Biological effects of carbon nanotubes generated in forest wildfire ecosystems rich in resinous trees on native plants

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Carbon nanotubes (CNTs) have a broad range of applications and are generally considered human-engineered nanomaterials. However, carbon nanostructures have been found in ice cores and oil wells, suggesting that nature may provide appropriate conditions for CNT synthesis. During forest wildfires, materials such as turpentine and conifer tissues containing iron under high temperatures may create chemical conditions favorable for CNT generation, similar to those in synthetic methods. Here, we show evidence of naturally occurring multiwalled carbon nanotubes (MWCNTs) produced from *Pinus oocarpa* and *Pinus pseudostrobus,* following a forest wildfire. The MWCNTs showed an average of 10 walls, with internal diameters of ~2.5 nm and outer diameters of ~14.5 nm. To verify whether MWCNT generation during forest wildfires has a biological effect on some characteristic plant species of these ecosystems, germination and development of seedlings were conducted. Results show that the utilization of comparable synthetic MWCNTs increased seed germination rates and the development of *Lupinus elegans* and *Eysenhardtia polystachya*, two plants species found in the burned forest ecosystem. The finding provides evidence that supports the generation and possible ecological functions of MWCNTs in nature.

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2 **resinous trees on native plants**

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46   **Abstract**

47   Carbon nanotubes (CNTs) have a broad range of applications and are generally considered

48   human-engineered nanomaterials. However, carbon nanostructures have been found in ice cores

49   and oil wells, suggesting that nature may provide appropriate conditions for CNT synthesis.

50   During forest wildfires, materials such as turpentine and conifer tissues containing iron under

51   high temperatures may create chemical conditions favorable for CNT generation, similar to those

52   in synthetic methods. Here, we show evidence of naturally occurring multiwalled carbon

53   nanotubes (MWCNTs) produced from *Pinus oocarpa* and *Pinus pseudostrobus,* following a

54   forest wildfire. The MWCNTs showed an average of 10 walls, with internal diameters of ~2.5

55   nm and outer diameters of ~14.5 nm. To verify whether MWCNT generation during forest

56   wildfires has a biological effect on some characteristic plant species of these ecosystems,

57   germination and development of seedlings were conducted. Results show that the utilization of

58   comparable synthetic MWCNTs increased seed germination rates and the development

59   of *Lupinus elegans* and *Eysenhardtia polystachya*, two plants species found in the burned forest

60   ecosystem. The finding provides evidence that supports the generation and possible ecological

61   functions of MWCNTs in nature.

62

63   **Introduction**

64   Carbon nanotubes (CNTs) have been the subject of extensive research in recent years because of

65   their extraordinary properties and broad range of biotechnological applications. Although CNTs

66   are commonly considered human-engineered nanomaterials, it has been generally accepted that

67   nature may provide appropriate conditions for their synthesis. CNT occurrences have usually

68   been sought in extreme environments (*e.g.,* at high temperatures and pressures), where evidence

69   has suggested their formation. For example, encapsulated CNTs have been found in the coal-

70   petroleum mix of oil wells (Velasco-Santos et al. 2003) and in Greenland ice-core samples dated

71   from the Neolithic Stone Age (10,000 years ago) (Esquivel & Murr 2004); however, the source

72   of these CNTs has not yet been identified. There have also been questions regarding the validity

73   of these reports because of the lack of clear high-resolution transmission electron microscopy

74   (HR-TEM) images, Raman analysis, or diffraction patterns (Mackenzie et al. 2008).

75   Previous studies have speculated that CNTs can form in volcanoes, based on the observation that

76   Mount Etna's lava can catalyze the synthesis of multiwalled CNTs (MWCNTs) (Su et al. 2008;

77   Su & Chen 2007). However, no direct evidence of the formation of CNTs within volcanoes has

78   been confirmed. Further, plant products such as turpentine, eucalyptus oil, neem oil, palm oil,

79   and olive oil have been used as raw materials for chemical vapor deposition (CVD) in CNT

80   synthesis (Afre et al. 2005; Ghosh et al. 2007; Kumar et al. 2011; Suriani et al. 2009). In

81   addition, plant and fungal tissues containing transition metals have been used as natural catalyst

82   precursors in the production of CNTs by CVD (Zhao et al. 2011).

83   Oleoresin extraction is commonly performed in the forestlands of Michoacán, México, where

84   oleoresin is collected from the trunks of living pines, and turpentine is obtained from steam

85   distillation. Alpha-pinene, which is used as a raw material for solvent production, is one of the

86   most important components of turpentine documented as an effective compound from which

87   high-quality and high-yield MWCNTs can be synthesized by CVD (Lara-Romero et al. 2011).

88   Pine species such as *Pinus leiophylla*, *Pinus oocarpa*, *Pinus montezumae*, *Pinus pseudostrobus*,

89   and *Pinus teocote* are considered the most important tree species for oleoresin extraction in the

90   Mexican industry. The ecosystems in Michoacán, México, associated with these species of

91   conifers are prone to wildfires. During the drought season, wildfires can cause temperatures

92   between 600 and 900 °C; this, coupled with the presence of turpentines (or alpha-pinene) and

93   conifer tissues containing iron, provides conditions similar to those required for CNT formation

94   in a process like CVD.

95   Moreover, MWCNTs have also been described as plant growth promoters, favoring seed

96   germination and an increase in the fresh weight of tomato plants (Khodakovskaya et al. 2012;

97   Yang et al. 2017). Recently, nanotechnology tools have developed CNTs for potential

98   applications in agriculture, including crop protection, pollution control, waste management,

99   pesticide detection, nanosensing, and as nanofertilizers (De La Torre-Roche et al. 2012; Gogos et

100   al. 2012; Hong et al. 2013; Khodakovskaya et al. 2012; Yang et al. 2017). Contrary to the

101   beneficial applications of CNTs, negative effects of nanoparticles on edible plants have also been

102   discussed (Miralles et al. 2012); thus, the known effects of MWCNTs on plants are still limited,

103   as are the responses of the natural and agricultural ecosystems to human-engineered

104   nanomaterials (Yang et al. 2017).

