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Metabolomics of wheat grains generationally-exposed to cerium oxide nanoparticles

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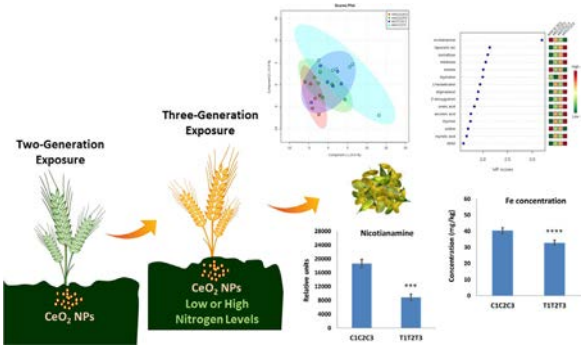
HIGHLIGHTS

- Generational exposure to CeO_2-NPs decreased nicotianamine in wheat grains.
- Decreased nicotianamine abundance was accompanied by decreased Fe concentration.
- More metabolites were affected when wheat was exposed to CeO_2-NPs at low N soil.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigated changes in metabolite compositions over three generation exposure of wheat (*Triticum aestivum*) to cerium oxide nanoparticles (CeO_2-NPs) in low or high nitrogen soil. The goal was to determine if CeO_2-NPs affects grains/seeds quality across generational exposure. Seeds from plants exposed for two generations to 0 or 500 mg CeO_2-NPs per kg soil treatment were cultivated for third year in low or high nitrogen soil amended with 0 or 500 mg CeO_2-NPs per kg soil. Metabolomics identified 180 metabolites. Multivariate analysis showed that continuous generational exposure to CeO_2-NPs altered 18 and 11 metabolites in low N and high N grains, respectively. Interestingly, DNA/RNA metabolites such as thymidine, uracil, guanosine, deoxyguanosine, adenosine monophosphate were affected; a finding that has not been observed on DNA/RNA metabolites of plants exposed to nanoparticles. Nicotianamine, a metabolite playing crucial role in Fe storage in grains, decreased by 33% in grains continuously exposed for three generations to CeO_2-NPs at high N soil. Notably, these grains also exhibited a concomitant decrease of 13–16% in Fe concentration. Together these changes suggest alterations in grain quality or implications in ecosystem processes (i.e., productivity, nutrient cycling, ecosystem stability) of progeny plants generationally-exposed to CeO_2-NPs.

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1. Introduction

Engineered nanoparticles supplied to plants modify their metabolite profile. Metabolomics studies have revealed changes in metabolite

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levels of cucumber, spinach, and maize (i.e. leaves or fruits) exposed to cerium oxide, yttrium oxide, or copper nanoparticles (Zhao et al., 2016; Zhang et al., 2019; Gong et al., 2019). Proteomic study also showed that cerium oxide nanoparticles altered major proteins for nutrient storage and carbohydrate metabolism in bean seeds (*Phaseolus vulgaris*) (Majumdar et al., 2015). Using microarray and quantitative real-time polymerase chain reaction techniques, Thumburu et al. (2015) also found that cerium oxide nanoparticles affected metabolic processes related to DNA and hormone metabolism, tetrapyrrole synthesis, and photosynthesis in *Arabidopsis thaliana*. However, changes in metabolomes of plant generationally-exposed to ENPs remain a major gap in nanophytotoxicity research.

Omics-based studies on generationally-exposed plants are missing in the literature despite several reports demonstrating nutritional, physiological, or biochemical changes in plants exposed to nanoparticles for several generations. We have found changes in physiological and agronomic growth patterns as well as variations in grains macro- and micro-nutrient profile of wheat (*Triticum aestivum*) generationally exposed to cerium oxide nanoparticles (Rico et al., 2017). *Brassica rapa* that had been exposed to cerium oxide nanoparticles for three generations exhibited higher oxidative stress than first generation plants (Ma et al., 2016). Tan et al. (2018) also showed that the photosynthetic activity of basil (*Ocimum basilicum*) exposed to hydrophobic or hydrophilic titanium dioxide nanoparticles for two generations was adversely affected although plant growth was not affected in the end. Medina-Velo et al. (2018) found minimal transgenerational effects on the nutrient profile (i.e. sugar, starch, protein) of *P. vulgaris* seeds exposed to zinc oxide nanoparticles for two generations.

Soil nitrogen level could also modulate the effects of cerium oxide nanoparticles in plants. Majumdar et al. (2015) found that *P. vulgaris* cultivated in soil with higher organic matter exhibited greater susceptibility to alterations in nutrient quality. Related studies have shown that environmental factors such as fertilizer level affect development and protein or starch accumulation in wheat grains (Dupont and Altenbach, 2003). For example, soil nitrogen levels affected the metabolite compositions of oats (*Avena sativa* L.), durum wheat (*Triticum durum* Desf.) and maize (*Zea mays*) (Allwood et al., 2019; Beleggia et al., 2013; Rohlig and Engel, 2010). Similarly, organic matter amendments affected levels of metabolites in *Brassica rapa* leaves (Watanabe et al., 2013) while nitrogen source (organic vs. conventional fertilizer) was an important factor in altering metabolite levels of wheat grains (Zorb et al., 2006, 2009).

This study was performed under the hypothesis that generational exposure to cerium oxide nanoparticles (CeO_2-NPs) could alter the metabolite profile in wheat grains (*Triticum aestivum*) at low or high nitrogen level in soil. This was hypothesized because cerium oxide nanoparticles affected wheat growth and development that could potentially influence the biosynthesis, transport, and storage of metabolites similar to reports by Zhao et al., 2016. The goals were 1) to identify the effects of parental vs. current generation exposure to CeO_2-NPs on the metabolite composition of wheat grains; and 2) to determine the influence of low vs. high soil nitrogen level on metabolite profile of wheat grains. Metabolomics investigation was performed because metabolites provide information on ultimate end products of cellular regulatory processes as plant responds to environmental stress conditions (Fiehn et al., 2008). Metabolomics, compared to transcriptomics or proteomics, provides greater sensitivity in detection of smaller molecules serving key roles in seed development (Zhen et al., 2016). CeO_2-NPs are found in sludge and biosolids amended to agricultural field (Hoppe et al., 2019). These particles are resistant to dissolution and could persist as nanoparticles in soil that plants could potentially be exposed repeatedly for several generations. For example, our previous study revealed that cerium oxide nanoparticles remained intact as nanoparticles on the root surface of wheat (Rico et al., 2017). This study provides information for assessing seed quality with implications to both nutritional status and long-term ecological behavior of plants.

