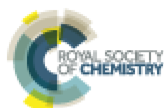

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Metabolomics Reveal that Engineered Nanomaterial Exposure in Soil Alters Both Soil Rhizosphere Metabolite Profiles and Maize Metabolic Pathways

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Accurate risk assessment of engineered nanomaterials (ENMs) in the environment is important for sustainable development and application of nanotechnology. However, previous studies have focused on the response of either plants or soil microbial communities separately. Soil metabolomics, which reflects the integrated response of both the plant and microbial communities to ENMs exposure, has not been used extensively. Here, maize plants were grown in soil amended with SiO_2, TiO_2, or Fe_3O_4 ENMs (100 mg/kg soil) for four weeks. Metabolomics analysis revealed that all ENMs treatments altered the leaf, root and soil metabolite profiles in a ENM-dependent manner. Integration of leaf, root and soil metabolomics enable a thorough characterization of plant metabolism and soil chemistry that can be a powerful tool for ENMs risk assessment.

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Metabolomics Reveal that Engineered Nanomaterial Exposure in Soil Alters Both Soil Rhizosphere Metabolite Profiles and Maize Metabolic Pathways

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ABSTRACT

Accurate risk assessment of engineered nanomaterials (ENMs) in the environment is important for sustainable development and application of nanotechnology. Soil metabolomics, which reflects the integrated response of both the plant and microbial communities to ENMs exposure, has not been used extensively. Moreover, since microbe- and plant-released metabolites contribute to the formation and accumulation of soil organic carbon (SOC), soil metabolite profile alteration from impacted plant and microbial activity may change SOC pool enrichment. Here, maize plants were grown in soil amended with SiO_2, TiO_2, or Fe_3O_4 ENMs (100 mg/kg soil) for four weeks. Plant and soil metabolomics were then used to investigate the global metabolic response of both the plant and soil to ENMs exposure. None of the tested ENMs showed negative impacts on plant growth. However, metabolomics analysis revealed that all ENMs treatments altered the leaf, root and soil metabolite profiles in a ENM-dependent manner. Fe_3O_4 and TiO_2 ENMs exposure induced stronger metabolic reprogramming in leaves, roots and soil compared to SiO_2 ENMs. Interestingly, leaf tissues, which is not the organ directly exposed to ENMs, showed significant amino acid pool alteration upon exposure to ENMs. In soil, levoglucosan, linolenic acid, 4-hydroxycinnamic acid and allo-inositol were significantly increased in response to ENMs. Alteration of the soil metabolite profile indicates that ENMs changed the SOC pool. Integration of leaf, root and soil metabolomics enable a thorough characterization of plant metabolism and soil chemistry that can be a powerful tool for ENMs risk assessment.

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INTRODUCTION

Although engineered nanomaterials (ENMs) have tremendous potential for beneficial impacts in a wide range of sectors, the risk associated with these uses should still be thoroughly evaluated.[1] Agricultural soil will be a primary sink for many ENMs through a number of input pathways. A robust literature on the safety assessment of ENMs to crop plants has developed, including endpoints from phenotypic, metabolomic, proteomic and transcriptomic levels using systems biology approches. Meanwhile, the impact of ENMs exposure on microbial communities in soil or sludge has also been studied using high-throughput sequencing platforms.[2-6] Importantly, soil is the place where plant roots and important rhizosphere microbes co-exist and engage in symbiotic relationships critical to ecosystem function. However, few studies have sought to integrate plant and soil response to ENMs exposure.

Plant and soil microbial communities are connected by a number of pathways, including surfaces and by root exudation.[7] Plant transport 5%-21% of their photosynthetically fixed carbon to the roots for excudation as soluble sugars, amino acids, carboxylic acids, as well as a diverse set of secondary metabolites. These organic constituents enter the rhizosphere, and are used by the microbes as carbon and energy sources, as well as signaling molecules.[8, 9] Soil is the critical location of plant root and soil microbe interaction. Plant and soil microbial metabolic activities ultimately govern the soil metabolic profile. Quantifying low molecular weight metabolites in soil can provide an integrated assessment of both plant and soil simultaneously. On the other hand, soil organic matter is the largest terrestrial carbon pool,[10, 11] and both microbes and plants contribute to the formation and accumulation of this SOC.[12] ENMs can indirectly impact

the SOC pool by modulating the metabolic activities of both microbes and plant. Evaluating the soil metabolite composition and the amounts of individual metabolites will reveal how the SOC pool are impacted by ENMs exposure.

Metabolomics is a powerful and high-throughput tool to capture and analyze a snapshot of the metabolic status of a plant at a given point in time, often in an untargeted manner.[13] Similarly, metabolomics can be used to quantify soil metabolites (soil metabolomics) and provide a similar profile of soil chemical composition status under various conditions, including stress from contaminant exposure. Swenson et al. proposed “soil metabolomics” methods for analysis soil organic matter pool.[14] Soil metabolites largely consist of small molecules of plant and microbial origin resulting from lysed cells and released metabolites.[15] Jones et al.[16] proposed the concept of “community metabolomics”, which is the application of metabolomics techniques to study the entire community of a soil sample. The application of soil metabolomics as part of a comprehensive safety assessment of ENMs has been less studied.

Fe_3O_4 magnetic nanoparticles (NPs) have catalytic and absorbent activity, as well as antimicrobial properties, and thus have wide use in biomedical applications, water treatment and soil remediation. Nanoparticle TiO_2 also has wide use in personal care products, antimicrobial products and as a catalyst.[17] In addition, both TiO_2 and SiO_2 have shown potential as agricultural amendments for pest and fungi control.[18] Thus, application of these ENMs in environmental remediation and nano-enabled agriculture will lead to their accumulation in soil, making an assessment of risk of the use of these materials critical to their safe and sustainable application.

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Maize (*Zea mays*) is a globally important crop with an estimated global production of 1.05 billion tonnes in 2017.[19] Therefore, we chose maize as model crop to evaluate the fate and effects of ENMs. Here, maize plants were cultivated in soil amended with different ENMs (Fe_3O_4, SiO_2 and TiO_2) at a dose of 100 mg/kg, which are likely to produce changes in the soil microbial community and that was also within the realm of environmental relevance. Upon exposure to ENMs, the leaf, root and soil metabolite profile was evaluated using gas chromatography-mass spectrometry (GC-MS). This molecular approach provides a new level of insight into ENMs effects and will significantly inform risk assessment efforts for ENMs and can be used for other contaminant systems.

MATERIALS AND METHODS

Nanoparticles and Plants. SiO_2 and TiO_2 NPs were purchased from Pantian Nano Material Inc. (99.9%, Shanghai, China) and Fe_3O_4 NPs were purchased from Xiangtian Nano Material Inc. (99.9%, Shanghai, China). Transmission electron microscopy (TEM) images of the NPs are shown in **Figure S1**. The original size for SiO_2, TiO_2 and Fe_3O_4 NPs are approximately 20, 5-10, and 30 nm, respectively (**Table S1**). To determine particle hydrodynamic size and zeta potential (Malvern, Nano Series ZS90) in nanopure water, 100 mg/L ENMs stock solutions were prepared and bath-sonicated (KH-140 100DB, Hechuang Utrasonic, Jiangsu, China) at 45 kHz for 30 min before determination. The average hydrodynamic sizes for SiO_2, TiO_2 and Fe_3O_4 were 876±41, 592±7, and 1230±56 nm, respectively, with the corresponding zeta potentials of 19.5±0.37, -

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21.4±0.64 and 11.6±0.64 mV, respectively (**Table S1**). Maize (*Zea Mays*) seeds were obtained from Hezhiyuan Seed Corporation (Shandong, China).