105   This report, as a first attempt to understand the roles of crystalline nanomaterials in plant

106   ecosystems and to scarce evidence of naturally-formed MWCNTs in the biosphere. The main

107   objective of this study was to provide evidence of spontaneously and naturally occurring

108   MWCNTs from *Pinus* species following a forest wildfire event, and their possible effects on

109   germination and development of species found in the burned forest ecosystem.

110

111   **Materials & Methods**

112   ***Sample collection from a pine forest***

113   During the dry season (June 2012), samples of burned wood were randomly collected from

114   mature trees of two different pine forest sites in Michoacán, west-central México, which had

115   been recently affected by forest wildfires. The sites were 'Cerro Huashan, Nahuatzen' (19°38'35"

116   N, 101°56'46" W; sampling *P. oocarpa* 2 weeks after fire extinguishment) and 'Cerro de la Cruz,

117   Uruapan' (19°26'40" N, 102°2'56" W; sampling *P. pseudostrobus* and *P. montezumae* 8 weeks

118   after fire extinguishment). At least 20 samples of each pinus species were collected from each

119   forest wildfire site. Sampling was collected under the supervision of the Ministry of Environment

120   and Natural Resources specifications (Nom-059-SEMARNAT-2010) and the conservation

121   program for flora and fauna of the Pico de Tancítaro (APFFPT) from Michoacán, México;

122   established by the Mexican decree law of august 19, 2009; and the Program for the Sustainable

123   Management of Mountain Ecosystems Pico de Tancítaro, Michoacán, México (APFFPT-2009).

124   Wood samples were ground and thoroughly mixed for further analyses.

125

126   ***CNT analysis***

127   Samples of burned wood from various types of pine trees were characterized by Raman

128   spectroscopy, thermogravimetry (TGA), and high-resolution transmission electron microscopy

129   (HR-TEM), at least 20 samples of each pinus species were analyzed. Raman spectroscopy was

130   performed using a micro-Raman spectrometer (Labram System model Dilor) equipped with a 20

131   mW He-Ne laser emitting at 514 nm, a holographic notch filter (supertNotch-Plus, Kaiser

132   Optical Systems, Inc.), and a 256×1024 pixel charge-coupled device (CCD) image recorder. All

133   measurements were carried out at room temperature with no special sample preparation.

134   TGA was carried out using a microbalance (Chan D-200) (Doudrick et al. 2012), where 40–50

135   mg samples of burned wood from the different pine species collected after a natural fire and

136   MWCNTs synthesized by spray pyrolysis of α-pinene/ferrocene were air-heated between 25 to

137   700 °C at a rate of 5 °C/min, to obtain TGA combustion curves of the samples.

138   HR-TEM micrographs were obtained from a Philips CM-200 analytical TEM operating at 200

139   kV. Specimens for HR-TEM analysis were prepared by dispersing the samples in acetone

140   through sonication for 2 min and air-drying a drop of the suspension on a perforated, carbon-

141   coated Cu° grid.

142

143   ***Seed germination and plant pot-growing using synthetic MWCNTs***

144   Seeds of *Lupinus elegans* and *Eysenhardtia polystachya*, collected from the pine forest of

145   Michoacán, México, were sterilized with 95% sulfuric acid for 20 min and by soaking in 1%

146   sodium hypochlorite (NaOCl) for 3 min, respectively; both were then rinsed with sterile distilled

147   water. The seeds of each species were divided into six separate sets of 100 seeds and incubated

148   in a suspension of 0 (Control), 10, 20, 30, 40, or 50 μg/mL MWCNTs (Sigma-Aldrich, St. Louis,

149   MO, USA; Cat. No. 698849; CVD-produced synthetic multiwalled CNTs, OD = 6.0–13.0 nm,

150   ID = 2.0–6.0 nm length = 2.5–20 μm, average wall thickness 7–13 graphene layers, > 98%

151   purity) for 10 min. The MWCNTs were dispersed in water by a three-step acid treatment. First

152   step consists on ultrasonic mixing the MWCNTs with concentrated HCl for 4 h, after refluxing

153   MWCNTs in nitric acid for 8 h at 80°C, and finally refluxing sample in a 1:1 mixture of sulfuric

154   and nitric acids for 4 h at 80°C. Each seed set was then placed on moistened filter papers in five

155   Petri plates (20–30 seeds per plate) and randomly distributed in a germination chamber.

156   Germination was evaluated after 10 days of incubation at 26 °C with a 12:12 light/dark cycles.

157   Pot-growing tests examined samples from all six treatments, each with 12 replicates (72 plants in

158   total for each seed type). Previously sterilized seeds (as described above) were directly planted in

159   5-cm-diameter polyethylene containers filled with 375 mL of the growth medium (Creci-root)

160   provided by a local nursery. These containers were then divided into six separate sets and seeds

161   were treated directly with 1.0 mL of a suspension of either 0 (Control), 10, 20, 30, 40, or 50

162   μg/mL MWCNTs, then covered with ~1.0 cm of plant growth substrate. The containers were

163   arranged at random in trays and watered on alternate days for 5 weeks. At the end of the 5-week

164   period, the plants were harvested, and biometric variables (leaf area and, fresh and dry weights of

165   shoots and roots) were recorded. Data were statistically analyzed using Graph Pad software with

166   an analysis of variance (one-way ANOVA), and mean were compared using Tukey's post hoc

167   tests at a significance level of $p < 0.05$.