2. Materials and methods

2.1. Generational treatments

The grain was obtained from full life cycle exposure of wheat to cerium oxide nanoparticles (CeO_2-NPs) as previously reported (Rico et al., 2020). The plants were cultivated in a randomized $2 \times 2 \times 2$ treatment combinations of soil N (low vs. high N soil), generationally-exposed seeds (seeds not previously exposed vs previously exposed), and CeO_2-NPs exposure (0 or 500 mg CeO_2-NPs per kg soil). Seeds from plants exposed for two generations to 0 or 500 mg CeO_2-NPs per kg soil (C1C2 = 0 mg CeO_2-NPs per kg soil in 1st and 2nd generations; T1T2 = 500 mg CeO_2-NPs per kg soil in 1st and 2nd generations, respectively) were grown for third generation in low or high N soil amended with 0 or 500 mg CeO_2-NPs per kg soil (C3 = 0 mg CeO_2-NPs per kg soil in 3nd generation; T3 = 500 mg CeO_2-NPs per kg soil in 3nd generation, respectively). The factorial combinations gave four treatments (C1C2C3, C1C2T3, T1T2C3, and T1T2T3) each for low N or high N soil. The treatments were replicated six times giving 24 samples each for low N or high N treatment. Low or high N soil received a total of 48 or 112 mg N of ammonium nitrate (NH_4NO_3) by adding ammonium nitrate modified Yoshida nutrient solution (Yoshida et al., 1976) during the duration of the experiment (SI provides schedule of addition of Yoshida nutrient solution). At the end of experiment, the concentration of nitrogen in low or high N treatment was 240 and 560 μg/g soil dry weight, respectively.

2.2. Plant cultivation and grain production

A 95.14% pure CeO_2-NPs were purchased from Meliorum Technologies (Rochester, NY). These particles were rods with primary size of $67 \pm 8 \times 8 \pm 1$ nm, particle size of 231 ± 16 nm in DI water, and surface area of 93.8 m^2/g (Keller et al., 2010). A 100 mg CeO_2-NPs in 50 mL Millipore water was sonicated in water bath (Branson Ultrasonics, Danbury, CT) at 25 °C for 30 min. The CeO_2-NPs suspension was poured evenly in each pot containing 200-gram dry weight equivalent of soil mix (3:1 (v:v) mixture of Sunshine Mix #2 and sand) to give the necessary 500 mg CeO_2-NPs per kg soil treatment. The pots were aged in the growth chamber for three days before seedlings were transplanted.

Two nine-day-old seedlings were transplanted in each pot (one seedling/100 g dry weight soil) and allowed to grow in growth chamber (Environmental Growth Chamber, Chagrin Falls, OH). The growth chamber conditions were 16-h photoperiod, 20/10 °C, 70% humidity, 300 μmol/m^2-s for the first 40 days, after which the conditions were kept at 16-h photoperiod, 25/15 °C, 70% humidity, 600 μmol/m^2-s until harvest. Soil was kept saturated with water all throughout the duration of the experiment. Ladybugs (family Coccinellidae) were released in the chamber to prevent possible occurrence of wheat green bug (*Schizaphis graminum*).

2.3. Metabolite analysis

Wheat grains were collected 18 days before harvest. Four spikes were collected from each pot, dehulled grains were pooled together, frozen in liquid nitrogen, and kept at −80 °C. Grain samples were sent to Fiehn Lab at West Coast Metabolomics Center at UC Davis (http://metabolomics.ucdavis.edu/) for analysis of primary metabolites using GC-QTOF-MS. Fiehn et al. (2008) provided a brief description of the sample treatment, instrumentations, and analytical method. SI also provides details on data acquisition, data processing, and data reporting. Data analysis followed that of Zhao et al. (2016). The actual measured data (e.g. no data transformation) was subjected to multivariate and univariate analyses using the online software Metaboanalyst (http://www.metaboanalyst.ca/). Multivariate analysis (i.e. Partial Least Square analysis) assigned Variable Importance in Projection (VIP) value on metabolites which indicates its importance to group clustering or

separation. A VIP value of above 1 assigned on a metabolite is regarded as significant. Univariate analyses between C1C2C3 and each generational treatments (C1C2T3, T1T2C3, and T1T2T3) were performed to identify metabolites that changed significantly at $p \leq 0.05$, 0.01, and 0.001 ($p > 0.05$ was excluded).

The metabolites identified from multivariate and univariate analyses were used for a two-way ANOVA. Two-way ANOVA was performed separately for low N and high N treatments to determine the statistical significance of parental exposure [parental exposure at first and second generations (C1C2 vs. T1T2)], current exposure at third generation [third generation exposure (C3 vs. T3)], and their interactions. ANOVA was performed using General Linear Model in SAS statistical package (SAS Institute, Cary, NC). Two-way ANOVA comparison between parental exposure indicates comparing C1C2 (mean of C1C2C3 and C1C2T3) with T1T2 (mean of T1T2C3 and T1T2T3), and comparison between current exposure indicates comparing C3 (mean of C1C2C3 and T1T2C3) with T3 (mean of C1C2T3 and T1T2T3).

2.4. Micro-nutrient analysis of grains

Micro-nutrient analysis of young and mature grains was performed to determine if changes in elemental contents occurred during the last stage of grain formation. Young grains were the same grains used in the metabolite analysis while mature grains were obtained when the plants reached the full life cycle. Elemental analysis was performed using Agilent 7900 Inductively Coupled Plasma - Mass Spectroscopy (Agilent Technologies, Palo Alto, CA). Plant materials were digested in 5 mL concentrated plasma pure nitric acid (SCP Sciences, Champlain, NY) using microwave system (CEM Mars 6, Matthews, NC). The digestate was diluted to 50 mL using Millipore water. Blanks, duplicates, and NIST-1547 peach leaves as reference standard (NIST, Gaithersburg, MD) were used to validate the digestion and analytical methods. Blank and spiked samples were analyzed between 12 samples to ensure instrument stability and performance. ANOVA comparison was also performed.

3. Results and discussion

3.1. Metabolite profile of grains

There were over 500 metabolites detected in wheat grains, but only 180 metabolites were identified using the KEGG Pathway Database (https://www.genome.jp/kegg/pathway.html). Multivariate analysis (i.e. Partial Least Square analysis) using MetaboAnalyst was performed on all 180 metabolites, and results of the analysis revealed separation between treatments (C1C2C3, C1C2T3, T1T2C3, T1T2T3) (Fig. 1A,C; SI Figs. 1, 2). The metabolites with VIP >1 are considered responsible for the separation (Fig. 1B,D). Several metabolites also showed significant differences at Fisher's test $p \leq 0.05$, 0.01, and 0.001 ($p > 0.05$ was excluded) when each generational treatment (C1C2T3, T1T2C3, T1T2T3) was individually compared to C1C2C3 using MetaboAnalyst. Interestingly, Fisher's test showed that compared to the control (C1C2C3), the number of metabolites altered in T1T2T3 in both low and high N greatly increased than C1C2T3 and T1T2C3 (Tables 1, 2; SI Figs. 1, 2). This result indicates that continued exposure for three generations to CeO_2-NPs increased plants susceptibility to metabolic changes in grains. Ma et al. (2016) also observed increased toxicity in *Arabidopsis thaliana* from first to third year exposure to CeO_2-NPs.