Exposure Assay. The agricultural soil was collected from agricultural experimental stations of Chinese Academy of Sciences at Hailun (site N, 126° 38′ E and 47° 26′ N), from the top 20 cm. The stock solutions of 1000 mg/L of SiO_2, TiO_2 and Fe_3O_4 were prepared in nanopure water. Before application to soil, the suspension were bath-sonicated at 45 kHz for 30 min in cool water until stable dispersion was achieved. The final doses for all ENMs was 100 mg/kg soil. Plastic containers (9cm×9cm×7cm) were then filled with 100 g growth media (40 g agricultural soil and 60 g of potting soil). The potting soil (0.68% N, 0.27% P_2O_5, and 0.36% K_2O, pH 4.28) (Miracle-Gro, Beijing, China) was added to ensure adequate nutrient supply during the cultivation time, specifically seeking to avoid nutrient-stress that might skew plant response. In total, there were four treatments: control (no ENMs), 100 mg/kg SiO_2, 100 mg/kg TiO_2 and 100 mg/kg Fe_3O_4. Four replicate plants (two plants per pot) were grown for each treatment. The plants were cultivated in a green house for 28 days at 25 °C during the day and 20 °C at night. The daily light integral was 180μmol·m^{-2}·s^{-1}. During the growth period, the plants were watered as needed and no additional fertilizers were applied.

Biomass and Chlorophyll Content Analysis. At harvest, maize plants were thoroughly rinsed with tap water for 5 min followed by deionized water for 3 times. The fresh biomass of root, stem and leaf tissues was determined before oven-drying (60 °C for 72 h). Photosynthetic pigment analysis was performed to determine the levels of chlorophyll a and b and total carotenoids. The pigments were extracted following the protocol of Sesták et al.[20]. Briefly, 0.01 g of maize leaves were mixed with 5 mL of 80% methanol

for 12 h, and then the mixture was centrifuged for 10 min at 3000 rpm. A microplate spectrophotometer (Biotek Synergy H1, America) was used to measure the absorbance of chlorophyll a and b and carotenoids in the methanolic extracts at 663, 645, and 470 nm, respectively.

ICP-MS Analysis for Element Content. Tissues for elemental analysis were dried at 60 °C for 72 h. A sample of approximately 0.02 g of dried tissue was microwave-digested (Milestone, Ethos Up, Germany) in a mixture of 8 mL of H_2O_2 and 2 mL of HNO_3 (4/1 v/v) at 160 °C for 40 min. In terms of Ti, 0.1 g of dried tissues were digested in a mixture of 1 mL of H_2O_2, 2 mL of HNO_3 and 5 mL of H_2SO_4 at 180 °C for 15 min according to the sample digestion method described by Larue et al. [21] The resulting digest solution was diluted to a final volume of 50 mL prior to analysis. The content of Fe, Si, Ti and other macro- and micro- nutrients (K, Ca, Mg, Cu and Mn), were quantified by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Optima 8300, Perkin Elmer, U.S.A) or ICP-mass spectrometry (MS) (NexION-300, PerkinElmer).

Metabolite Analysis in Maize Tissues and Soil. The freeze-dried leaf, root and soil samples were subjected to GC-MS based metabolomics analysis. Details on metabolites extraction, GC-MS analysis, and multivariate analysis are described below.

Metabolite Extraction. At harvest, maize plants were thoroughly rinsed with tap water and nanopure water to remove the residual soil or particles from the surfaces. The plants were blotted dry with kimwipes. The fresh leaves and roots were ground into powder in liquid nitrogen and were stored -80 °C until use. Metabolites in maize tissues were extracted with methanol and chloroform. In terms of soil, a 1000 mg soil sample from each treatment (pot) was sieved to 2 mm and then ground to a fine powder in liquid N_2

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and stored at -80 °C for later extraction. Briefly, soil was extracted with the methanol/water (1:1) by sonicating at 60 HZ for 2 min. Additional details regarding the extraction of metabolites from tissues and soil are described in Supporting Information.

GC-MS Analysis. The derivatized sample extracts were analyzed on an Agilent 7890B gas chromatography system coupled to an Agilent 5977A mass selective detector (single quadrupole) (Agilent Technologies Inc., CA, USA). The column employed was a DB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm; Agilent J & W Scientific, Folsom, CA, USA Agilent Technologies, Santa Clara, CA). Helium (> 99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL/min through the column. The initial oven temperature was 60 °C, ramped to 125 °C at a rate of 8 °C/min, to 210 °C at a rate of 4 °C/min, to 270 °C at a rate of 5 °C/min, to 305 °C at a rate of 10 °C/min, and finally, held at 305 °C for 3 min. The injection volume was 1 μL with the injector temperature 260 °C in splitless mode. The temperature of MS quadrupole and ion source (electron ionisation) was set to 150 and 230 °C, respectively. The ionisation energy was 70 eV. Mass data was acquired in a full-scan mode (m/z 50-500), and the solvent delay time was set to 5 min. Quality control samples, which were prepared by applying small aliquots from each sample with L-2-Chlorophenylalanine as internal standard, were injected at regular intervals (every 10 samples) throughout the analytical run.

Multivariate Statistical Analysis. A supervised partial least-squares discriminant analysis (PLS-DA) clustering method was conducted on the GC-MS data via online resources (http://www.metaboanalyst.ca/).[22] Before PLS-DA analysis, data normalization was performed (normalization by sum) for general-purpose adjustment for difference

among samples, and data transformation (log transformation) was conducted to make individual features more comparable. Variable Importance in Projection (VIP) is the weighted sum of the squares of the PLS-DA analysis, and indicates the importance of a variable to the entire model.[23] A variable with a VIP greater than 1 is regarded as responsible for separation, and is defined as a discriminating metabolite in this study.[24] Biological pathway analysis was performed based on GC-MS data using MetaboAnalyst 4.0.[25] The impact value threshold calculated for pathway identification was set at 0.1.[24]

Univariate Statistical Analysis. For the assay of biomass, MDA, photosynthetic pigments and metal content, significant differences between treatment and control means were avaluated using an independent two sample *t*-test. Reference to a significant difference between treatment means is based on a probability of $p<0.05$, unless otherwise stated. Data are presented as mean±standard errors ($n=4$).