168

169   **Results**

170   ***Identification and characterization of CNTs in the burned wood of resinous forests, after***

171   ***wildfire***

172   Burned wood samples were collected after an intense wildfire in a resinous pine forest in the

173   Michoacán state of Mexico (June 2012). This forest mainly comprised *P. oocarpa*, *P.*

174   *pseudostrobus*, and *P. montezumae*. Samples of the carbonized trees of these species were first

175   analyzed by Raman spectroscopy. The Raman spectra of three different burned wood samples

176   indicate that *P. oocarpa* and *P. pseudostrobus* samples show characteristic bands for CNTs, *i.e.,*

177   the *D* and *G* bands (Fig. 1A). The *D* band was observed at approximately 1370 cm^{-1}, and the *G*

178   band, also known as the tangential band, was observed at approximately 1600 cm^{-1}, which arises

179   from the E_{2g} mode of the graphite plane and confirms the presence of sp^2 electronic hybridization

180   in the carbon bond network. Unexpectedly, the 2*D* (*G'*) band, which is associated with the source

181   or metal load and temperature during synthesis, was not found in the Raman spectra. Moreover,

182   no CNT signals were detected in the samples of burned tree bark obtained from *P. montezumae*

183   (Fig. 1A).

184   Thermogravimetric analysis (TGA) was used to determinate the amount of MWCNTs in the

185   burned wood of *P. oocarpa*, *P. pseudostrobus*, and *P. montezumae* (Fig. 1B). Weight losses up to

186   ~150 °C correspond to the release of water contained in the samples, whereas weight losses in

187   the range of 200–300 °C and 300–400 °C are attributed to the degradation of hemicellulose and

188   cellulose, respectively. Weight losses in the range of 370–550 °C are attributed to the ligneous

189   components such as biochar (Esquivel & Murr 2004; Mackenzie et al. 2008; Velasco-Santos et

190   al. 2003). Relevantly, the weight loss detected at 610 °C in the *P. oocarpa* samples, which

191   coincides with that in a synthetic-origin MWCNTs sample, corresponds to CNT combustion.

192   Thus, according to the TGA analysis, *P. montezumae* contains approximately 10% (w/w)

193   moisture, 38% (w/w) hemicellulose, 46% (w/w) cellulose, and 4% (w/w) ligneous species; *P.*

194   *pseudostrobus* is composed of approximately 5% (w/w) moisture, 7% (w/w) hemicellulose, 22%

195   (w/w) cellulose, and 66% (w/w) of ligneous components; and *P. oocarpa* is composed of

196   approximately 14% (w/w) moisture, 18% (w/w) hemicellulose and cellulose, and 60% (w/w)

197   ligneous components. Relevantly, the TGA plot indicates that the burned wood samples of *P.*

198   *oocarpa* contained ~2.8% (w/w) of CNTs and *P. pseudostrobus* less than 0.1% (w/w), and the

199   remaining weight of ~5–10% (w/w) is attributed to metals and elements.

200   HR-TEM images and fast Fourier transforms (FFTs) of the *P. oocarpa* samples clearly indicated

201   the presence of CNTs. HR-TEM images and their corresponding FFTs show diffraction patterns

202   characteristic of graphitic crystalline carbon (Fig. 2A–C). HR-TEM data obtained from *P.*

203   *oocarpa* samples revealed the presence of highly crystalline MWCNTs, consisting of 10 walls

204   with inner and outer diameters of ~2.52 nm and ~12–15 nm, respectively (Fig. 2C). The FFT

205   image displayed one pair of sharp spots, and a line scan along those spots confirmed the presence

206   of sharp spots corresponding to highly ordered carbon (narrow spots). The estimated plane-to-

207   plane distance between the walls is 0.335 nm, which is in agreement with the nominal distance

208   between the planes in crystalline CNTs (Fig. 2D–E). The bright spots in the dark-field HR-TEM

209   images indicate the presence of metals on the carbon tubes, and the corresponding energy-

210   dispersive X-ray spectroscopy (EDS) analysis confirmed the presence of iron (Fig. 2F),

211   suggesting that this iron could have acted as a catalyst during CNT formation.

212   For the burned wood samples of *P. pseudostrobus*, HR-TEM images and the corresponding FFT

213   data show preferential formation of coil-shaped nanoparticles consisting of curved crystalline

214   multiwalled carbon layers (Fig. 3A). The FFT images of these MWCNTs reveal one pair of

215   sharp spots and a line scan along those spots confirmed the presence of highly ordered carbon.

216   The estimated distance between the lattice fringes of the carbon walls is 0.335 nm, which is in

217   agreement with the nominal distance between the planes of graphite (Fig. 3B–C). The

218   corresponding EDS analysis reveals the presence of several elements such as calcium, potassium,

219   and phosphorous, but no evidence of the presence of iron or other transition metals is found (Fig.

220   3D). Unexpectedly, no evidence of CNT structures was found in *P. montezumae* samples;

221   however, amorphous carbon structures were abundant (Fig. 4A–B). The FFT spectrum displayed

222   diffuse spots, characteristic of amorphous carbon, and EDS analysis confirmed the presence of

223   iron, calcium, and phosphorous (Fig. 4C–E). These findings provided evidence of naturally

224   occurring MWCNTs from *Pinus* species after forest wildfire events.

225

226   ***Synthetic multiwalled CNTs increase seed germination in plants growing in resinous Pinus***

227   ***forests***

228   The MWCNTs found in burned *P. oocarpa* and *P. pseudostrobus* wood samples had ~10 layers,

229   with an inner diameter of ~2.52 nm and an outer diameter of ~14.59 nm. To investigate if

230   MWCNTs with structural features similar to those found in the natural samples could have a

231   biological effect over some plants species characteristic of these ecosystems (*L. elegans* and *E.*

232   *polystachya*), we conducted a germination and development of seedlings test. This assay was

233   based on previous studies on the positive or negative effects on plant germination and the

234   development of seedlings grown by MWCNT treatment (Hong et al. 2013; Khodakovskaya et al.

235   2012; Miralles et al. 2012). We supplemented the seed germination and early seedling growth

236   with 10–50 µg/mL of the synthetic MWCNTs with structural features similar to those found in

237   the *P. oocarpa* and *P. pseudostrobus* wood samples of burned forest (average wall thickness ~7–

238   13 layers; inner diameter of ~2–6 nm; outer diameter of ~12–20 nm; length of 2.5–20 µm).