Metabolites are the end products of cellular processes (Fiehn et al., 2008; Watanabe et al., 2013; Das et al., 2017); thus, CeO_2-NPs clearly induced changes in plant metabolic processes that eventually altered the metabolite components of the grains/seeds. The metabolites that were significant in both Partial Least Square and Fisher's *t*-test were combined to give 31 metabolites at low N treatment and 24 metabolites at high N treatment (Table 3; SI Tables 1, 2). These metabolites were used in further analysis by two-way ANOVA to determine the effects of parental (C1C2 vs T1T2) or current (C3 vs T3) generation exposure to cerium oxide nanoparticles on the metabolite profile of grains.

3.2. Generational exposure and soil N level affected the metabolite profile of grains

Two-way ANOVA revealed that parental (C1C2 vs T1T2) or current (C3 vs T3) exposure to cerium oxide nanoparticles altered the metabolite compositions of wheat grains at the late developmental stage of grain development. Results of the two-way ANOVA showed that current generation exposure at T3 (mean of C1C2T3 and T1T2T3 vs. mean of C1C2C3 and T1T2C3) affected more grain metabolites than parental exposure at T1T2 (mean of T1T2C3 and T1T2T3 vs. mean of C1C2C3 and C1C2T3) compared to their relative controls (Figs. 2, 3, 4; SI Tables 1, 2). This trend was due to much greater degree of changes in metabolite levels at T1T2T3 exposure (Tables 1, 2). When wheat has been exposed in previous generations (T1T2) only a few metabolites were affected at low N (acetylserine, homoserine, aconitic, and 6-deoxyglucitol) and high N (thymidine, lignoceric acid, isomaltose, melibiose, and nicotianamine) (Figs. 2, 3). Contrary to this, current exposure to cerium oxide nanoparticles (T3) altered the relative abundances of 18 out of 31 metabolites at low N; 11 metabolites (mannose-6-phosphate, ribose, thymidine, uracil, guanosine, 2-deoxyguanosine, fumaric acid, 2-ketoisocaproic, oleamide, glycyl-proline, and spermidine) decreased while 7 metabolites (glucose-6-phosphate, erythritol, adenosine-5-monophosphate, pyrophosphate, phosphoethanolamine, pipecolinic acid, and aconitic acid) increased compared to C3 (Figs. 2, 3). At high N, seven out of 24 metabolites were modified: five metabolites (glucose-6-phosphate, uridine, hexose-6-phosphate, inosine, and nicotianamine) decreased while two metabolites (i.e., 1-hexadecanol and 1-monopalmitin) increased in T3 compared to C3 (Figs. 2, 4).

Notably, the results also revealed that more metabolites were affected at low N compared to high N and that changes at low N were at higher significance levels ($p \leq 0.05$ and 0.01) as opposed to low significance level ($p \leq 0.10$) at high N (SI Tables 2, 3). ANOVA showed 22 metabolites altered in both T1T2 and T3 at low N but only 12 metabolites changed at high N (Figs. 2, 3, 4). Fisher's test also showed that relative to C1C2C3, T1T2T3 disturbed 18 metabolites in low N soil compared to 11 metabolites at high N soil (Tables 1, 2). These findings evidently demonstrated the greater susceptibility of plants to metabolic changes at lower soil N treatment. The amount of soil organic matter also modulated the effects of CeO_2-NPs on protein expression in kidney beans (Majumdar et al., 2015). Similar studies have found greater alterations in metabolite profile of plants cultivated at N deficient soil (Allwood et al., 2019; Lemoine et al., 2013; Watanabe et al., 2013).

3.3. Metabolites affected at third generation exposure that share common metabolic pathways

Several of the metabolites disturbed in grains exposed to CeO_2-NPs at third generation (T3) were related by pentose phosphate, phosphoribosyl pyrophosphate, and fructose-mannose pathways (Fig. 2). At low N, T3 increased glucose-6-phosphate and erythritol by 49 and 56% and decreased mannose-6-phosphate, ribose, guanosine, 2′-deoxyguanosine, thymidine, and uracil by 44, 12, 23, 64, 40, 56%, respectively, compared to C3. In the case of high N, the relative abundances of glucose-6-phosphate, uridine, and inosine decreased (37, 22, 26%, respectively) while thymidine increased (36%) in T3 grains compared to C3. There were more metabolites that decreased at low N suggesting more significant alterations in the physiological and metabolic pathways during grain development.

Glucose-6-phosphate is a precursor for starch synthesis. Direction of changes in this metabolite could indicate direction in storage of starch in grains. Both the increase in glucose-6-phosphate and pyrophosphate suggest starch formation was strongly dominant in low N treatment (Rahdhawa and Singh, 1998). Alternatively, the decrease in glucose-6-

