RESPONSE OF TOMATO PLANTS EXPOSED TO TREATMENT WITH NANOPARTICLES

RÉPONSE DE PLANTS DE TOMATE EXPOSÉES A DES TRAITEMENTS AVEC NANOPARTICULES

RISPOSTA DI PIANTE DI POMODORO ESPOSTE A TRATTAMENTI CON NANOPARTICELLE

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Summary

In this work the response of Tomato plants cv. Micro-Tom to nanoparticles (NPs) treatment was investigated. Tomato seedlings were grown in hydroponic condition and NPs treatments were carried out by adding Fe_3O_4 or TiO_2 NPs to nutrient solution. At the end of treatments, NPs root uptake and tissue deposition were investigated using Environmental Scanning Electron Microscope, equipped with energy dispersive spectroscopy for chemical identification. At morphological level, one week after the beginning of NP treatment, seedlings grown with high concentration of TiO_2 NPs showed an abnormal proliferation of root hairs, as compared to the control seedlings and to the seedlings exposed to Fe_3O_4 NPs, Shoot morphology did not differ in tomato seedlings grown under different conditions and no symptoms of toxicity were observed in NP-treated plants. In order to analyse genetic effects of NPs treatments, RNA transcription was studied in roots of NP-exposed and control plants by Illumina RNA sequencing, evidencing the induction of transposable elements.
Key-words: *nanoparticles; tomato; hydroponic; root uptake*

Résumé

Dans ce travail nous avons étudié la réponse des plantes de tomate cv. Micro-Tom à des traitements avec des nanoparticules (NPs). Plants de tomates ont été cultivées dans des conditions hydroponiques et des traitements ont été effectués en ajoutant des NPs de TiO_2 ou Fe_3O_4 à la solution nutritive. À la fin des traitements, nous avons mesuré l'absorption de NPs par les racines et leur dépôt dans les tissues radicaux, utilisant un microscope électronique à balayage, équipé de spectroscopie à dispersion d'énergie pour l'identification chimique des matériaux. Par rapport aux plantes non traitées et a celles exposées à des NPs de Fe_3O_4, les plantes cultivées avec des concentrations élevées de nanoparticules de TiO_2 ont montré une prolifération anormale de poils racinaires. La morphologie des bourgeons ne

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différait pas dans les plantes cultivées dans des conditions différentes et on n'ai été observé aucun symptôme de toxicité chez les plantes traitées avec des NPs.
Afin d'analyser les effets génétiques du traitement avec NPs, nous avons étudié l'expression de l'ARN dans les racines par séquençage Illumina de l'ARN, attestant l'induction des éléments transposables.
Mots-clés: *nanoparticules; tomate; hydroponique; absorption radical*

Riassunto

In questo lavoro è stata studiata la risposta di piante di pomodoro al trattamento con nanoparticelle (NP). Piantine di pomodoro sono state coltivate in idroponica e i trattamenti sono stati eseguiti aggiungendo NP di Fe_3O_4 o di TiO_2 alla soluzione nutritiva. Al termine degli esperimenti sono stati esaminati l'assorbimento radicale di NP e la loro deposizione sui tessuti radicali usando un microscopio elettronico a scansione, dotato di spettroscopio a dispersione di energia per l'identificazione chimica dei materiali. Rispetto ai controlli e a piantine esposte a nanoparticelle di Fe_3O_4, piantine cresciute con alte concentrazioni di nanoparticelle di TiO_2 hanno mostrato una anormale proliferazione di peli radicali. La morfologia dei germogli non differiva in piantine coltivate nelle diverse condizioni e non sono stati osservati sintomi di tossicità in piante trattate con NP. Al fine di analizzare la risposta ai trattamenti con NP a livello genetico, è stata studiata la trascrizione dell'RNA nelle radici, attraverso sequenziamento Illumina, evidenziando l'attivazione di elementi trasponibili.
Parole chiave: *nanoparticelle; pomodoro; idroponica; assorbimento radicale*