239   Seed germination results showed that the addition of MWCNTs increased the number of

240   germinated seeds and significantly shortened the germination period (Fig. 5A). Seeds of *L.*

241   *elegans* and *E. polystachya* treated with MWCNTs exhibited increased germination rates

242   compared to untreated seeds. For *L. elegans*, a prolific plant in this forest, seed germination rates

243   were 62.5% higher after the addition of 30 µg/mL of the synthetic MWCNTs compared to those

244   of untreated plants. Moreover, *E. polystachya* seeds treated with MWCNTs reached germination

245   rates 40% higher than those of the untreated seeds (Fig. 5A–C).

246   We further investigated the effects of MWCNTs on the growth and development of *L. elegans*

247   and *E. polystachya* seedlings by growing them in a medium supplemented with different

248   concentrations of the synthetic nanoparticles and measuring the yield of variables such as fresh

249   and dry plant biomass, number of lateral roots, and foliar area (Fig. 6A–B). *L. elegans* plants

250   germinated and grown in the MWCNT dose range of 10 to 50 µg/L exhibited a significant

251   amount of vegetative biomass at 30 µg/L and a decrease at 50 µg/L of MWCNTs (Fig. 6A).

252   Significant increases in the fresh weight of the shoot and root, dry weight of the shoot, number of

253   lateral roots, and foliar area (90.23%, 132.59%, 84.51%, 91.05%, and 93.72%, respectively)

254   were observed in treated plants, compared to the untreated plants (Fig. 6C–H). Results also

255   suggest that the plant growth stimulation correlates with the increment in the shoot and root dry

256   weights; when plants were treated with 30 µg/L of MWCNTs these variables reached a

257   maximum of 45.2% and 120.46%, respectively, compared to those of the untreated plants (Fig.

258   6E–F). *E. polystachya* plants grown in media supplemented with increasing doses of MWCNTs

259   (10–50 µg/L) showed a large vegetative biomass at 50 µg/L and no negative effects of these

260   nanotubes were recorded at any dosage level tested (Fig. 6B). The maximum increases in the

261   shoot and root fresh and dry weight, number of lateral roots, and foliar area (87.8%, 302.78%,

262   148%, 114.54%, 313.66%, and 150.39%, respectively) were observed in treated plants,

263   compared to the untreated ones (Fig. 6C–H). These results show that synthetic MWCNTs

264   increase seed germination and plant growth in two plant species growing in the studied resinous

265   *Pinus* forests ecosystem.

266

267   **Discussion**

268   Our observations clearly support the hypothesis that MWCNTs can be formed spontaneously in

269   nature and are capable of self-assemble without human interference. Although the process was

270   not directly studied, the formation of MWCNTs during a resinous forest wildfire could be the

271   consequence of a synthetic CVD-like mechanism. Production of MWCNTs by CVD requires the

272   presence of volatile carbon compounds, which may act as precursors, in the gaseous state. *P.*

273   *oocarpa* is a species rich in turpentine, and its oleoresin is a mixture of highly volatile

274   monoterpenes, including *α*- and *β*-pinenes, which have been identified as highly effective

275   MWCNT precursors capable of providing a high yield (Lara-Romero et al. 2011). According to

276 previous studies, coil-shaped crystalline nanoparticles cannot be synthesized by processes other

277 than CVD (Fejes & Hernádi 2010; Mhlanga et al. 2011). Therefore, the above hypothesis is also

278 supported by the detection of coil-shaped crystalline carbon nanoparticles in the HR-TEM

279 images of the burned *P. oocarpa* and *P. pseudostrobus* wood. In addition, as mentioned above,

280 the impossibility to find MWCNTs into the *P. montezumae* samples and the sole presence of

281 amorphous carbon structures in this species, indicates that *P. montezumae* trees lacks either the

282 concentration of catalystics or the type of metal required for an effective synthesis of these

283 structures by a CVD like method (Lara-Romero et al. 2011; Zhao et al. 2011).

284 The presence of iron in the samples of *P. oocarpa* containing MWCNTs suggests that this metal

285 provided catalytically active sites for the CNT synthesis. Previous studies that used plant tissue

286 precursors to catalyze CNT growth have suggested that iron catalytic sites are uniformly

287 distributed in plant cells (Zhao et al. 2011). Consequently, CNTs formed in plant tissues could be

288 expected to have uniform diameters. This is consistent with our HR-TEM observations (average

289 wall thickness ~7–13 layers; inner diameter of ~2–6 nm; outer diameter of ~12–20 nm; length of

290 2.5–20 μm), which revealed that the MWCNTs had homogeneous number of layers and external

291 diameters. In addition, the TGA results indicated that the burned wood samples of *P. oocarpa*

292 after pyrolysis degradation contain ~2.8% wt of CNTs. Although it has been generally accepted

293 that nature could provide the conditions for their synthesis, there is scarce evidence of naturally

294 formed MWCNTs in the biosphere. Therefore, we provide evidence of spontaneously and

295 naturally produced MWCNTs from *Pinus* species, following forest wildfires.

296 In another context, the effects of nanomaterials such as CNTs on plant growth and development

297 has been documented, and it has been suggested that their effects are because of factors such as

298 the type of nanoparticles, concentration, plant species, and experimental conditions, including

299   the method of nanoparticle uptake (Tiwari et al. 2014); in contrast, studies indicate that some

300   CNTs nanomaterials show toxic effects on several plant models (Miralles et al. 2012). With

301   respect to human engineered MWCNTs, several molecular mechanisms involved in their

302   biological effects have been described. Genomic analyses of *Lycopersicon esculentum* have

303   indicated that exposure to MWCNTs altered the total gene expression, with up-regulation of

304   stress-related genes (Lahiani et al. 2015; Mohamed et al. 2016), while that in *Nicotiana tabacum*

305   has been found to cause alterations in total gene expression, with up-regulation of genes related

306   to cell-wall assembly/cell growth, regulation of cell cycle progression, and aquaporin production

307   (Lahiani et al. 2015; Miralles et al. 2012; Mukherjee et al. 2016; Yang et al. 2017). Thus, the