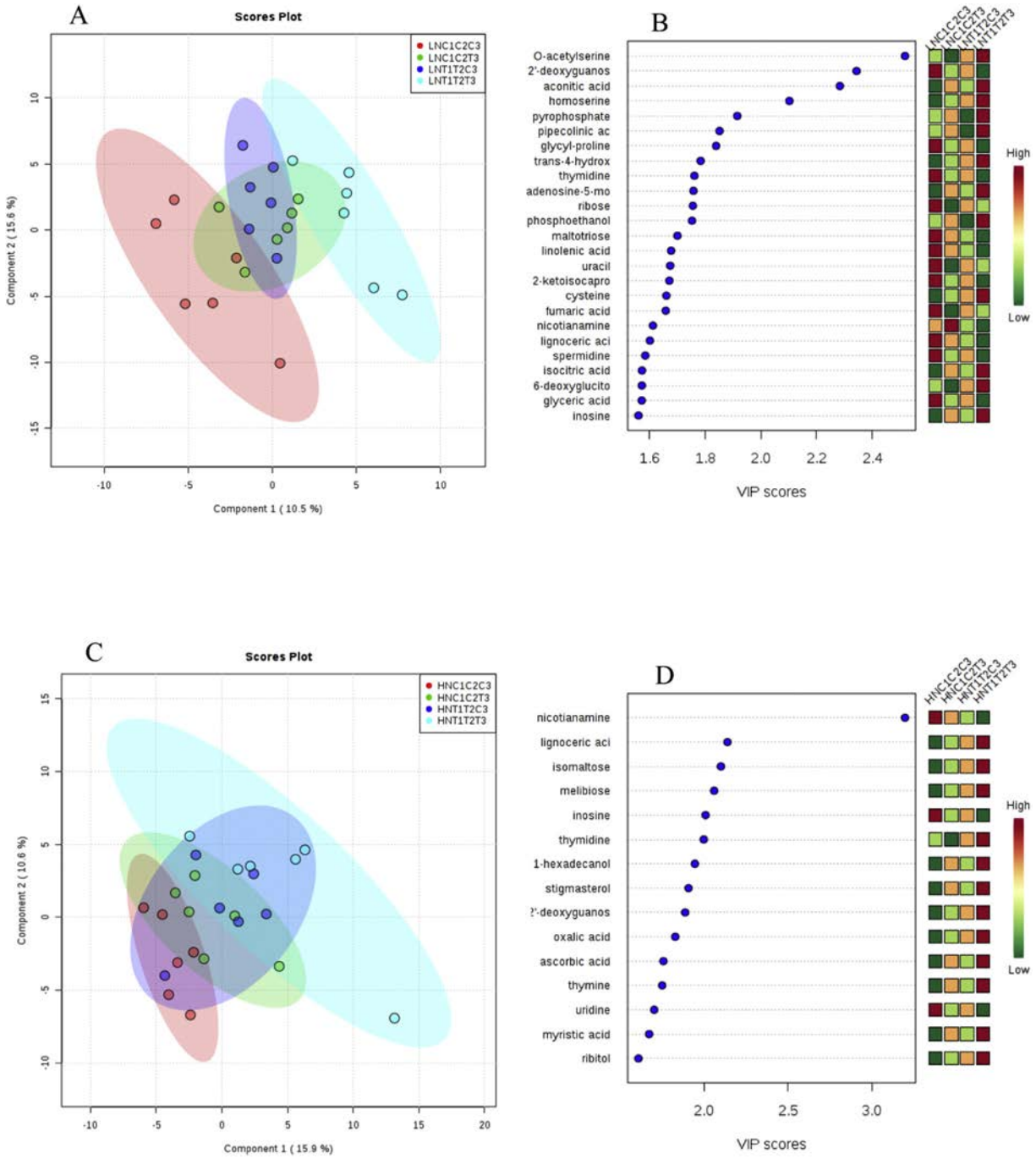

Fig. 1. Partial least square (PLS) analysis and VIP scores of metabolites from wheat grains as affected by generational exposure to CeO_2-NPs at low N (A, B) or high N soil (C, D). Data are based on semiquantitative analysis of 180 metabolites from GC-TOF-MS; quantitative variances of the relative abundances were clustered to reveal the relative similarities of metabolite profiles of wheat grains. 95% confidence regions are displayed for each treatment. VIP scores show the discriminating metabolites leading to the separation between generational treatments (red = up-regulated, green = down-regulated). N level in soil: LN = low nitrogen, HN = high nitrogen; LN or HN indicates total addition of 48 or 112 mg N as nutrient solution throughout the duration of the experiment. CeO_2-NPs treatment: C = 0 mg CeO_2-NPs kg^{-1} soil T = 500 mg CeO_2-NPs kg^{-1} soil; generational exposure: 1 = 1st generation, 2 = 2nd generation, 3 = 3rd generation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phosphate at high N T3 grains compared to C3 could indicate redirection of carbon flux from glycolysis to pentose phosphate pathway (Fraire-Velazquez and Balderas-Hernandez, 2013). Erythritol, a low molecular weight sugar alcohol, is widely used as stress indicator in plants since it functions as osmoprotectant and antioxidant for protection against salt and photooxidative stress (Williamson et al., 2002). The increase in erythritol could suggest stressed metabolic pathways at low N T3 grains compared to C3 grains.

At low N, the DNA/RNA metabolites thymidine, uracil, guanosine, and 2′-deoxyguanosine decreased concomitantly with mannose-6-phosphate and ribose sugar, which are the precursors of these nucleotides/nucleosides (Das et al., 2017; Fraire-Velazquez and Balderas-Hernandez, 2013). At high N, inosine and uridine also decreased at T3, which was consistent with the decrease in glucose-6-phosphate. These metabolites are precursors for DNA synthesis and many other biochemical reactions. Interestingly, Thumburu et al. (2015) found that CeO_2-NPs affected genes involved in DNA metabolism. In contrast, these findings are different from those reported by Zhao et al. (2016) who found no changes in DNA/RNA metabolites of cucumber fruits or spinach leaves exposed to nano-copper or CeO_2-NPs, respectively.

Table 1
Univariate analysis of grain metabolites that changed significantly in relative abundances in treated grains (C1C2T3, T1T2C3, and T1T2T3) compared to control (i.e. C1C2C3) at low N. Low N indicates total addition of 48 mg N as nutrient solution throughout the duration of the experiment. Values are means ± SE (n = 6). Up (↑) or down (↓) arrows indicate increase or decrease relative to control (C1C2C3). **, *** = p ≤ 0.05 and 0.01, respectively.

Metabolites	KEGG	C1C2C3	C1C2T3	T1T2C3	T1T2T3
Glucose 6-phosphate	C00092	40,146 ± 8852	96,017 ± 22937**↑	75,465 ± 12,935	76,215 ± 10009**↑
Fructose 6-phosphate	C05345	20,302 ± 3964	43,112 ± 10,165	35,590 ± 5490	34,702 ± 4167**↑
Mannose 6-phosphate	C00275	1390 ± 187	697 ± 187**↓	1563 ± 308	959 ± 201**↓
Ribose	C00121	86,795 ± 10,966	52,062 ± 6978**↓	65,322 ± 6412	56,638 ± 7000**↓
Erythritol	C00503	18,413 ± 2080	47,292 ± 7102***↑	27,936 ± 1347***↑	24,895 ± 3015
Isocitric acid	C00451	13,706 ± 2620	21,079 ± 4344	18,829 ± 2756	22,528 ± 3098**↑
Aconitic acid	C00417	2534 ± 519	4225 ± 544	3917 ± 540	5266 ± 857**↑
Fumaric acid	C00122	619,901 ± 80,017	347,329 ± 61,800	557,742 ± 47,567	358,832 ± 35147**↓
2-Ketoisocaproic acid	C00233	60,597 ± 6439	41,326 ± 5135	57,954 ± 3813	38,939 ± 3648**↓
Inosine	C00294	2509 ± 323	3136 ± 531	2931 ± 474	3767 ± 418**↑
Guanosine	C00387	10,288 ± 1123	7891 ± 840**↓	9116 ± 1372	7092 ± 786**↓
2′-Deoxyguanosine	C00330	6148 ± 1487	2350 ± 566	4024 ± 1117	1323 ± 144**↓
Adenosine 5-monophosphate	C00020	1757 ± 319	2698 ± 252	2001 ± 227	2950 ± 285**↑
Uracil	C00106	27,400 ± 5836	11,262 ± 1705**↓	24,052 ± 3639	11,335 ± 1048**↓
Pyrophosphate	C00013	99,695 ± 9581	123,889 ± 20,760	94,493 ± 10,333	182,961 ± 29761**↑
Phosphoethanolamine	C00346	7092 ± 872	7756 ± 1379	5510 ± 854	12,400 ± 1873**↑
Acetylserine	C00979	2053 ± 384	1850 ± 196	2604 ± 211	3480 ± 479**↑
Oleamide	C19670	2533 ± 395	1621 ± 310	2527 ± 347	1473 ± 253**↓
Glycyl-proline		3371 ± 278	3074 ± 405	3461 ± 276	2277 ± 260**↓