Introduction

Nano-sized TiO_2 is widely used in cosmetic and skin care products, in antibacterial and cleaning air products, and for decomposing organic matter in wastewater. Due to the increased demand and production volume, the environmental exposure to nanoscale TiO_2 has been predicted and measured to be higher than the exposure to other frequently used NMs such as ZnO, nano-Ag, fullerenes and carbon nanotubes (Gottschalk *et al.*, 2009).
On their side, Iron oxide (Fe_3O_4) nanoparticles have wide applications several fields of technology (Yi *et al.*, 2006) and these magnetic NPs are used as carriers for controlled delivery of drugs in anticancer applications (Lin *et al.*, 2007).
Increasing application of nanotechnology highlights the need to clarify nanoparticles (NPs) biological effects in organisms and nanotoxicity (Handy *et al.*, 2008a, b). Actually, in spite of increasing amount of research on the toxicity of NPs to animal kingdom and bacteria, limited studies are available in higher plants. Phytotoxicity studies reported both positive and negative effects of NPs on higher plants on seed germination, root elongation, growth, and metabolic processes, such as photosynthesis (Yang *et al.*, 2006, Lin and Xing, 2007 and 2008).
What remains unclear, however, are issues regarding the uptake, accumulation, translocation, and transmission of NPs in plant cells and tissues, and the impact of

these processes on plant development and genetics (Yang *et al.*, 2006, Navarro *et al.*, 2008,).

As for Nano-sized TiO_2, phytotoxicity studies showed both negative and positive effects on plants (Wang *et al.*, 2011, Feizi *et al.*, 2012). In a similar way, Corredor *et al.* (2010) recently tested the effects of applications of carboncoated iron nanoparticles to pumpkin tissue, to evaluate inter- and intra cellular NPs delivery and how plant cells respond to the presence of a high density of nanoparticles by changing their subcellular organization. A phytotoxic effect on root elongation due to Fe_3O_4 treatments was observed in *Arabidopsis* (Lee *et al.*, 2010).

In this work, we have analysed the effects of different concentrations of nanosized TiO_2 and Fe_3O_4 on seedling growth of tomato. We used a hydroponic culture system and examined tomato plant cv Micro-Tom, a dwarf tomato cultivar widely used in plant physiology studies (Meissner *et al.*, 1997). In particular we focused our attention to possible morphological alterations caused by NPs as well as tissue internalization and possible upward translocation of Fe_3O_4 and TiO_2 nanoparticles. Finally, in order to analyse the genetic response of tomato plants to NPs treatment, we began an RNA sequencing approach which allows a comprehensive study of the RNA expressed in given tissues and in given growth conditions. In particular we evaluated the expression of transposable elements (TEs) in TiO_2 treated plants. TEs are the mobile component of the genome, potentially able to change their chromosomal location (transposition) through different mechanisms so determining heritable mutations, genome rearrangements, and epigenetic variations (Lisch, 2009). A few examples correlate TEs activity in the genome to a stress mediated reaction, for example, retrotransposons *Tnt1* and *Tto1* in *Nicotiana* and Tos17 in rice showed stress induced (by tissue culture) transcription and transposition (Hirochika 1993; Hirochika *et al.* 1996; Grandbastien 1998) while these elements are not transcribed in unstressed culture conditions.

Materials and Methods

Seeds of tomato (*Lycopersicum esculentum*) cv Micro-Tom (Meissner *et al.*, 1997) were germinated on wet cotton wool and seedlings were grown in hydroponic condition in a growth cabinet at 25/20°C day/night air temperature. Nutrient solution was a 1:3 dilution of this medium: Ca 4.0 mM; K 7.0 mM; Mg 0.75 mM; N-NO_3 11.0 mM; P-H_2PO_4 1.2 mM; S-SO_4 2.41 µM; Fe 17.8 µM; Zn 5.0 µM; Cu 2.7 µM; Mn 10.0 µM. Twenty uniform ten days old seedlings per treatment were transferred to a small plastic vessel (Fig. 1). For NPs treatments Fe_3O_4 or TiO_2 NPs of 10-30 nm size were used. These were first suspended in nutrient solution for 1 h in a sonication bath, and then were added to nutrient solution at the concentration of 50 mg/L or 500 mg/L. In order to discriminate between a Ti effect due to the presence of NPs or dissolved ions in the nutrient solution, control plants were grown in the same condition as above, but nutrient solution enriched of NPs were first centrifuged then filtered through Anatop syringe filter 0.02 µm to remove all NPs from medium and presumably kept Ti ions. Each experiment was replicated twice.