308   authors have suggested that size, composition, and specific surface characteristics of the

309   engineered nanomaterials may play important roles in their phytotoxicity (Hong et al. 2013;

310   Mukherjee et al. 2016).

311   In our work, the effect of MWCNTs was evaluated using two plant species found in the burned

312   forest ecosystem, although the MWCNTs utilized are of synthetic origin; these were acquired

313   with structural characteristics similar to those of CNTs found in burned wood samples from the

314   resinous forest. Interestingly, seed germination and growth promotion were observed in both the

315   *L. elegans* and *E. polystachya* plant species tested, with the influence of all the quantified

316   biometrical plant variables; it was unlikely that seed germination and growth promotion occurred

317   exclusively owing to water retention in the plant tissues (Fig. 6). In addition, the results did not

318   indicate that CNTs had toxic effects on seed germination or plant development in the

319   concentration range used, suggesting that at low dosages, MWCNTs function as plant-growth

320   promoters. The plant growth dose-dependence also suggests that the concentrations at which

321   CNTs exert their maximum plant growth-promoting effect depend on the plant species. Although

322   the mechanisms of the observed biological effects were not investigated, the findings indicate

323   that the seed germination and plant-growth promotion was due to the activation of the cell

324   division and nutrient uptake and also increased water influx as previously suggested , rather than

325   an increase in the cell volume.

326   Forest fires are known to enhance the recruitment of a number of important native species

327   associated with forest ecosystems (Keeley & Fotheringham 1998; Keeley et al. ; Turner et al.

328   1997), including *E. polystachya* (Orozco 2008) and *L. elegans* (Díaz-Rodriguez B 2013). In

329   addition, products resulting from the combustion of wood, such as ash (Keeley & Fotheringham

330   1998) and charred wood (Roy & Sonie 1992), have also been shown to trigger germination and

331   plant growth after forest fire events. The influence of MWCNTs formed in burned wood after

332   forest wildfires on plant growth or other post-fire characteristic events in terrestrial ecosystems

333   requires extensive studies.

334   CNT formation has usually been associated with extreme environments; however, we have

335   provided evidence that MWCNTs can be found in biotic environments after atmospheric events.

336   MWCNTs, formed in forest wildfires could be introduced into the soil by burned plant material

337   such as smoke or solid particles. If this is true, then MWCNTs have been interacting with soil

338   organisms and plant species since a long time. This may explain our findings, which strongly

339   suggest that MWCNTs produced in resinous forest wildfires promote seed germination and

340   growth of native plants in forest ecosystems.

341

342   **Conclusion**

343   This study shows direct evidence of MWCNT generation during forest wildfires as a natural

344   phenomenon, strongly suggesting a possible impact on natural plants of the resinous forest

345   ecosystems through their effects on seed germination and plant growth promotion.

346

347

348   **References**

349

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Figure 1

Analysis of the burned wood samples of *Pinus* species collected after a forest wildfire event.

(**A**) Raman scattering spectra (He-Ne laser emitting at 514 nm) of the *Pinus* burned wood samples of: (**black**) MWCNTs produced by chemical vapor deposition method using alpha-pinene/ferrocene as raw material, (**red**) *P. oocarpa*, (**blue**) *P. pseudostrobus*, and (**green**) *P. montezumae*. The characteristic bands of CNTs, *i.e.*, the D band (1370 cm^{-1}), G band (1600 cm^{-1}), and G' band (2640 cm^{-1}) are shown. (**B**) Thermogravimetric analysis (TGA) of the burned wood samples from *P. oocarpa*, *P. pseudostrobus*, *P. montezumae*, and synthetic MWCNTs (pyrolyzed at 610 °C).
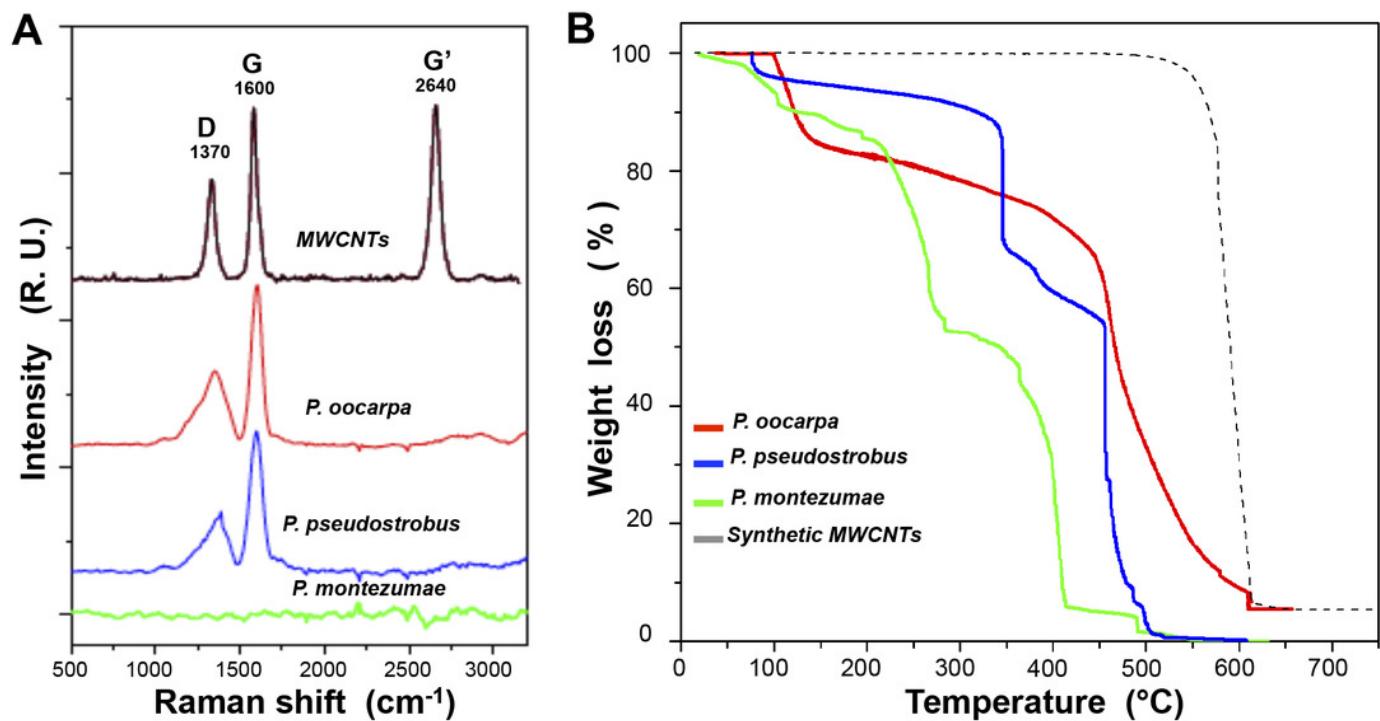
Figure 2