3.4. Metabolites affected at both previous and third generation exposures to CeO_2-NPs

At low N, aconitic acid was upregulated in both T1T2 and T3 by 56 and 36%, respectively, compared to respective controls (Fig. 3). Igamberdiev and Eprintsev (2016) noted that aconitic acid is a prevalently accumulating organic acid in plants and increase in this metabolite would indicate metabolic shifts in amino acid synthesis. Since there were no changes on the relative abundances of amino acids, these results may suggest accumulation of fixed carbon pools or alternative pools of tricaboxylic acids in grains (Igamberdiev and Eprintsev, 2016). Aconitic acid is also an intermediate in the isomerization of citric to isocitric acid which probably explains the simultaneous increase in both aconitic and isocitric acids in low N T1T2T3 treatment (Table 1). These metabolites (aconitic and isocitric acids) also play important role in metal chelation for nutrient acquisition or metal detoxification in plants (Jones, 1998).

In case of high N, nicotianamine decreased by 33% in both T1T2 and T3 (Fig. 4). It is interesting to note also that nicotianamine is the only metabolite that progressively decreased in relative abundance across generational treatments (C1C2T3, T1T2C3, T1T2T3) compared to control (C1C2C3) (Table 2). Nicotianamine is a known chelator of metal ions. Studies have documented the role of nicotianamine in the long distance transport of metals in plants and in Fe(II) scavenging to protect cells from oxidative stress (Takahashi et al., 2003). Nicotianamine is also the precursor of mugineic acid, a family of phytosiderophores that graminaceous plants secrete to solubilize Fe in the soil (Takahashi et al., 2003). Zheng et al. (2010) also reported that nicotianamine enhances rice grains Fe bioavailability to humans. The reduction in nicotianamine levels in T1T2 may have implications on elemental uptake in the succeeding generations.

3.5. Changes in other metabolites at low N

Stress marker metabolites were affected in wheat grains from low N soil. Pipecolinic acid increased by 115% in T3 compared to C3. Similar to erythritol, pipecolinic acid is a low molecular weight solute used as osmoprotectants in plants (Gouffi et al., 2000). On the other hand, glycyl-proline and spermidine both decreased (22 and 36%, respectively) in T3 grains compared to C3. Glycyl-proline is related to major organic osmolytes glycine and proline, while spermidine is low molecular weight polyamines that enhance plants tolerance to environmental stress (Das et al., 2017; Liu et al., 2015). The simultaneous increases in erythritol and pipecolinic acid and decreases in glycyl-proline and

Table 2
Univariate analysis of grain metabolites that changed significantly in relative abundances in treated grains (C1C2T3, T1T2C3, and T1T2T3) compared to control (C1C2C3) at high N. High N indicates total addition of 112 mg N as nutrient solution throughout the duration of the experiment. Values are means ± SE (n = 6). Up (↑) or down (↓) arrows indicate increase or decrease relative to control (C1C2C3). **, ***, **** = p ≤ 0.05, 0.01, and 0.001 respectively.

Metabolites	KEGG	C1C2C3	C1C2T3	T1T2C3	T1T2T3
Glucose 6-Phosphate	C00092	98,401 ± 11,437	76,829 ± 11,532	107,620 ± 34,799	53,626 ± 13939**↓
Hexose 6-Phosphate	C02965	34,369 ± 3008	28,224 ± 4001	34,956 ± 8461	19,988 ± 3347**↓
Isomaltose	C00252	563 ± 131	830 ± 204	1043 ± 129**↑	1337 ± 412
6-Deoxyglucitol	C21416	30,476 ± 2742	24,314 ± 2691	21,845 ± 1707**↓	22,863 ± 5726
1-Hexadecanol	C00823	1851 ± 200	2561 ± 619	2262 ± 171	5339 ± 1968**↑
Ascorbic acid	C00072	2037 ± 365	2601 ± 842	2589 ± 468	3699 ± 599**↑
Palmitic acid	C00249	108,023 ± 4773	140,486 ± 30,827	129,928 ± 15,358	231,496 ± 102384***↑
Uridine	C00299	30,034 ± 1438	24,911 ± 4296	28,074 ± 2091	20,397 ± 2748**↓
Inosine	C00294	4613 ± 131	3333 ± 487	3630 ± 722	2728 ± 377**↓
Guanosine	C00387	6614 ± 656	8662 ± 1030	9301 ± 769**↑	8780 ± 1166**↑
1-Monopalmitin	C01885	7798 ± 903	8859 ± 2788	5513 ± 623**↓	14,155 ± 4457
Alpha-Tocopherol	C00376	1516 ± 238	3478 ± 774**↑	2814 ± 425	2191 ± 435
Stigmasterol	C05442	886 ± 79	1807 ± 339**↑	1857 ± 422	2115 ± 560
Phosphate	C00009	731,398 ± 41,953	702,671 ± 149,611	899,534 ± 95,280	870,242 ± 69813**↑
Nicotianamine	C05324	18,626 ± 1180	10,613 ± 1366***↓	10,601 ± 935***↓	8864 ± 908****↓
Levoglucosan		2746 ± 297	3709 ± 398	3163 ± 195	5759 ± 2093**↑

Table 3
Metabolites from wheat grains that showed significant differences in multivariate and univariate analyses using Metaboanalyst (http://www.metaboanalyst.ca/).[a]

	Sugars	Sugars alcohols	Organic acids	Others
Wheat grains from low N soil	Fructose-6-phosphate Glucose-6-phosphate Mannose-6-phosphate Ribose	Erythritol Maltotriose 6-Deoxyglucitol	Aconitic acid Fumaric acid Glyceric acid Isocitric acid Lignoceric acid Linolenic acid Pipecolinic acid 2-Ketocaproic acid	Adenosine-5-phosphate Cysteine Glycyl-proline Guanosine Homoserine Hydroxyproline Inosine Nicotianamine Oleamide O-acetylserine Phosphoethanolamine Pyrophosphate Spermidine Thymidine Uracil 2-Deoxyguanosine
Wheat grains from high N soil	Glucose-6-phosphate Hexose-6-phosphate Isomaltose Melibiose	Ribitol 1-Hexadecanol 6-Deoxyglucitol	Palmitic acid Ascorbic acid Lignoceric acid Oxalic acid Myristic acid	Alpha-Tocopherol Guanosine Inosine Uridine Levoglucosan Nicotianamine Phosphate Stigmasterol Thymidine Thymine 1-monopalmitin 2′-Deoxyguanosine

[a] Low or High N soil indicates total addition of 48 or 112 mg N as nutrient solution throughout the duration of the experiment.

spermidine could suggest greater stress experienced in T3 grains compared to C3 at low N. Likewise, the data suggests that wheat grains at T1T2 experienced enhanced stress compared to C1C2 as indicated by higher levels of acetylserine, homoserine, and 6-deoxyglucitol (56, 41, and 33%, respectively) at T1T2 compared to C1C2 (Fig. 3). Studies have shown increased levels of serine in plants under abiotic stress (e.g. salt stress) (Das et al., 2017; Sanchez et al., 2010).