RESULTS AND DISCUSSION

Chlorophyll Content and Biomass. Exposure to the ENMs did not result in signs of overt toxicity or stress. **(Figure 1A).** Similarly, the levels of photosynthetic pigments (chlorophyll a, b and carotenoid) were unchanged upon SiO_2 and TiO_2 ENMs exposure. However, Fe_3O_4 ENMs resulted in a significant ($p<0.05$) increase (22.7%) in chlorophyll b content (**Figure 1B**). This is consistent with previous studies demonstrating that superparamagnetic iron oxide increased the chlorophyll content of soybean.[26] In addition, Fe_3O_4 ENMs significantly ($p<0.01$) increased leaf biomass by 15% compared to control; root and stem biomass were not significantly impacted. However, total fresh biomass was significantly ($p<0.05$) increased by Fe_3O_4 NPs as well (**Figure 1C**). Similarly, Rui et al

reported that nano-Fe_2O_3 enhance peanut biomass and chlorophyll content at 1000 mg/kg.[27] The mechanism for Fe_3O_4 enhancing chlorophyll b is still unknown. In contrast, SiO_2 and TiO_2 NPs did not significantly impact maize biomass, which is different from previous reports. Zahra et al.[28] reported that 50 mg/kg TiO_2 significantly increased shoot dry weight and length of *Lactuca sativa*. Rafique et al.[29] also observed positive impact of TiO_2 NPs (up to 100 mg/kg) on root length and biomass of wheat seedlings. It is assumed that the observed phytoxicity of ENMs depends on the plant species, particle size, surface charge.

Malondialdehyde (MDA) content, which is an indicator of membrane lipid peroxidation, was determined to evaluate the influence of ENMs exposure on cell membrane integrity. The results show that none of the ENMs increased the MDA levels in either the root or leaf tissues of the maize plant (**Figure 1D**), indicating no lipid peroxidation had been induced. Conversely, TiO_2 exposure significantly decreased MDA content ($p<0.05$) in maize leaves. This may indicate that TiO_2 ENMs have a protective role in alleviating plant oxidative stress. A decreased MDA level in *Coriandrum sativum* was also observed in presence of 400 mg/kg ZnO NPs by Pullagurala et al.[30]

Distribution of Si, Ti, Fe and Nutrient Elements in Plant and Soil. SiO_2 ENMs are known to be relatively stable in the rhizosphere, whereas TiO_2 and Fe_3O_4 are more likely to undergo dissolution, releasing Ti (Ti^{4+}) and Fe ions (Fe^{3+} and Fe^{2+}) into the soil solution and rhizosphere.[31] We found that Si, Fe and Ti content in maize tissues (leaf and root) and their water soluble content (bioavailable) in soil, were unchanged compared to controls (**Table 1**), indicating no uptake and translocation of Si/SiO_2, Ti/TiO_2 and Fe/Fe_3O_4 NPs in maize plant. These results also indicate that the release of Fe ions from

Fe_3O_4 NPs is negligible, although the soil pH significantly decreased from 5.35 to 5.13 (p<0.05) in presence of Fe_3O_4 NPs during four weeks cultivation. This result is consistent with Antisari et al.[32] who reported that the solubility of Fe_3O_4 NPs in soil is low.

Interestingly, exposure to ENMs (SiO_2, TiO_2, Fe_3O_4) significantly (p<0.05) decreased soil water soluble Cu by 47-64% compared to control (**Table 1**). It is possible that ENMs act as a adsorbent or chelator for copper ions and reduced soil soluble amount of the element. An alternative explanation is that these NMs triggered some metabolites release to soil as root exudates, which chelate with Cu ions and lowered the bioavailability of Cu. For example, organic acid and nicotianamin have been found to be able to binding with metal ions. In root tissues, ENMs exposure had no impact on the level of mineral nutrients. However, Mg and Mn content in the leaves under Fe_3O_4 treatment was significantly (p<0.05) increased by 18% and 5%, respectively, compared with the control. Magnesium is the central atom of chlorophyll molecule and many key chloroplast enzymes are strongly affected by magnesium ions presence.[33] Manganese, as an activator for a number of enzyme reactions in plant, activates several important metabolic reactions and plays an important role in photosynthesis. Therefore, increases in leaf Mg and Mn likely contributed directly to the increased chlorophyll content and leaf biomass. However, the underlying mechanism for Fe_3O_4 increased Mg and Mn content in leaves is still under investigation.

Impact of ENMs on Leaf Metabolome. Using GC-MS, 287 metabolites were identified and semi-quantified in maize leaves. To visualize general grouping information as a function of treatment, PLS-DA analysis was performed. The loading plot (**Figure 2 A**) reveals a clear separation in the first principle component for the composition of leaf

metabolites basd on exposure to different ENMs compared to control, explaining 14.7 % of the variation in the data. These results clearly indicate that ENMs exposure induced alteration in the maize leaf metabolite profile, which is somewhat unexpected given the lack of direct exposure to this tissue and the lack of observed phenotypic changes. Bezemer and Dam showed that belowground pathogenic microorganisms can also induce defense responses aboveground tissues and vice versa.[34] It is clear that below ground tissues transport and deliver the long-distance signal to the upper tissues, resulting in metabolic alterations in leaves. We also note that the metabolic changes are ENM-dependent, with Fe_3O_4 inducing the most noticeable metabolic changes, followed by TiO_2, and SiO_2 (**Figure 2 A**).

In order to discern potential patterns underlying the tested ENMs, a univariate analysis (one-way ANOVA) was run and revealed that 49 metabolites (**Table S2**) were found to be significantly changed by exposure. Interestingly, all ENMs resulted in some common generalized metabolic changes; specifically, the triggering of strong amino acid perturbations in the maize leaves. This is particularly interesting given that the ENMs have substantially different physiochemical characteristics. A number of amino acids were either up-regulated (glutamic acid, isoleucine, serine, valine, tyrosine, phenylalanine, threonine) or down-regulated (glutamine, aspartic acid, glycine, proline) upon exposure to ENMs (**Figure 3**). The reason why ENMs induced pronounced amino acids pool changes is still unknown, however, amino acids play a central role in a wide variety of plant physiological processes, including providing the building blocks of proteins, osmolytes, regulating ion transport; participating in heavy metal detoxification and affecting the synthesis and activity of many critical cellular enzymes.[35, 36] Tyrosine

and phenylalanine are precursor compounds for a variety of secondary metabolites such as phenylpropanoids, alkaloids and glucosinolates,[36] the up-regulation of these two amino acids is a likely indicator of leaf activated defense response. Furthermore, the amino acids glutamine[37] and leucine[38] have been reported to function as a signaling molecules and to regulate important stress-responsive genes. Hence, amino acid profile changes may indicate a reprogramming of nitrogen metabolism to modulate carbon and nitrogen status, to manage plant growth or development or to stimulate defense upon exposure and/or stress.[36]

Additionally, the nitrogen-containing compound 4-aminobutyric acid (GABA) and its precursor (glutamic acid) significantly accumulated in maize leaves when grown in soil amended with ENMs (**Figure S2**). GABA is a non-protein amino acid that plays an essential role in signal transduction, pH regulation, nitrogen storage, plant development and stress defense.[39] Here, we speculate that GABA may play a stress-signaling role in response to ENMs exposure. Succinate semialdehyde (SSA), which is formed from GABA by transaminase, increased by up to 5-8 fold when compared to controls (**Figure S2**). SSA is a mitochondrially-generated intermediate in GABA metabolism and will accumulate intracellularly in response to a variety of biotic and abiotic stressors.[40] Thus, the up-regulation of GABA and its up/down stream metabolites may indicate that the plant was able to sense root stress upon ENMs exposure and and the signal was transduced to the shoot tissues through signaling compounds.