Identification of MWCNTs in the burned wood samples of *Pinus oocarpa* collected after a forest wildfire event.

(**A–C**) HR-TEM images of the burned wood samples at different magnifications, (**D**) FFT image, (**E**) analysis of the FFT image, and (**F**) EDS analysis. Representative images are shown.
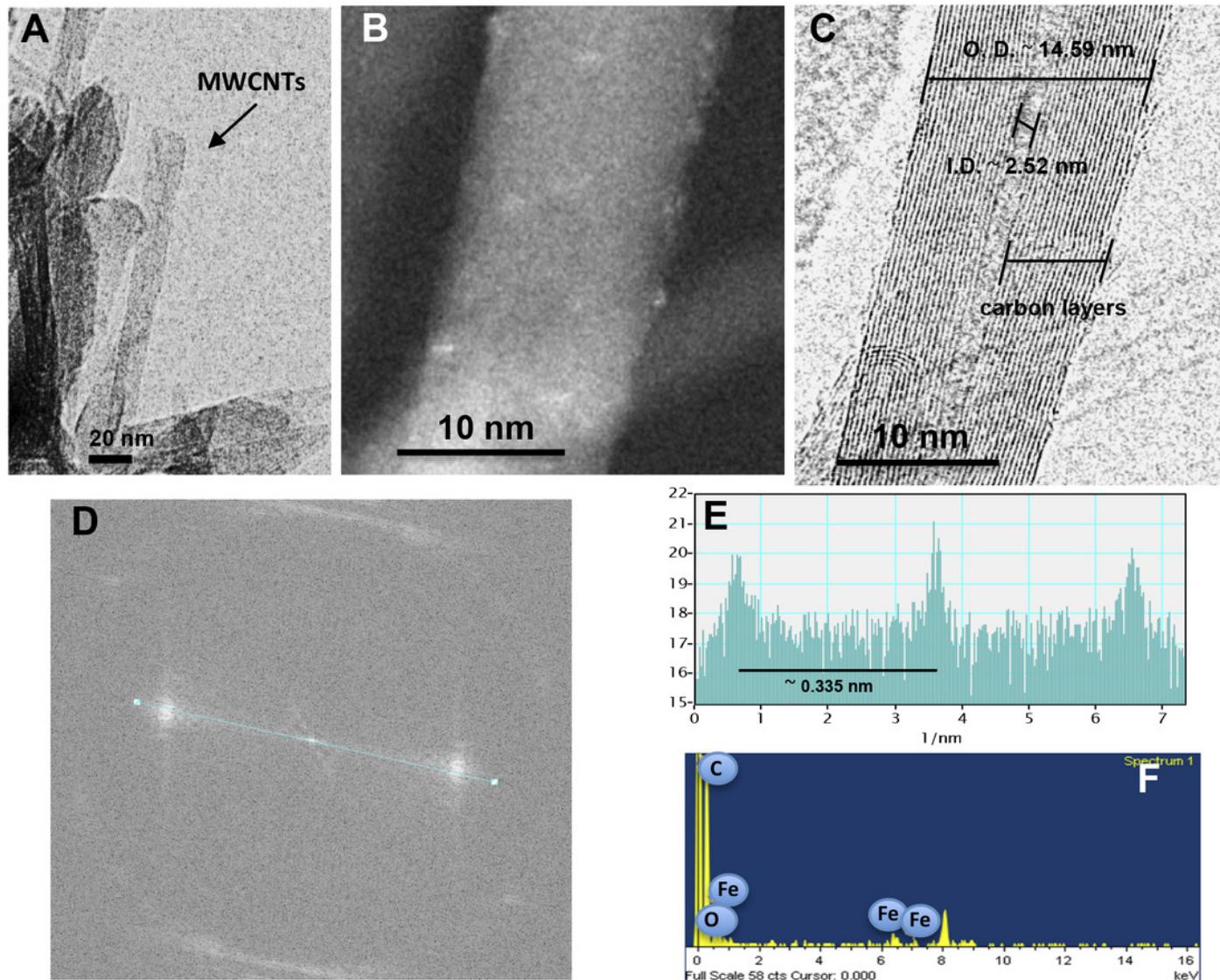
Figure 3

Identification of MWCNTs in the burned wood samples of *Pinus pseudostrobus* collected after a forest wildfire event.

