytokinins, and sugars in the plant cell cycle, Plant Biol. 8 (2006) 389–396. doi:10.1055/s-2006-923797.
[155] F. Turck, F. Zilbermann, S.C. Kozma, G. Thomas, F. Nagy, C.H. Basel, P. Biology, Phytohormones Participate in an S6 Kinase Signal Transduction Pathway in Arabidopsis 1, Plant Physiol. 134 (2004) 1527–1535. doi:10.1104/pp.103.035873.1.
[156] M. Schepetilnikov, L.A. Ryabova, Recent discoveries on the role of tor (Target of rapamycin) signaling in translation in plants, Plant Physiol. 176 (2018) 1095–1105. doi:10.1104/pp.17.01243.

[157] K. Yokawa, F. Balusˇka, The TOR complex: An emergency switch for root behavior, Plant Cell Physiol. (2016). doi:10.1093/pcp/pcv191.