In addition to GABA, a substantial increase of putrescine, another nitrogen-containing compound, was observed in maize leaves exposed to TiO_2 ($p<0.05$) and Fe_3O_4 ($p<0.01$) (**Figure S2**). Putrescine is an important component of polyamines, which have also been

reported to involved in response to a variety of abiotic stresses.[41] In addition to stress response, polyamines are involved in a number of physiological processes such as organogenesis, embryogenesis, floral initiation and development, leaf senescence and fruit development.[42] Additionally, polyamine signaling is directly involved in different complex metabolic routes and intricate hormonal cross-talks.[42] In addition, another polyamine known as spermine and its citrulline precursor were significantly up-regulated upon Fe_3O_4 ENMs exposure. In summary, the up-regulation of GABA and a number of polyamines in maize may be a defense or coping strategy in response to ENMs exposure.

Amino acid and carbohydrate metabolism are closely linked through glycolysis and the citric acid cycle (TCA cycle). In addition to amino acid profile alteration, a number of intermediates from glycolysis and the TCA cycle are precursors for amino acid biosynthesis, including pyruvic acid, oxaloacetate, and α-ketoglutarate. We observed that three TCA cycle intermediates, citric acid, alpha-ketoglutaric acid, and succinic acid were significantly up- or down- regulated in an ENM-dependent manner (**Figure S3**). This demonstrates that ENMs exposure not only induced nitrogen metabolism perturbation, but also altered carbon metabolism.

Perturbed Biological Pathways in Maize Leaves. The significantly changed metabolites above are involved in a number of important metabolic pathways. The perturbed biological pathways were characterized by MetaboAnalyst 4.0. The results of this analysis reveal that there are 11 altered pathways in the leaves of maize plants exposed to Fe_3O_4 ENMs (**Table S3, Figure 4 A**). It is noteworthy that a number of the disturbed pathways are related to nitrogen metabolism. In addition, carbohydrate-related pathways such as the TCA cycle, glycolysis and gluconeogenesis were also significantly

altered by Fe_3O_4 exposure (**Table S3**). The TCA cycle is a key pathway for the biosynthesis of plant hormones such as salicylic acid, ethylene, and auxin;[43] TCA cycle intermediates are precursors for the synthesis of a variety of amino acids. The significant changes of TCA cycle metabolites suggest a significant metabolic reprogramming. Additionally, pyrimidine metabolism; which is involved in a number of important developmental processes such as germination, pollen tube growth, and flowering,[44] was altered by Fe_3O_4 exposure. Inhibition of the pyrimidine pathway may be a strategy for the plant to re-allocate energy and resources for other stress-related processes.

In contrast, TiO_2 ENMs induced perturbations in 9 biological pathways, seven of which overlapped with Fe_3O_4 exposure (**Table S3** and **Figure 4 A**). The TiO_2-specific pathways were glycerophospholipid, glyoxylate and dicarboxylate metabolism, in which succinic acid and isocitric acid were significantly changed. In addition, SiO_2 ENMs induced changes in 6 pathways (**Table S3** and **Figure 4 A**). Importantly, there are three biological pathways commonly altered across all ENMs (**Table S3 and Figure 4 A**); arginine and proline metabolism, methane metabolism, and pantothenate and CoA biosynthesis. These metabolic pathway changes are central to nitrogen and carbohydrate metabolism.

Impact of ENMs on Root Metabolome. In maize root tissues, 360 metabolites were identified and semi-quantified by GC-MS. The PLS-DA loading plot (**Figure 2 B**) shows clear ENM-dependent separation from the untreated controls, particularly for Fe_3O_4 and TiO_2, clearly suggesting metabolite profile alteration in below-ground tissue as well. The magnitude of metabolic changes followed the order of $Fe_3O_4 > TiO_2 > SiO_2$, which shares

the similar pattern to that in the leaves (**Figure 2 A**), and highlights the observation that Fe_3O_4 induced the most noticeable changes in exposed plant tissues. Meanwhile, a one-way ANOVA analysis revealed that only 7 metabolites (homovanillic acid, phosphate, D-glyceric acid, mannose, beta-hydroxypyruvate, ornithine and adenine, **Figure S4 and Table S2**) were significantly changed compared to the control, which is much less than that 49 metabolites significantly changed in exposed leaf tissues. This observation that metabolic perturbation in the roots is less pronounced than the leaves is particularly interesting given that the roots were directly exposed to the ENMs-amended soil. Across these metabolites, decreases in the simple polysaccharide mannose was found across all ENM treatments. An increase of mannose levels was reported in plants under cold stress.[45] It has been reported that mannose governs the expression of the enzymatic antioxidant defense system.[46] In addition, the up-regulation of phosphate, homovanillic acid, and D-glyceric acid and the down-regulation of ornithine and beta-hydroxypyruvate were Fe_3O_4-specific effects (**Figure S4 and Table S2**).

Notably, Fe_3O_4 induced greater metabolic reprogramming than did TiO_2 and SiO_2 NPs and a number of metabolites were only responsive to Fe_3O_4 exposure. For example, 1-Hydroxyanthraquinone, 4-hydroxycinnamic acid, caffeic acid and ascorbate, which are reactive oxygen species scavengers, were significantly increased in Fe_3O_4-exposed root tissue (**Figure S5**). Moreover, phenylalanine, a precursor of antioxidant phenolic acids, was also up-regulated by Fe_3O_4. The up-regulation of antioxidant metabolites may indicate that Fe_3O_4 ENMs induced oxidatives stress in maize roots. Our previous studies revealed that $Cu(OH)_2$ NPs and Ag NPs also caused oxidative stress and upon exposure, plants activated low molecular weight antioxidant compounds to cope with this stress.[47,]

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[48] In addition to the up-regulation of antioxidants, amino acids such as aspartic acid, lysine, serine and valine were also increased in response of Fe_3O_4 ENMs. Interestingly, serine and valine were also increased in maize leaves exposed to Fe_3O_4 ENMs. In addition, phenylalanine and tyrosine, which were increased in maize leaves exposed to Fe_3O_4 ENMs, were increased in the roots as well. As mentioned before, phenylalanine and tyrosine are precursors of defense related secondary metabolites. Their up-regulation in whole plant tissues indicates the activation of defensive systems.

Perturbed Biological Pathways in Maize Roots. A biological pathway analysis showed that there are only 4 pathways perturbed by Fe_3O_4 in the roots, which is significantly less than that in the leaves (11 pathways). As shown in **Table S4** and **Figure 4 B**, the disturbed pathways include inositol phosphate metabolism, ascorbate and aldarate metabolism, glycerolipid metabolism and the TCA cycle. Interestingly, the TCA cycle is the only pathway which was significantly disrupted in both root and leaf tissues. Citric acid and isocitric acid, two important intermediates in the TCA cycle, were up-regulated in both tissues, indicating that energy-related metabolism was altered throughout the plant. Previous study has shown that both fatty acids and glycerolipid metabolism play an important role in plant defense.[49] Ascorbate and aldarate metabolism is also a well known as an antioxidant defense-related pathway. In addition, inositol phosphate functions as a secondary messenger for a variety of extracellular signals.[50] Taken together, the activation of defense and antioxidant-related biological pathways clearly suggests that Fe_3O_4 induced a significant stress response.