(**A**) HR-TEM images of the burned wood samples at several magnifications, (**B**) FFT image, (**C**) FFT analysis, and (**D**) EDS analysis. Representative images are shown.
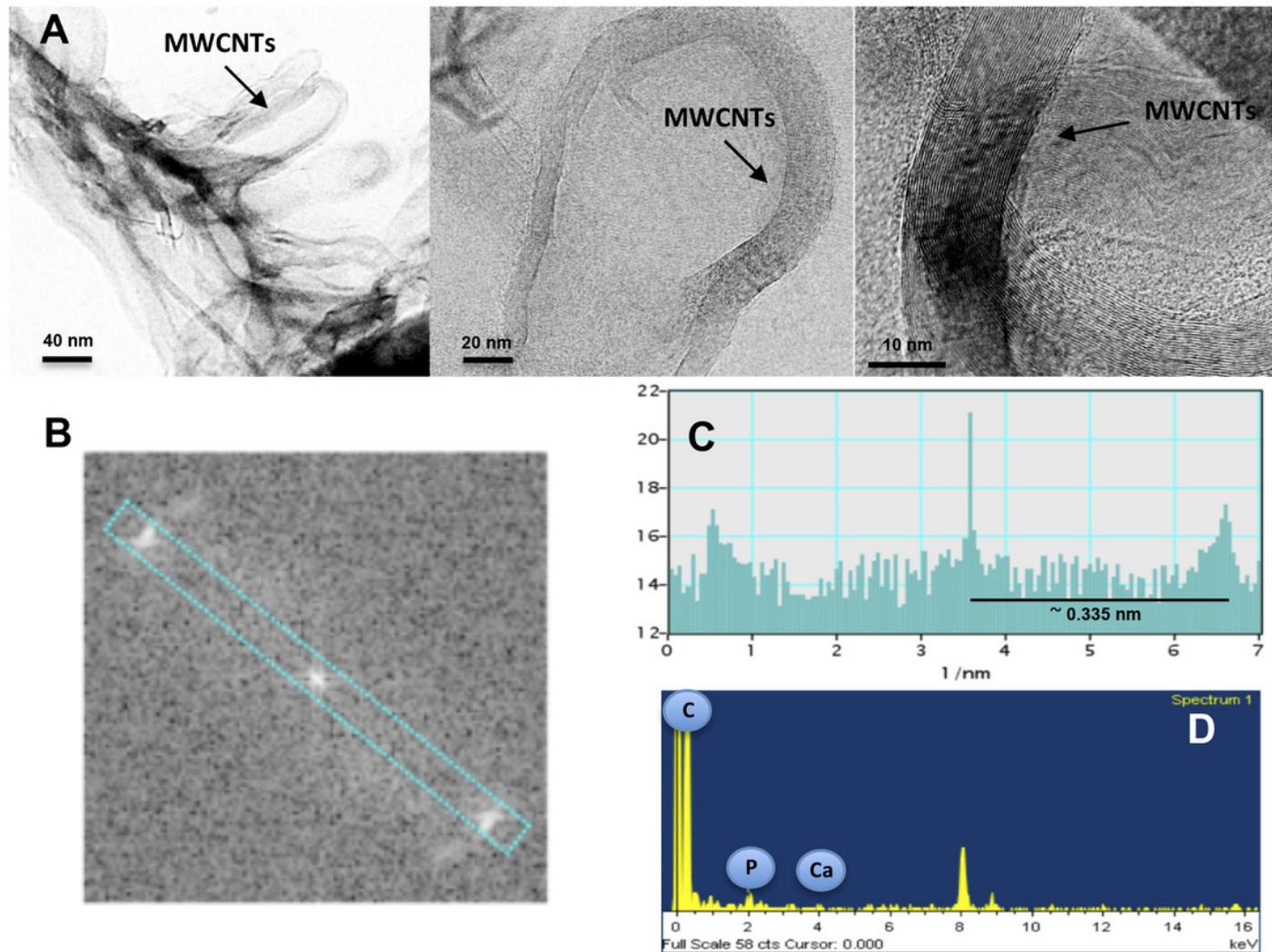
Figure 4

Identification of the carbon structures in the burned wood samples of *Pinus montezumae* collected after a forest wildfire event.

(**A**) HR-TEM images of the burned wood samples at several magnifications, (**B**) FFT image, (**C**) FFT analysis, and (**D**) EDS analysis. Representative images are shown.
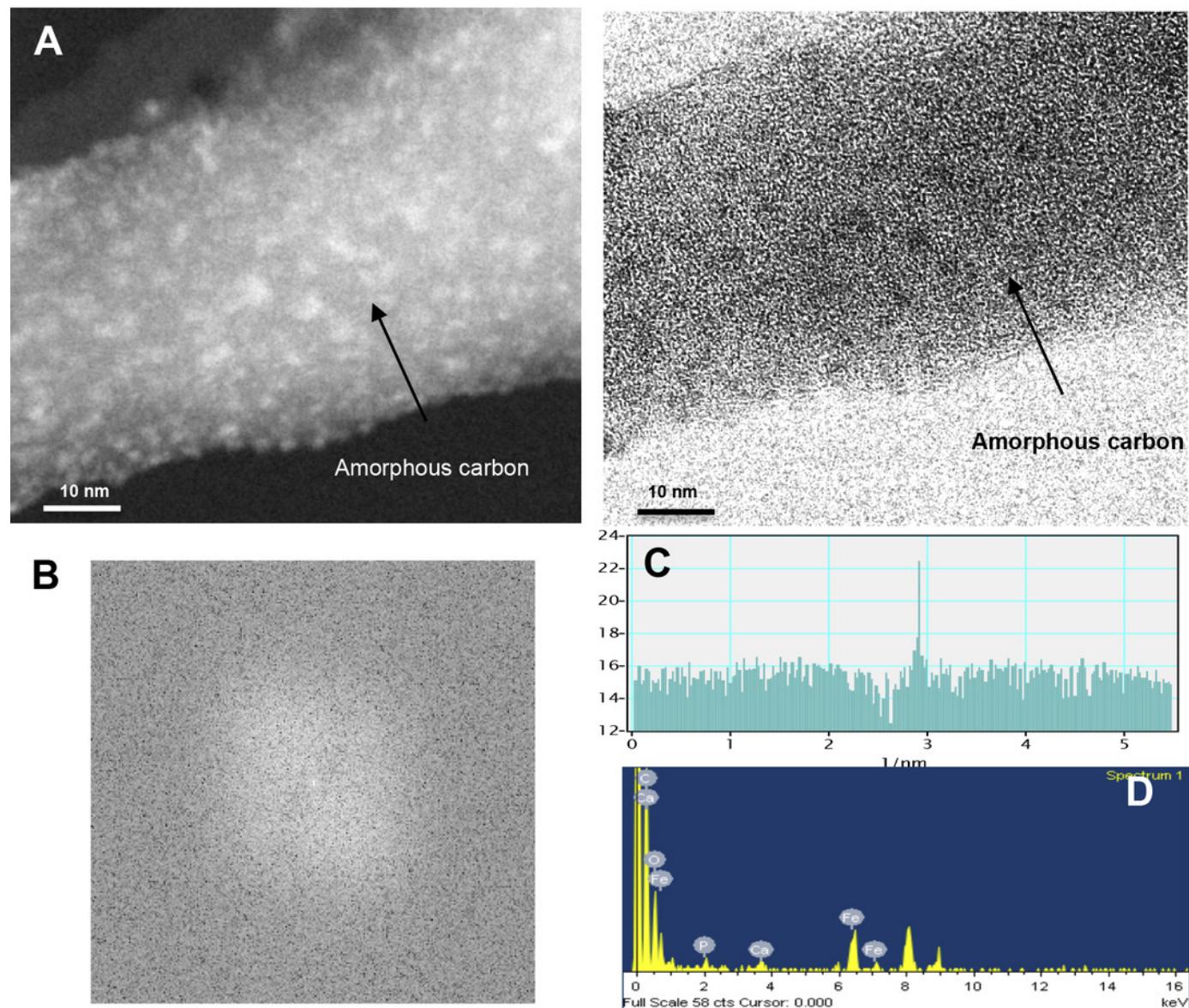
Figure 5