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Figure 1

Hydroponic culture of Micro-Tom seedlings.

After 7 days of treatment, plants were observed and root apices, seedling hypocotyls and first true leaves were collected and fixed in buffered formalin. Formalin-fixed tissues were paraffin embedded and sectioned (thickness: 20 μm); the sections were placed on acetate slides, and paraffin was removed by washing in xylene.

Root uptake and NPs deposition over roots were investigated using Environmental Scanning Electron Microscope (ESEM) FEI Quanta 200 ®, equipped with energy dispersive spectroscopy (EDS) for chemical identification.

For molecular analysis 1 d- and 7 d-old seedling roots were collected. Total RNA was isolated from roots of 3-4 plants for each of two replicate experiments, according to the method described by Logemann *et al.* (1987) followed by DNAse I (Roche) treatments. RNA-Seq library was generated using the TruSeq RNA-Seq Sample Prep kit according to the manufacturer's protocol (Illumina Inc., San Diego, CA). The library was quantified using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and run on the Illumina HiSeq2000 (Illumina Inc.) using version 3 reagents. Single-read sequences of length 50 bp were collected.

Sequence alignments of cDNAs were generated with CLC-BIO Genomic Workbench 4.9. The TIGR *Solanum* Repeats v3.2 database (ftp://ftp.plantbiology.msu.edu/pub/data/TIGR_Plant_Repeats/) was used for alignments after removing all repeats not belonging to *Lycopersicum esculentum* and after adding 7 available tomato actin encoding sequences to be used as standard.

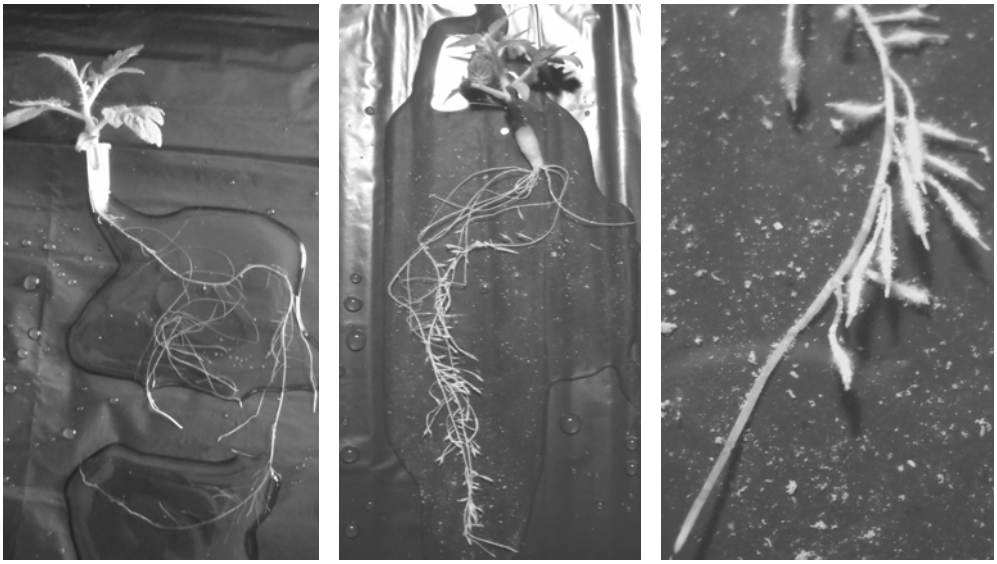
The evaluation of DNA repeats expression in tomato roots was performed with the same software, that reports the number of mapped Reads Per Kilobase per Million of mapped reads (RPKM), measuring the transcriptional activity for each repeat.

CLC-BIO Genomic Workbench computes this normalized expression level by assigning reads to a sequence in the database and counting them.
Only repeats showing a significant expression in at least one of the samples (at least one mapped read per million, according to Mortazavi *et al.* (2008) were considered for analysis. Expression induction or repression was evaluated after 1 and 7 days, considering RPKM values in control and treated plants. RPKM ratios between NPs treated plants and control plants were evaluated using Baggerly's test (Baggerly *et al.*, 2003) using $p < 0.05$.