In contrast, TiO_2 and SiO_2 induced perturbation in five (metabolism of inositol phosphate, ascorbate/aldarate, methane, glyoxylate and dicarboxylate, and TCA cycle)

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and two (inositol phosphate metabolism, TCA cycle) biological pathways, respectively (**Table S4** and **Figure 4 B**). It is noteworthy that inositol phosphate metabolism and TCA cycle are pathways that were perturbed in maize roots by all tested ENMs, indicating that carbohydrate metabolism is a sensitive target in root tissues, which is different from leaf where nitrogen and carbohydrate metabolism were the primary targets.

Soil Metabolite Profile. Rhizosphere chemistry is the result of root exuded chemicals, including their breakdown metabolites, as well as microbially released compounds and breakdown products.[51] Root exudates are actively or passively secreted from root tissues and serve numerous functions to control abiotic and biotic processes, including changing the chemical and physical properties of the soil, inhibiting the growth of competing plants, combatting herbivores, and regulating the microbial community.[52] GC-MS based metabolomics identified and semi-quantified 300 soil-derived metabolites, ranging from hydrophilic metabolites (e.g. sugar, organic acid, amino acids and nucleotides) to hydropobic analytes (e.g. lipids and phenylpropanoids). The PLS-DA loading plot shows that the ENMs groups (SiO_2, TiO_2, Fe_3O_4) were clearly separated from the control group in a ENM-dependent manner along PC1. (**Figure 2 C**). This indicates that ENMs clearly altered the soil metabolite profile. Interestingly, Fe_3O_4 and TiO_2 ENMs induced more pronounced metabolic response compared to SiO_2 ENMs, which is similar with that in the plant leaf and root. Importantly, dissolved soil organic matter (DOM) is composed of small molecules of plant and microbial origin resulting from lysed cells and released metabolites.[14] Thus, the altered soil metabolite profile may indicate altered DOM

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composition caused by ENMs exposure, although the total content of DOM was unchanged (**Figure S6**).

A one-way ANOVA analysis reveals that levoglucosan was significantly increased by 1.5-2 fold ($p<0.05$) in planted soil amended with ENMs as compared to controls (**Figure 5 and Table S2**). Levoglucosan is a bioactive compound that is exuded into the rhizosphere, and has been associated with significantly increased root length in *Arabidopsis*. We speculate that levoglucosan may be acting as signaling compound in soil in response to ENMs exposure. In addition to levoglucosan, a number of metabolites, including linolenic acid, 4-hydroxycinnamic acid, allo-inositol, beta-mannosylglycerate, gluconic acid, methyl mhosphate, and methyl-beta-D-galactopyranoside were all significantly ($p<0.05$) increased by ENMs amendment (**Figure 5 and Table S2**). These significantly modulated metabolites are classified as sugars, amino acids and amides, aliphatic and aromatic acids, phenolics and fatty acids; all of which stimulate microbial growth or regulate plant growth.[53] As mentioned above, exuded root metabolites act in various roles in plant-plant, plant-microbe (including pathogens) and plant-pest interactions.[52] For example, sugars and amino acids act as chemoattractants for other microbes.[54] In addition, maize has been reported to up-regulate some metabolites, such as phenolics and flavonoids,[55] to attract plant-beneficial rhizobacteria,[56] including nitrogen fixing and growth promoting species.[52] Zhalnina et al.[57] observed that rhizosphere microbes showed affinity preference for the consumption of root secreted aromatic organic acids, such as nicotinic, salicylic and cinnamic acid. Conversely, some phenolic compounds (chlorogenic, caffeic and cinnamic acid) have been reported to enhance plant resistance to soilborne disease (*Fusarium oxysporum f.sp. niveum*).[58] Dakora et al.

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demonstrated that phenolic compounds exuded by roots of N_2-fixing legumes serve as primary signaling molecules to Rhizobia, bacterial which form root nodules and reduce nitrogen to ammonia.[59] Therefore, select actively released compounds may be part of a strategy plants employ to sense or cope with the the ENMs-induced stress. Since these metabolites serve as carbon and energy sources for the microbial community, the altered soil metabolite profile or composition could subsequently impact soil microbial community composition. Further soil microbial analysis are needed to verify this hypothesis.

It must be noted that the observed soil metabolite changes are not completely the result of root exudated metabolites; the contribution from the soil microbial community can not be neglected. Very likely, ENMs, especially those with antibacterial propertes, can impact soil microbial metabolic activity, and thereby trigger the up- or down- regulation of extracellular metabolite release. A number of ENMs have been reported to impact soil microbial community structure and composition, including as AgNPs,[60, 61] TiO_2 and ZnO NPs.[62] Thus, an altered soil metabolite profile may partially attributed to passively released extracellular comounds by microbes. Unfortunately, the current experimental design does not enable elucidation of the relative contribution of the plant and microbes to the altered soil metabolites.

CONCLUSIONS

As the use of nanomaterials in the environment and nano-enabled agriculture continues to increase, a thorough understanding of their environmental impact will be critical to sustainable design and application. In this study, the response of both maize plants and the soil to engineered nanomaterials was investigated using a metabolomic strategy. The

results provide a comprehensive perspective on molecular changes in the leaf, root and soil compartments as a function of ENM exposure. Importantly, although the exposure was in the soil, more pronounced metabolic changes were observed in plant leaves, highlighting the importance of *in planta* sensing and stress signaling originating in the root zone. The application of soil metabolomics enabled a single frame snapshot of soil chemical composition changes upon exposure to ENMs. This approach provides an integrated and simultaneous response of the soil and plant compartments. Our results demonstrate that soil metabolomics can be a powerful tool identify the soil biotic responses to ENMs, and that this approach is transferrable to other contaminant groups. Additionally, this study observed the early response of maize seedlings to ENMs, the full life cycle studies are being planned to assess the impacts of ENMs on food yield and quality.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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Supporting Information. Characteristics of ENMs (Table S1); significantly changed metabolites in maize tissues and soil exposure to different NPs (Table S2); perturbed biological pathways in leaf and root tissues (Table S3 and S4); TEM images of SiO_2 and Fe_3O_4 NPs (Figure S1); the relative abundance of four nitrogen-containing compounds (Figure S2); significantly changed carbohydrates in maize leaves in response to ENMs (Figure S3); significantly changed metabolites in maize root by ENMs (Figure S4); metabolites in maize roots that only respond to Fe_3O_4 ENMs (Figure S5); total organic carbon content (Figure S6).