Fumaric acid decreased by 40% in T3 grains compared to C3 (Fig. 3). The decrease in fumaric acid could be related with the increase in aconitic acid. Igamberdiev and Eprintsev (2016) showed competing

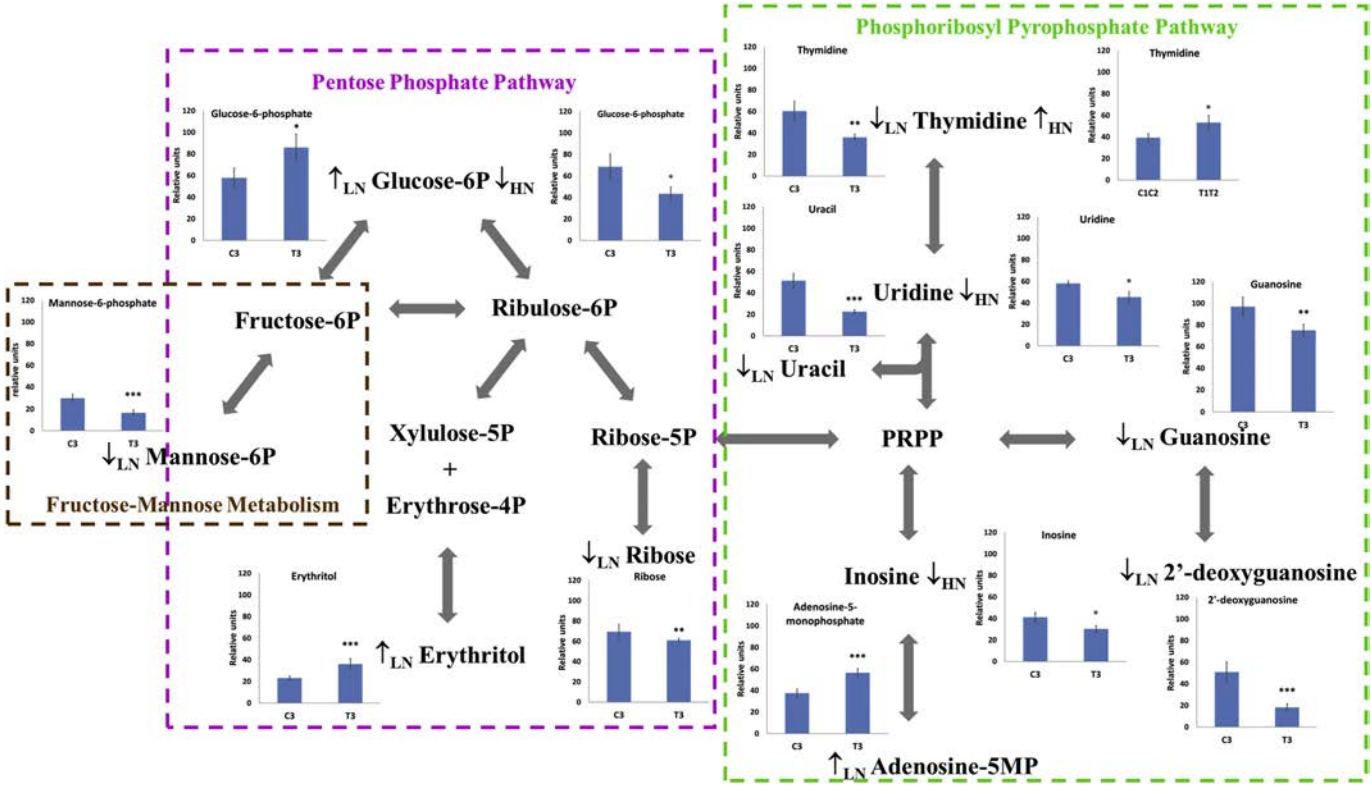

Fig. 2. Common metabolic pathways shared by the metabolites that were altered at T3 (third generation exposure to CeO_2-NPs). LN (low nitrogen) and HN (high nitrogen) indicate nitrogen treatment in soil. LN or HN indicates total addition of 48 or 112 mg N as nutrient solution throughout the duration of the experiment. Up (↑) or down (↓) arrows indicate direction of change relative to control (C3). Values are means ± SE (n = 6). Statistical significance at $p \leq 0.10$, 0.05, and 0.01 was indicated as *, **, and ***, respectively.