Effect of synthetic MWCNTs on the seed germination rate of *Lupinus elegans* and *Eysenhardtia polystachya*.

Seed germination of the native plants from the *Pinus* forest was evaluated after 10 days with MWCNTs treatment and recorded after 5-week of cultivation. (**A**) *L. elegans* seed germination, (**B**) *E. polystachya* seed germination, (**C**) quantitative data (**A**) and (**B**) assays. Bars represent mean ± standard error of three independent experiments, 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 with respect to control is indicated with different lowercase letters.
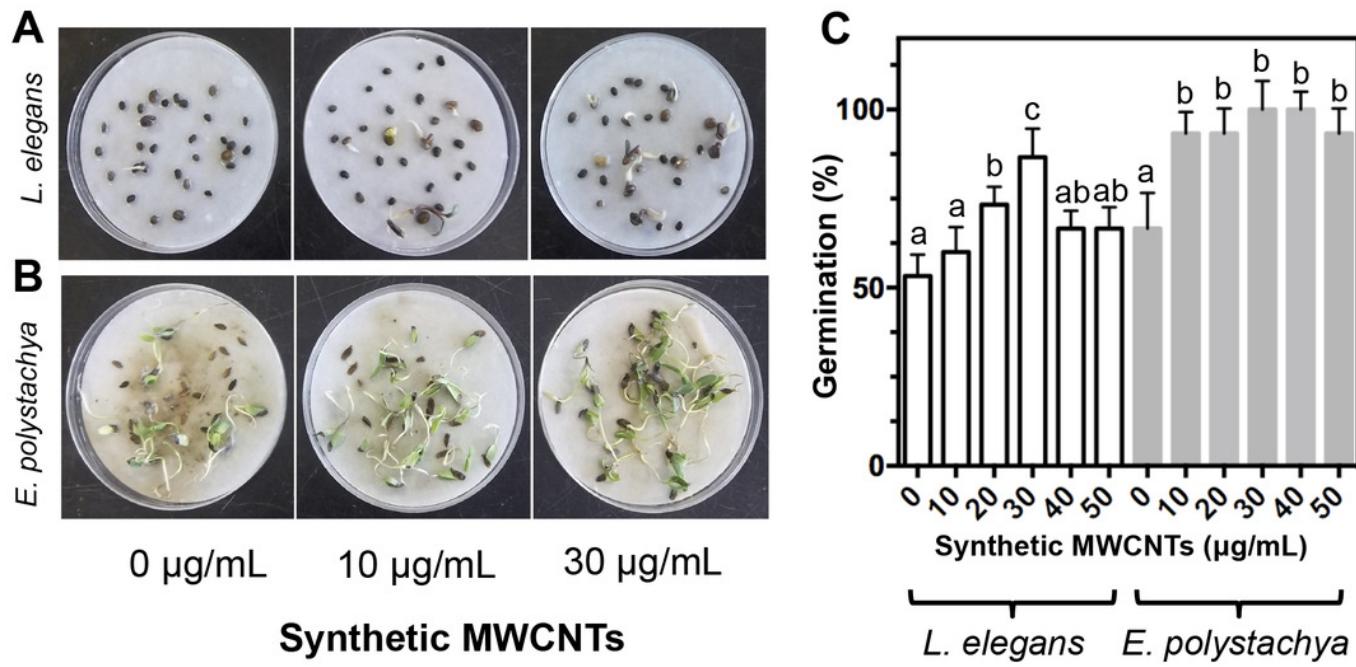
Figure 6

Effect of synthetic MWCNTs on the plant growth rate of *Lupinus elegans* and *Eysenhardtia polystachya*.

After seed germination of the native plants from the *Pinus* forest (as described above), they were planted in 5–cm-diameter polyethylene containers filled with growth medium (Creci-root). These containers were then divided into six separate sets and the seedlings were treated directly with 1.0 mL of the suspension consisting of either 0 (control), 10, 20, 30, 40, or 50 µg/mL of synthetic MWCNTs. At the end of the 5-week period, the plants were harvested, and biometric variables were recorded. (**A**) *L. elegans* plant growth, (**B**) *E. polystachya* plant growth, (**C**) determination of the growth variables (**A**) and (**B**) assays: (**C**) shoot in fresh weight, (**D**) root in fresh weight, (**E**) shoot in dry weight, (**F**) root in dry weight, (**G**), lateral roots number, and (**H**) foliar area. Bars represent mean ± standard error of three independent assays, *n* = 72. 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.