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Table 1. Element content in maize tissues and in the water soluble fraction in soil (mg/kg)

	K	Ca	Mg	Fe	Ti	Cu	Mn	Si
				Leaf				
Control	20191 ± 1451	2355 ± 392	2482 ± 218	127 ± 67	15 ± 3	66 ± 6	11 ± 1	711 ± 82
SiO_2	20848 ± 2525	2094 ± 176	2408 ± 242	106 ± 27	12 ± 3	61 ± 6	14 ± 7	609 ± 40
TiO_2	21109 ± 1690	2204 ± 355	2524 ± 320	99 ± 19	11 ± 0	67 ± 9	10 ± 1	704 ± 49
Fe_3O_4	21954 ± 2576	2669 ± 186	2904 ± 99*	90 ± 16	13 ± 2	74 ± 2	12 ± 1**	989 ± 227
				Root				
Control	5288 ± 403	3856 ± 318	1757 ± 152	1441 ± 244	103 ± 26	22 ± 9	86 ± 19	2772 ± 489
SiO_2	5363 ± 677	4026 ± 303	1860 ± 155	1417 ± 252	113 ± 39	15 ± 1	113 ± 26	2738 ± 534
TiO_2	4659 ± 699	3670 ± 227	1751 ± 159	1302 ± 357	102 ± 17	50 ± 56	101 ± 14	2898 ± 730
Fe_3O_4	5501 ± 487	4275 ± 408	1807 ± 166	1151 ± 332	77 ± 24	18 ± 1	98 ± 19	2234 ± 767
				Soil				
Control	7 ± 1.2	108 ± 63	31 ± 16	8 ± 2	0.25 ± 0.07	0.064 ± 0.015	0.47 ± 0.28	4.74 ± 0.13
SiO_2	7 ± 3.1	62 ± 8	20 ± 3	14 ± 6	0.30 ± 0.08	0.034 ± 0.005*	0.17 ± 0.03	4.93 ± 1.24
TiO_2	5 ± 0.8*	87 ± 26	25 ± 7	7 ± 2	0.24 ± 0.07	0.027 ± 0.007**	0.17 ± 0.07	3.39 ± 1.02
Fe_3O_4	7 ± 0.7	110 ± 38	31 ± 10	10 ± 5	0.14 ± 0.07	0.023 ± 0.010**	0.25 ± 0.07	4.47 ± 2.36

* indicates statistical difference compared to control based on *t*-test at $P \leq 0.05$, ** $P \leq 0.001$.

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Figure Legend

Figure 1. Maize plant images (**A**) and phenotypic changes, including chlorophyll (**B**), biomass (**C**) and MDA content (**D**) of maize in soil without (control) and with ENMs. Data are means of four replicates. Error bars represent standard deviation. *represent statistical significant at $p \leq 0.05$.

Figure 2. Scores plot (PC1 vs PC2) of partial least squares-discriminant analysis (PLS-DA) of metabolites in maize leaves (**a**), roots (**b**) and soil (**c**). Maize plants were exposed to different doses of engineered nanomaterials (SiO_2, TiO_2, Fe_3O_4) for 4 weeks at 100 mg/kg soil.

Figure 3. Up-regulated and down-regulated amino acids in maize leaves in response to ENMs exposure. Red, green, purple and blue represent control, SiO_2, TiO_2 and Fe_3O_4, respectively.

Figure 4. Venn diagrams showing the overlapping and interconnection between perturbed pathways in maize leaves (A) and roots (B) at different ENMs (SiO_2, TiO_2, Fe_3O_4) exposure at 100 mg/kg. Number represent the numbers of perturbed biological pathways.

Figure 5. Box-whisker plots of GC-MS data showing relative abundance of signicantly changed metabolites in soil. A, B, C and D represent control, SiO_2, TiO_2 and Fe_3O_4 respectively. Thy *y*-axis indicates the absolute signal from GC-MS.

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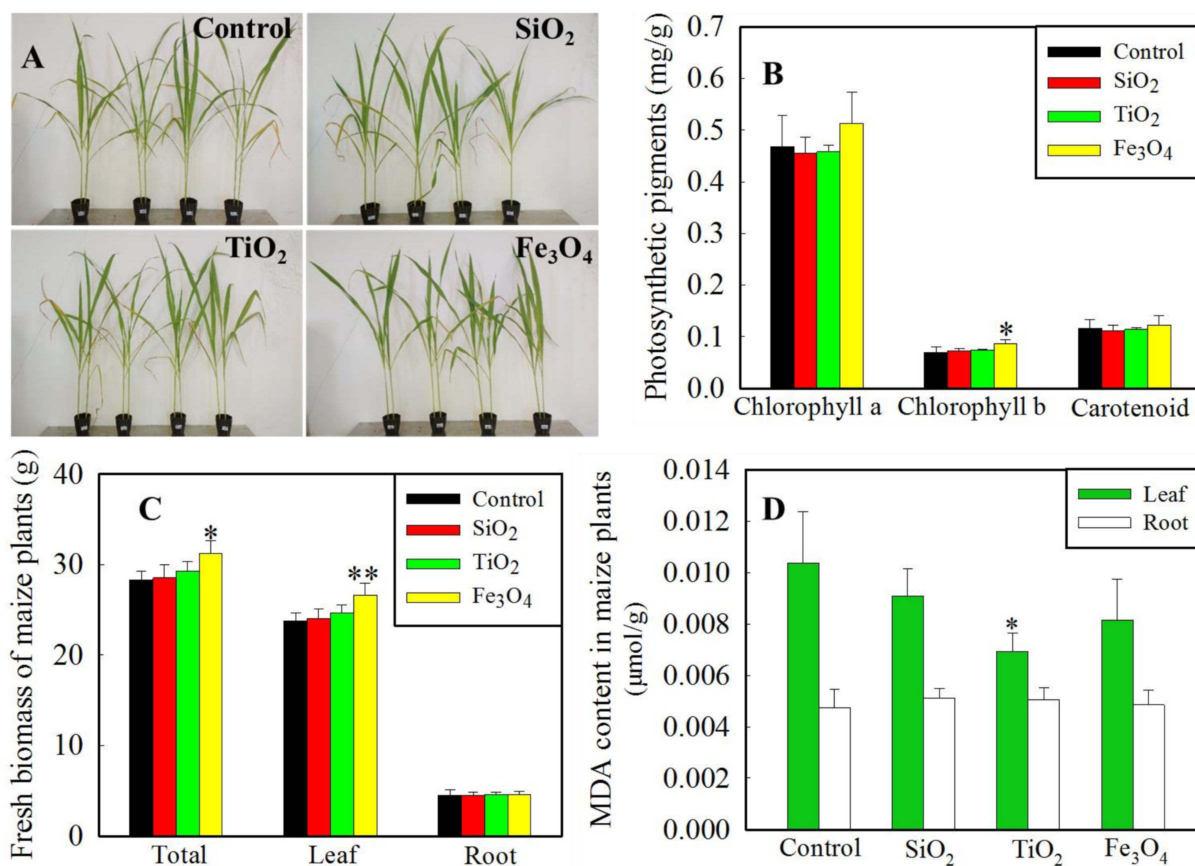

Figure 1.