Results

Compared to the control seedlings and to the seedlings exposed to 50 and 500 mg/L of Fe_3O_4 NPs and to 50 mg/L TiO_2 NPs, the roots developed at 500 mg/L of TiO_2 NPs showed higher number of root hairs one week after the beginning of NPs treatment (Fig. 2-4). Shoot morphology and growth did not differ in tomato seedlings grown under different conditions and no symptoms of toxicity were observed in NP-treated plants (data not shown).

Figure 2 - *Tomato seedlings grown in hydroponic nutrient solution containing no (a) or 500 mg/L TiO_2 NPs (b and c).*



a) **b)** **c)**

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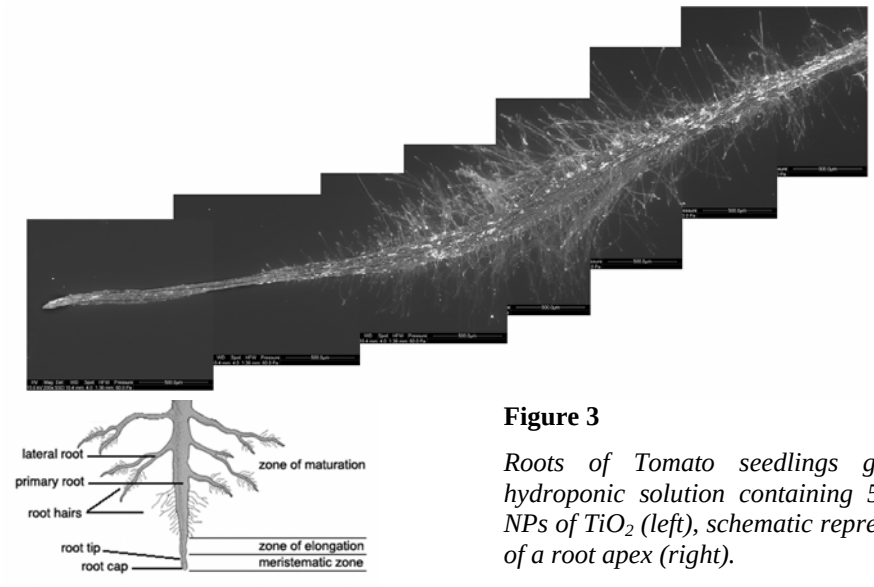

Figure 3

Roots of Tomato seedlings grown in hydroponic solution containing 500 mg/L NPs of TiO_2 (left), schematic representation of a root apex (right).

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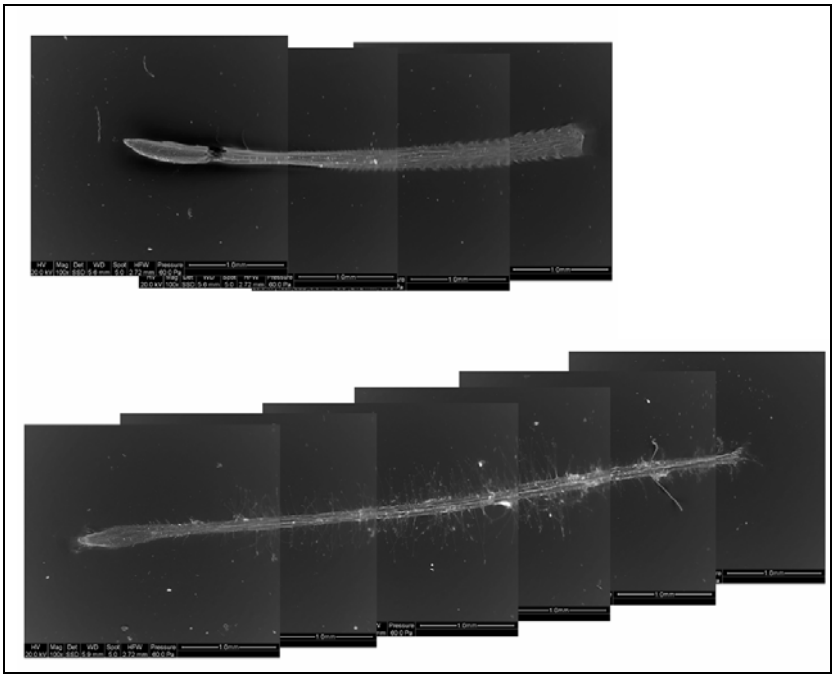


Figure 4

Roots of Tomato seedlings grown in hydroponic nutrient solution containing no (top) or 50 (bottom) mg/L NPs of TiO_2.