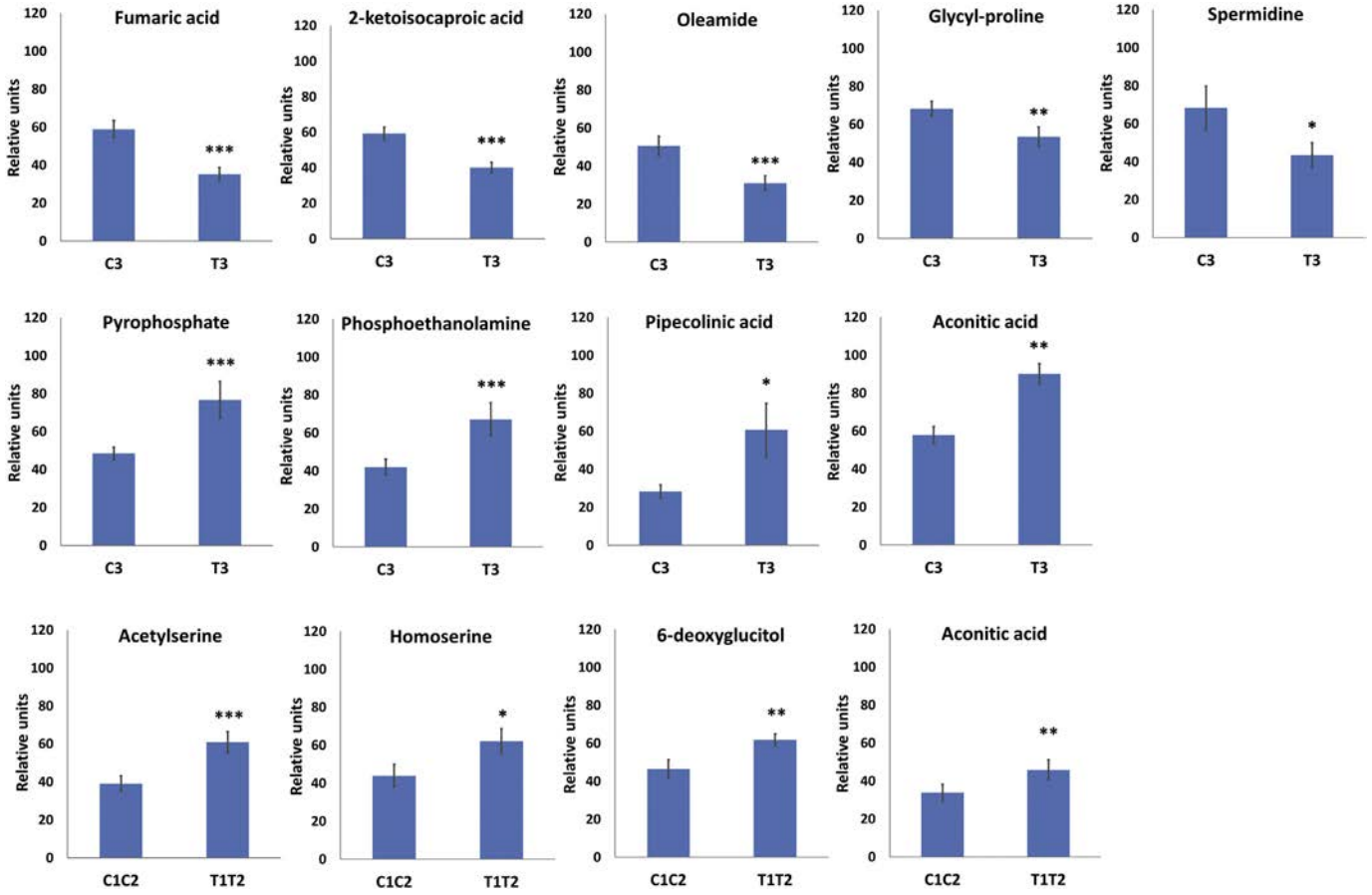

Fig. 3. Metabolites that were modified at third generation exposure (C3 vs. T3; top and middle panels) or parental exposure (C1C2 vs. T1T2; bottom panel) to CeO_2-NPs at low nitrogen soil. Low nitrogen soil indicates total addition of 48 mg N as nutrient solution throughout the duration of the experiment; CeO_2-NPs treatment: C = 0 mg CeO_2-NPs kg^{-1} soil T = 500 mg CeO_2-NPs kg^{-1} soil; generational exposure: 1 = 1st generation, 2 = 2nd generation, 3 = 3rd generation. Values are means ± SE (n = 6). Statistical significance at $p \leq 0.10$, 0.05, and 0.01 was indicated as *, **, and ***, respectively.

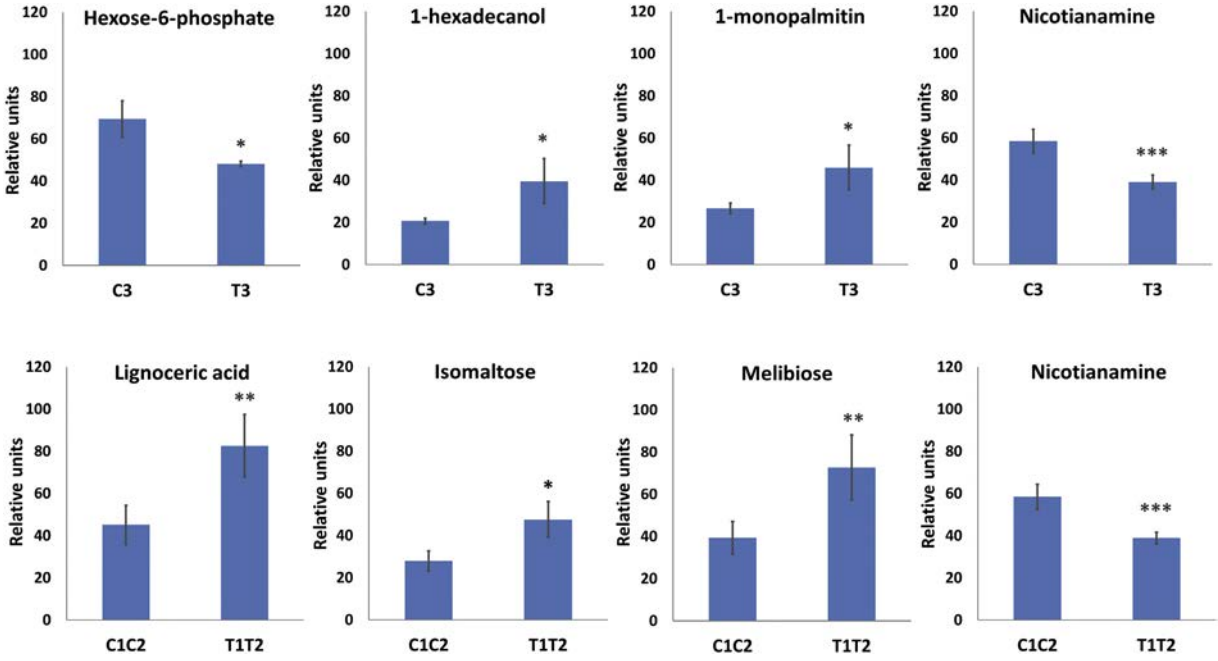

Fig. 4. Metabolites that were modified at third generation exposure (C3 vs. T3; top panel) or parental exposure (C1C2 vs. T1T2; bottom panel) to CeO_2-NPs at high nitrogen soil. High nitrogen soil indicates total addition of 112 mg N as nutrient solution throughout the duration of the experiment; CeO_2-NPs treatment: C = 0 mg CeO_2-NPs kg^{-1} soil T = 500 mg CeO_2-NPs kg^{-1} soil; generational exposure: 1 = 1st generation, 2 = 2nd generation, 3 = 3rd generation. Values are means ± SE (n = 6). Statistical significance at $p \leq 0.10$, 0.05, and 0.01 was indicated as *, **, and ***, respectively.

reactions in malate and citrate valves in the TCA cycle: fumaric acid is produced in the malate valve while aconitic acid in the citrate valve. Alternatively, a previous report has also shown that production of fumaric acid, which is used to produce energy and reducing power, decreased significantly at the late developmental stage of the grains (Zhen et al., 2016). Ketoisocaproic acid was also lower in T3 grains (32% lower) than C3 (Fig. 3). This organic acid is a precursor for leucine synthesis and has been shown to be likely precursor in volatile aroma compounds or flavors in tomato (*Lycopersicon esculentum*) and melon (*Cucumis melo*) (Kochevenko et al., 2012; Gonda et al., 2010). Binder (2010) noted a significant breakdown of branched-chain amino acids leucine to ketoacids (e.g., ketoisocaproic acid) under certain environmental conditions. Phosphoethanolamine, which increased by 60% in T3 compared to C3, is an intermediate product in the synthesis of phosphatidylcholine. Phosphatidylcholine is a major phospholipid that has important structural and signaling roles in plants (Chen et al., 2019). Chen et al. (2019) reported that significant loss in phosphatidylcholine could be lethal in plants. Oleamide, a fatty acid primary amide form of oleic acid, decreased notably (39%) in T3 grains compared to C3; it could signify decrease in nutritional quality of wheat grains since oleamide is an important nutritional component in plants (Cheng et al., 2010; Heo et al., 2003). Heo et al. (2003) reported that oleamide reverses cognitive impairment and could be useful against Alzheimer's disease.