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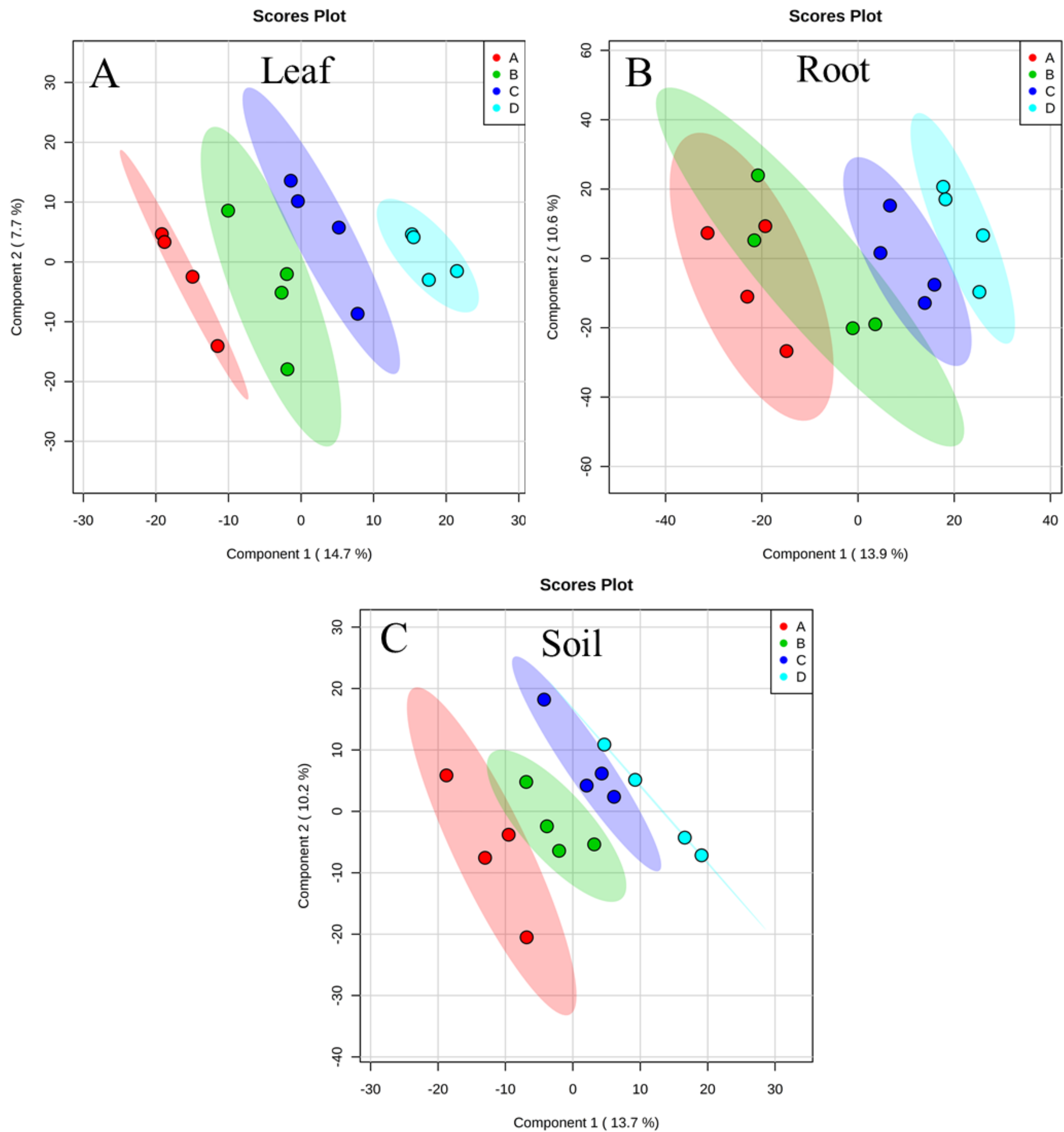

Figure 2.

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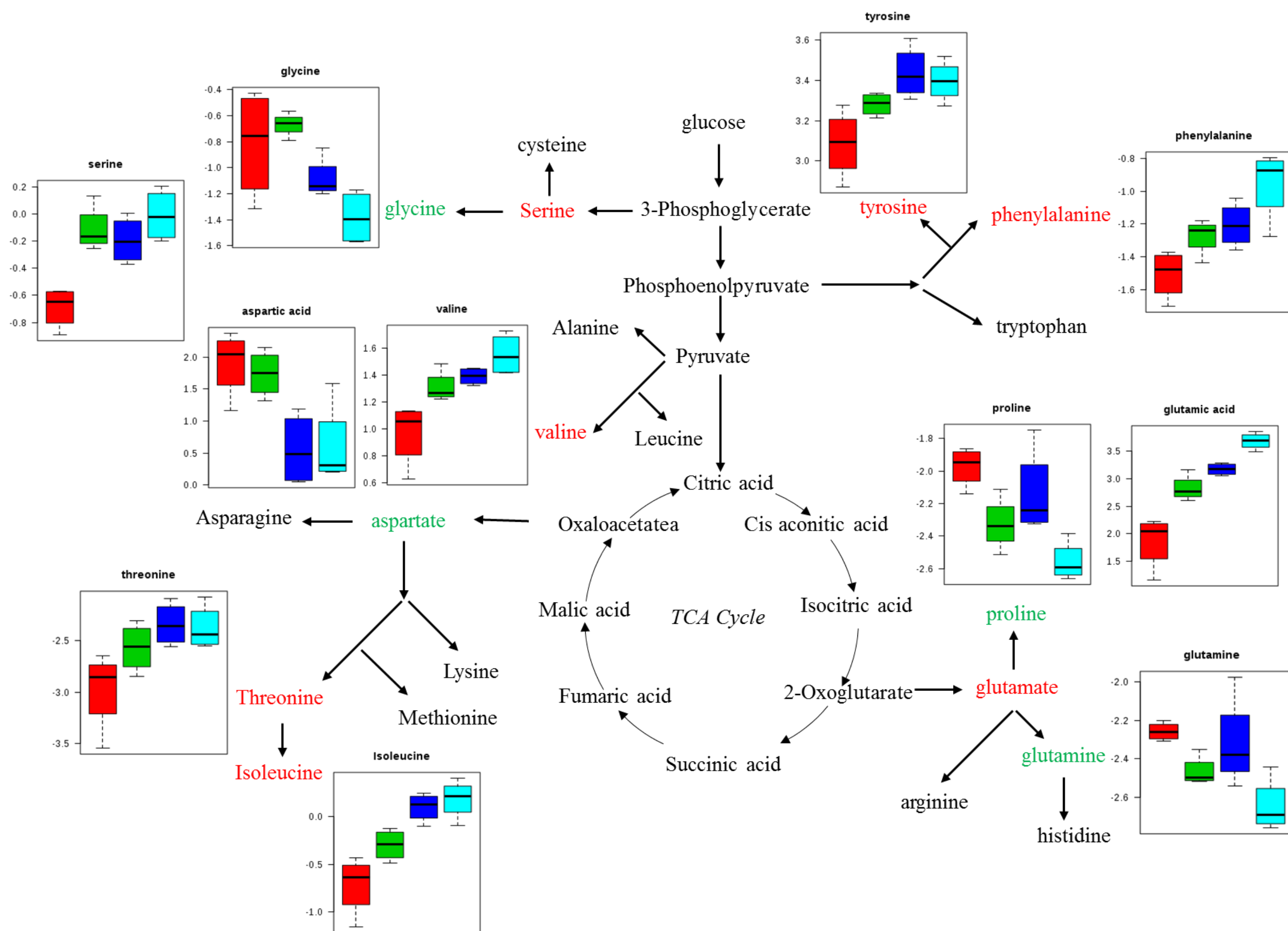

Figure 3.