ESEM analyses allowed micro structural investigation of root apex surface of plantlets treated with Fe_3O_4 or TiO_2. Nano-structured aggregations which cover root epidermis were apparent, especially when 500 mg/L concentration were used (see Fig. 5, as an example).

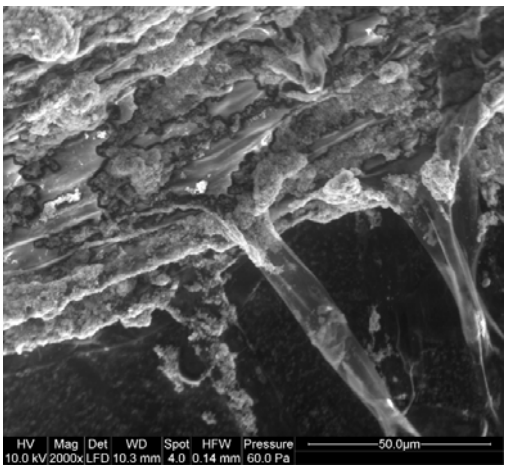
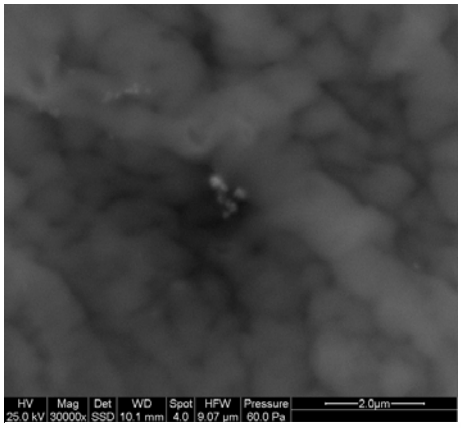


Figure 5

SEM image of root epidermis covered by NPs of TiO_2

ESEM images and EDS spectrum of root apex sections of the seedlings treated with Fe_3O_4 and TiO_2 are shown in Fig. 6 and Fig. 7 respectively, where EDS spectrum of NP aggregations is also reported.

a)



b)

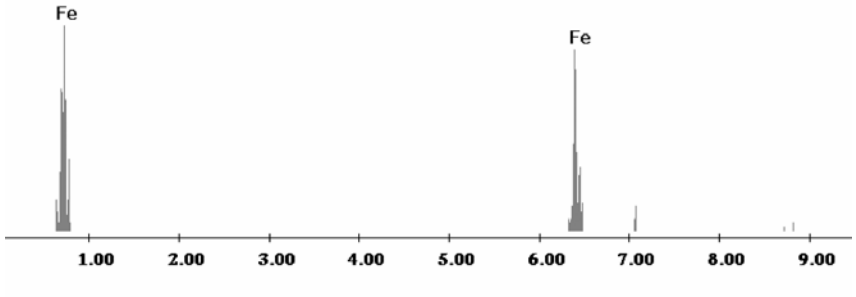


Figure 6

SEM image of root apex of tomato seedlings treated with Fe_3O_4.

a) Aggregated of NPs inside sample treated with Fe_3O_4;

b) EDS spectrum associated with NPs.

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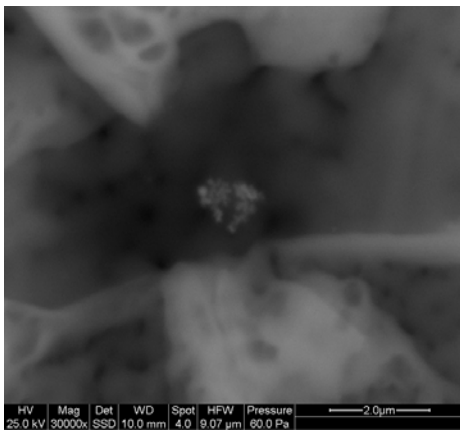
a)



b)