3.6. Changes in other metabolites at high N

At high soil N, hexose-6-phosphate decreased by 30% in T3 compared to C3 (Fig. 4), a trend that was in agreement with the decrease in glucose-6-phosphate. Hexose phosphates act as direct precursor of starch synthesis in wheat grain (Rahdhawa and Singh, 1998) indicating a possible decrease in starch content in wheat grains exposed to cerium oxide nanoparticles. The metabolites 1-hexadecanol and 1-monopalmitin, which are degradation products of fatty acid increased in T3 by 92 and 73%, respectively, compared to C3 (Fig. 4). These metabolites have been shown to affect the pasting properties of wheat starches (Blazek and Copeland, 2009). Isomaltose, melibiose, and lignoceric acid all increased also in T1T2 by 85, 71, and 83% compared to C1C2 (Fig. 4). Isomaltose and melibiose are both reducing disaccharide sugars; isomaltose has two glucose molecules while melibiose is composed of galactose and glucose. These metabolites are photosynthates translocated to the developing grains (Lemoine et al., 2013). Lignoceric acid is a saturated fatty acid byproduct of lignin production. Lignins are involved in seed protection, grain growth, and increased grain weight (Chateigner-Boutin et al., 2018; Beaugrand et al., 2004). This may be related to decrease in grain weight in T1T2 grains as we have previously reported (Rico et al., 2020).

3.7. Grain accumulations of iron and cerium

Analysis of macro- and micro-elements concentrations in grains showed that only Fe concentration significantly changed (Table 4). However, the presence of cerium in grains was not detected, a result that is in agreement with our previous studies on wheat exposure to cerium oxide nanoparticles (Rico et al., 2017, 2020). These findings suggest that CeO_2-NPs could induce changes in metabolite compositions and Fe accumulation in grains without Ce uptake in plants. Similar findings have been reported wherein exposure to CeO_2-NPs even without Ce accumulation in plant tissues could affect plant performance and behavior (Rico et al., 2017, 2020).

Nicotianamine is essential for transport and acquisition of Fe in vegetative and reproductive organs of wheat, rice, and barley (Takahashi et al., 2003; Zheng et al., 2010; Singh et al., 2017). Studies showed that transgenic grains overexpressing nicotianamine had twice as much Fe compared to control grains (Zheng et al., 2010; Singh et al., 2017). Current results showed corroborations in nicotianamine abundance and Fe concentrations in grains. Iron accumulation in grains was affected in high N grains but not altered in low N grains, a result that was in agreement with the changes in nicotianamine abundance in high N grains. Nicotianamine relative concentration in low N grains did not change but significantly decreased in both previously treated (T1T2) and third generation treated (T3) high N grains (Figs. 2, 3). The decrease in nicotianamine relative concentration at high N grains was accompanied by decreased Fe concentration. The data revealed a large decrease in Fe concentration (34.5–34.9 mg/kg) at high N third generation grains (T3) compared to control (40.1–41.1 mg/kg) of both young and mature grains. Conversely, there was an increase in Fe concentration (40.7–40.8 mg/kg) at low N third generation grains (T3) compared to its control (34.3–38.4 mg/kg) of both young and mature grains (Table 4).

Table 4
Effect of parental or current exposure to CeO_2-NPs on Fe concentrations (mg/kg) in wheat grains cultivated at low or high N soil.[a]

	Low N			High N		
	C1C2	T1T2	Mean	C1C2	T1T2	Mean
Young grains						
C3	35.7 ± 1.3	41.1 ± 2.0	38.4 ± 1.2	40.3 ± 1.8	41.8 ± 1.6	41.1 ± 1.2
T3	42.2 ± 2.0	39.5 ± 0.6	40.8 ± 1.1*	36.3 ± 1.4	32.8 ± 1.6	34.5 ± 1.2****
Mean	38.9 ± 1.5	40.3 ± 0.8		38.3 ± 1.2	37.3 ± 1.7	
Mature grains						
C3	32.3 ± 1.6	36.2 ± 2.1	34.3 ± 1.4	40.5 ± 2.3	39.6 ± 1.7	40.1 ± 1.4
T3	42.2 ± 3.4	39.2 ± 2.3	40.7 ± 2.0*	39.2 ± 2.9	30.6 ± 0.7	34.9 ± 1.9**
Mean	37.3 ± 2.3	37.7 ± 1.6		39.9 ± 1.8	35.1 ± 1.6	

[a] Low or High N indicates total addition of 48 or 112 mg N as nutrient solution throughout the duration of the experiment; C1C2 or T1T2 indicates parental exposure to 0 or 500 mg CeO_2-NPs per kg soil at 1st and 2nd generations; C3 or T3 denotes current or 3rd generation exposure to 0 or 500 mg CeO_2-NPs per kg soil. Values are means ± SE; n = 6 per treatment combination. *, **, **** represent significance at $p \leq 0.10, 0.05, 0.001$, respectively. Young grains were collected 18 days before fully matured grains were harvested.

Interestingly, Fe concentration at high N T1T2T3 in young and mature grains (32.8 and 30.6 mg/kg, respectively) was significantly lower than their controls (C1C2C3) (40.3 and 40.5 mg/kg, respectively) at $p \leq 0.01$ (Table 4). This trend was consistent with the very large decrease in nicotianamine abundance (8864 relative unit) in T1T2T3 grains compared to that in C1C2C3 (18626 relative unit) (Table 2). The simultaneous decrease in nicotianamine abundance and Fe concentration are in agreement with the role of nicotianamine in Fe accumulation in grains (Zheng et al., 2010; Singh et al., 2017).

4. Conclusion

This work provides evidence that previous generation exposure to CeO_2-NPs affects the metabolite composition in progeny plants. Wheat exposed continuously to cerium oxide nanoparticles for three generations exhibited greater degree in metabolite alterations. The offspring environment (i.e., soil nitrogen) also modulates the influence of parental exposure wherein more metabolites were significantly altered at lower soil N than at higher soil N. Cerium oxide nanoparticles altered more DNA/RNA metabolites (thymidine, uridine, guanosine, uracil, deoxyguanosine, adenosine) in grains from low N soil than in high N soil (thymidine, inosine). Nicotianamine, which plays crucial role in nutrient (i.e., Fe, Mn) storage in grains, significantly decreased with concomitant decrease in Fe concentration in grains from high N soil. These alterations in metabolite compositions have unknown implications in nutritional quality of wheat. These findings also suggest that continuous exposure to CeO_2-NPs may have implications on ecosystem processes (e.g., primary productivity, ecosystem stability, nutrient cycling) of progeny plants.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.136487.

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