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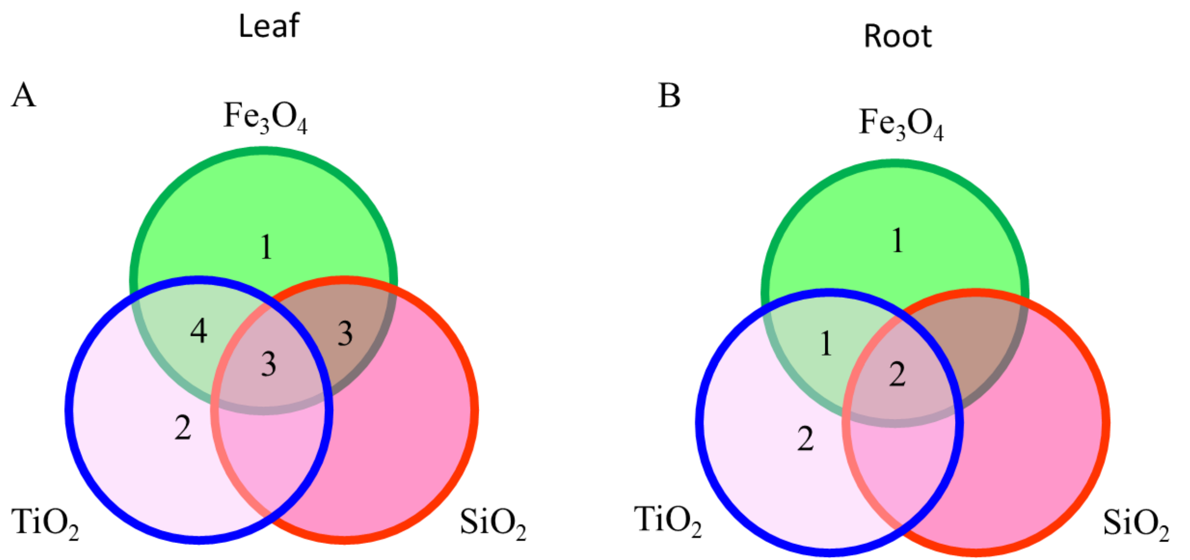

Figure 4.

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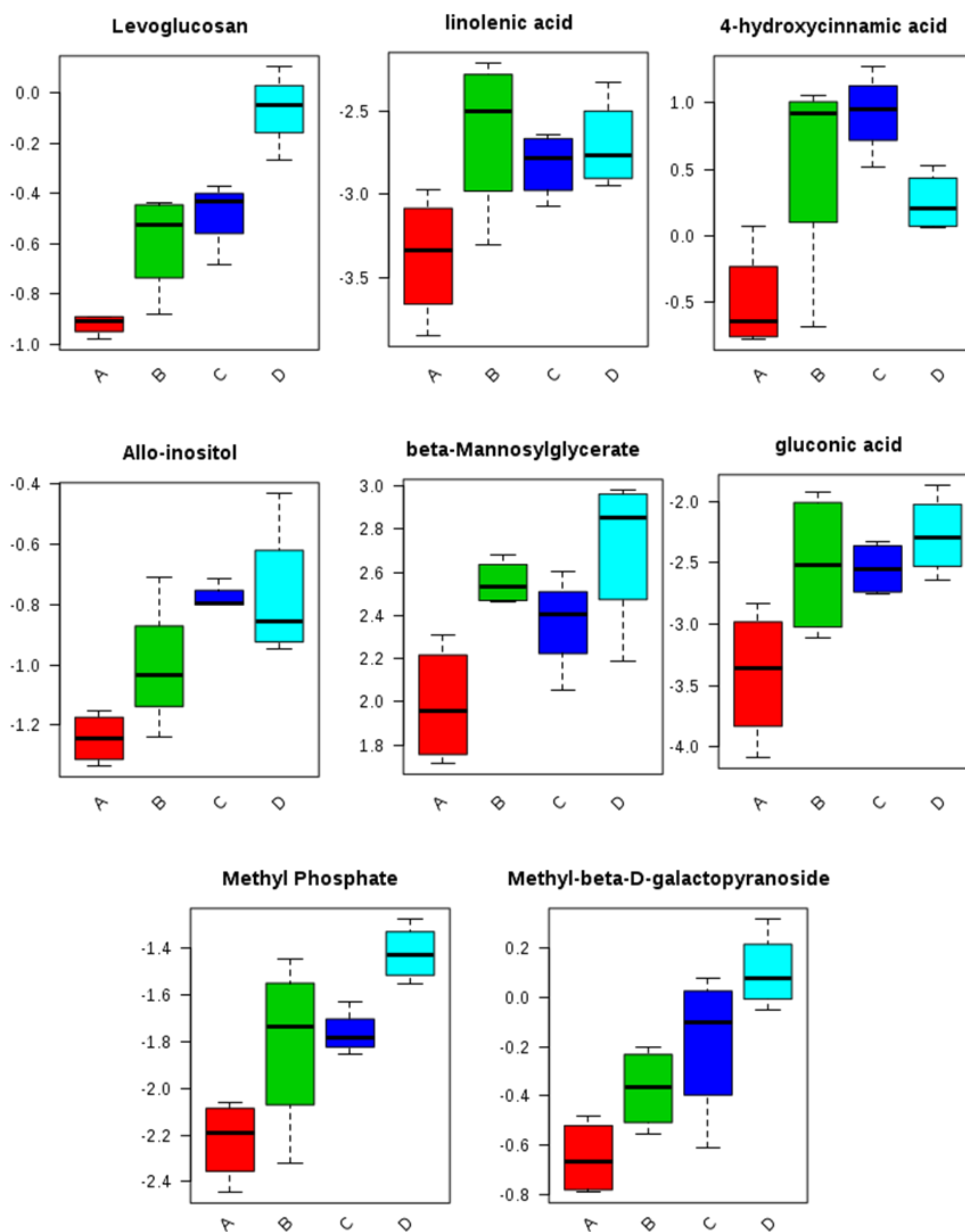

Figure 5.

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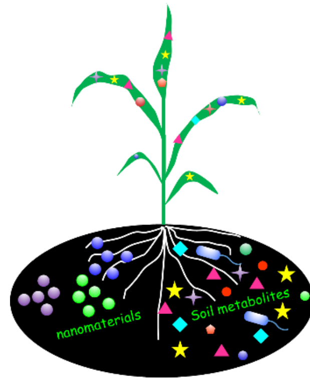

Soil metabolomics enabled a single frame snapshot of plant rhizosphere and soil chemical composition changes upon exposure to engineered nanomaterials.