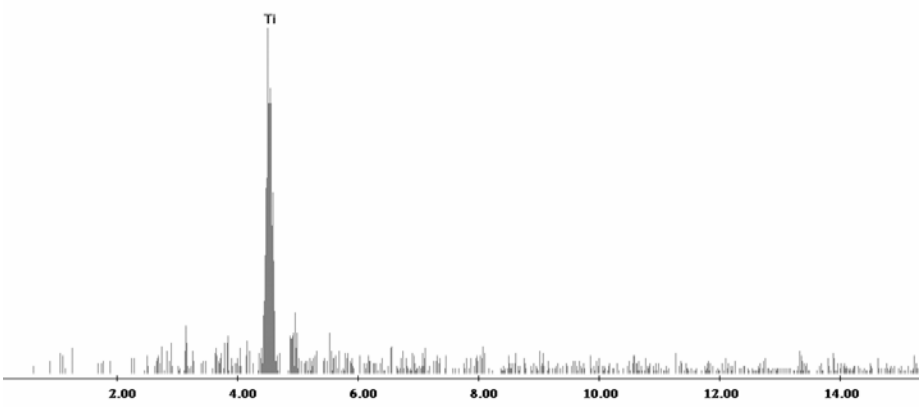


Figure 7

SEM image of root apex of tomato seedlings treated with TiO_2.

a) Aggregates of NPs inside sample treated with TiO_2;

b) EDS spectrum associated with NPs.

Small NPs aggregates with a clear nanostructure and the same chemical composition of the applied NPs were identified also in the hypocotyls while no NPs were found in true leaves (data not shown). For molecular analyses, an Illumina RNA sequencing approach was applied to study the genetic effects of treatments with NPs. The expression of transposable elements (TEs) was evaluated both in control and 500 mg/L TiO_2 NPs treated plants. We prepared and sequenced by Illumina technology eight libraries of cDNAprepared from RNA purified from roots of control and NPs treated tomato plants (for 1 day and 7 days, two replicates per treatment).

We generated 116.5 millions Illumina sequence reads, each 50 nt in length, encompassing 5,8 Gb of sequence data. Each sample (control or TiO_2 NPs treated plants) was represented by at least 20 million reads, a tag density sufficient for quantitative analysis of gene expression (Morin *et al.*, 2008). Sequence reads were aligned to the TIGR Tomato Repeats dataset, using CLC BIO Genomic Workbench software set to allow two base mismatches.

Of 6,486 tomato repeated sequences in the database only a few resulted significantly expressed in control or treated plants (Table 1), belonging to only five

categories. The most represented category was that of *Copia* LTR retrotransposons; a higher number of *Copia* RE resulted expressed in treated than in control roots. Differential expression (i.e. induction or repression) was observed only for five repeats after 1 day and for 9 repeats after 7 days of treatment. Differential expression was shown especially for Copia REs (Table 1). Again, these results are particularly evident after 7 days of treatment. Interestingly, the same unclassified DNA transposon resulted induced both after 1 day and after 7 days.

Table 1 - *Number and categories of DNA repeats in the TIGR tomato database that are significantly expressed in roots of control or treated (for 1 day or 7 days) plants. The number of repeat sequences differentially expressed ($p < 0.05$) in treated compared to control plants is also reported.*

		Expressed after 1 day				Expressed after 7 days			
Repeat category	**Nr.**	**Control**	**Treated**	**Induced**	**Repressed**	**Control**	**Treated**	**Induced**	**Repressed**
Telomeric repeats	**198**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Unclassified repeats	**1,857**	**5**	**5**	**-**	**2**	**4**	**4**	**1**	**-**
Copia REs	**1,333**	**7**	**11**	**1**	**1**	**11**	**13**	**6**	**1**
Gypsy REs	**331**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
LINE REs	**626**	**-**	**-**	**-**	**-**	**1**	**1**	**-**	**-**
SINE REs	**170**	**-**	**1**	**-**	**-**	**-**	**-**	**-**	**-**
Unclassified REs	**941**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
DNA transposons	**22**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Unclassified transpos.	**995**	**1**	**2**	**1**	**-**	**3**	**1**	**1**	**-**
Total	**6,473**	**13**	**19**	**2**	**3**	**19**	**19**	**8**	**1**

Discussion and conclusions

Both Fe_3O_4 and TiO_2 NPs dispersed in the nutrient solution were able to cover root epidermis and to form nanostructured aggregates. Even if these aggregates form quickly it is possible that small aggregates or individual NPs can stay bioavailable. In fact both Fe_3O_4 and TiO_2 NPs were absorbed by the roots and translocated to the hypocotyl but not to the leaves. There were no apparent visual differences in plants grown with or without Fe_3O_4 or TiO_2 NPs, indicating that the particles did not pose any toxicological effects to the plants at the concentration level tested. NPs uptake by roots and translocation to aerial organs including leaves were reported in pumpkin (*Cucurbita maxima*), using hydroponic condition and the same concentration of Fe_3O_4 NPs as in our experiments (Zhu *et al.*, 2008), and in *Arabidopsis thaliana* and *Triticum aestivum*, in which TiO_2-NPs were used (Kurepa *et al.*, 2010, Larue *et al.*, 2011). In other studies, Feizi *et al.* (2012) and Zheng *et al.* (2005) showed that nano TiO_2 improves growth seedling or promote germination and photosynthesis in wheat or spinach in comparison to untreated control plants. Different results concerning uptake or effects obtained by using the

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same species of NPs are not surprising, considering that NPs can explicate their actions depending on the size and/or the shape of the particles, but also on the applied concentrations, specific conditions of experiments, plant species and their mechanism of uptake (Ruffini Castiglione *et al.*, 2011).

In our experiments, relatively high NPs concentration (500 mg/L) in the nutrient solution induced a clear effect on root morphogenesis by stimulating hair formation. This response depends on NPs concentration and on chemical/physical features of NPs used, in fact only high TiO_2 determined this effect, while Fe_3O_4 NPs did not show any apparent effect. However, it remains to be elucidated whether this phenomenon resulted from mechanical perturbation (tigmomorphogenesis) and/or lack of oxygen or mineral uptake (suffocation) due to external (i.e. on the epidermis) TiO_2 NPs deposition or from genuine NPs uptake. One hypothesis is that root hairs proliferation was an adaptive response to the NPs deposition that reduced the root ability to absorb water and nutrients. Root hairs increase absorption area of the root; hairs are polarized outgrowths of root epidermal cells and their initiation and growth (elongation) are regulated by different sets of genes and sensitive to hormonal substances, in particular to auxin and ethylene (Schiefelbein, 2000), and environmental factors e.g. nutrients (Schmidt and Schikora, 2001). It is possible that the morphological response to 500 mg/L TiO_2 NPs observed in Micro-Tom plants is caused by low bioavailability of iron or other micronutrient due to NPs deposition over root tip surface. Further studies are in progress to elucidate the physiological nature of this response.

In order to study the genetic effects of 500 mg/L TiO_2 NPs treatment on tomato plants, Illumina's RNA sequencing were performed, and transcriptome analyses are in progress. First we have evaluated if NPs treatments could trigger the activity of TEs and consequent possible genome rearrangements. In fact, genotoxic effects like chromosomal aberrations have been observed in root meristems of maize and *Vicia narbonensis* exposed to TiO_2 NPs (Ruffini Castiglione *et al.*, 2011) and it is known that TE activation can result in chromosome rearrangements (Zhang *et al.*, 2011).

Our results on DNA repeats expression showed that only a few of the 6,486 tomato repeats analysed were differently expressed in control and in treated plants. Among these, the most common were TEs belonging to the superfamily of *Copia* LTR-retrotransposons. Interestingly, several of these *Copia* retroelements showed high similarity with *Tnt1* and *Tto1*, two retrotransposons found to be expressed in *Nicotiana tabacum* following different stress conditions (Grandbastien, 1998), indicating that these retroelements are induced in *Solanaceae* also by exposure to NPs.

We are now analysing RNA sequencing data to find genes involved in the morphological response of tomato root tip, as well as genes that are known to be activated during mineral deficiency, and genes of auxin and ethylene pathways. Transcriptome analyses in control and NPs treated plants will also allow to evaluate differential expression of other genes involved in the molecular response to both NPs deposition over root epidermis and to the uptake of these particles, as for example

those coding for antioxidant enzymes which provides the first defense mechanism against oxidative toxicity at the cellular level caused by NPs (Kim *et al.*, 2012).
Although our experiments and other similar studies likely represents a worst-case scenario (plants grown in a liquid medium containing high particle concentrations), they nevertheless provide convincing evidences that: i) plant uptake is a potential transport pathway of NPs in the environment, ii) NPs could trigger response at morphological and genetic level.

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