Nanoscale phosphorus-based agrochemicals enhance tomato and rice growth via positively modulating the growth-associated gene expression and endophytic microbial community

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Environmental significance

Due to the low use efficiency, increasing amounts of commercial P-based fertilizers have been applied annually to meet the demands for crops. However, excessive use of P-based fertilizers resulted in serves environmental problem, e.g. soil degradation and water pollution. Our research indicates that using nano-hydroxyapatite (nHA) as a substitute for traditional phosphorus-based fertilizers can significantly enhance agricultural productivity while decreasing the need for agrochemicals. Notably, 20 nm-nHA and 1% Cu-nHA can recruit the beneficial microbes and positively alter the endophytic microbial community in crops, thereby promoting plant growth, nutrient uptake, and stress resistance. Overall, the implementation of small-scale agricultural chemicals has significantly contributed to minimizing the consumption of agricultural inputs and mitigating environmental pollution in farming practices.

Nanoscale phosphorus-based agrochemicals enhance tomato and rice growth via positively modulating the growth-associated gene expression and endophytic microbial community

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Abstract

Nano-hydroxyapatite (nHA) has attracted increasing attention as a potential novel fertilizer. The present study investigated the effects of root exposure to nHAs (20 nm-nHA, 60 nm-nHA, 1% Cu-nHA, and 10% Cu-nHA) at 50 mg/kg on the growth and development of tomato (*Solanum lycopersicum* L.) and rice (*Oryza sativa* L.) seedlings for 50 days. Compared with the control, different types of 50 mg/kg nHA increased the biomass of seedlings by 10.7%-30.9%; for example, 20 nHA significantly increased the fresh weight of the two plant species by 17.2% and 29.2%, respectively. Additionally, 20 nm-nHA and 1% Cu-nHA altered the diversity of plant endophytic microbial communities and increased the abundance of plant-associated beneficial microorganisms, including *Glomeromycotina*, *Funneliformis*, and *Blastocladiomycota*. Transcriptomic analysis suggests that 20 nm-nHA and 1%Cu-nHA induced transcriptional reprogramming in exposed seedlings. KEGG pathway analysis shows that root exposure to 20 nm-nHA and 1% Cu-nHA promoted plant hormone signal transduction pathways in both tomato and rice roots; and, 1% Cu-nHA promoted photosynthesis and amino acid metabolism. Overall, this work demonstrates that root exposure to 50 mg/kg 20 nm-nHA significantly improves crop growth, and provides valuable insight into the development of novel nanoscale phosphorus fertilizers as a sustainable path for precision agriculture.

Keywords: nano-hydroxyapatite, nanofertilizer, phytohormones, transcriptional analysis, endophytic microbial community

1. Introduction

Global food production must increase significantly and cope with the confounding impacts of a rapidly changing climate to meet the food security needs of the growing population. The lack of sufficient soil nutrients can significantly restrict the production of agricultural crops and necessitates the application of exogenous nutrients in the form of synthetic fertilizers[1]. Phosphorus is among the nutrients required in greatest amounts and plants obtain elemental phosphorus (P) by accumulating plant-accessible water-soluble phosphorus salts[2]. Importantly, the delivery and utilization of commercial P-based fertilizers is highly inefficient, with approximately 20% of the applied conventional P fertilizers being utilized by crops[3]. The remaining fraction of the added P either becomes biologically unavailable in soil, representing a significant waste of resource inputs, or leaches to local water bodies, resulting in eutrophication and significant environmental damage[4, 5].

As such, the development of novel fertilizers that can increase crop yield and reduce P loss from agricultural fields is urgently needed. Rapid advances in nanotechnology have demonstrated significant potential to provide precise and efficient solutions to challenges in conventional agrochemical utilization. More specifically, engineered nanomaterials (NMs) can increase agricultural productivity and improve the efficiency of agrochemicals while minimizing negative environmental impacts[6]. NMs have a higher surface area to volume ratio and tunable surface chemistry that can be used to control the release of active ingredients[7, 8]. Nano-hydroxyapatite (nHA), with the molecular formula $Ca_{10}(PO_4)_6(OH)_2$, has been proposed as an alternative phosphate (pi) fertilizer. For example, in comparison with a conventional phosphorus (H_3PO_4-P), 200 mg/kg nHA significantly increased the biomass of lettuce plants by more than 3-fold and the P content by more than 50% in low calcium soils[9]. Similarly, the growth rate and seed yield of nHA treated soybean under greenhouse conditions was increased by 32% and 20%, respectively, as compared to commercial fertilizers[10]. Additionally, nHA has been shown to be a promising amendment for acidic soils contaminated with fluoride (F), primarily due to the induced reduction of soil bioavailable F and supplementation of Ca[11]. In addition, nHA has been effectively utilized as a soil amendment to mitigate heavy metal accumulation in crops. For instance, soil application of 1500 mg/kg nHA resulted in a 17% reduction in cadmium content in potato tubers, compared to unamended controls[12].

nHA has been shown to improve the utilization efficiency of P by mediating controlled release, thereby enhancing crop growth and development[9-11]. However, mechanistic relationship between phenotypic changes and underlying gene regulation as a function of nHA remains poorly understood. NMs can modulate metabolic pathways associated with biosynthesis and signal transduction of phytohormones, non-enzymatic biomolecules (ascorbic acid and carotenoids), and photosynthesis, subsequently stimulating plant growth and yield. For example, different concentrations of CeO_2 NPs significantly upregulated the expression of iron redox *Ferredoxin-NADP reductase (PetH),* a key gene for photosynthesis; in addition, the top 20 metabolic pathways affected by CeO_2 NPs included plant hormones, amino acids, and carbon metabolism[13]. Seed transcriptional reprogramming triggered by Ag NPs enhanced salt tolerance in rice (*Oryza sativa* L.). Ag NPs activated stress signaling and defense-related pathways, such as phytohormone signaling, glutathione metabolism, and plant-pathogen interaction[14]. Importantly, transcriptomic analysis can not only evaluate the differential expression of photosynthesis-related genes but also can provide insight into the function and specific regulatory mechanism of corresponding unknown genes[9, 10]. Therefore, a comprehensive understanding of the effects of nHA on plant growth and development should be investigated at the transcriptomic level.

Endophytic microbes colonize the internal tissues of plants by forming complicated relationships with hosts[1]. Endophytic microbes play an essential role in maintaining plant health and optimizing crop yield; a robust plant-associated microbiome can promote growth and development, and improve the efficiency of nutrient utilization and stress tolerance of plants[15]. For example, *Sphingomonas* SaMR12 can colonize and aggregate in the roots of the host plant *Brassica juncea* and then migrate to the shoots, where colonization was found to increase the shoot and root biomass by 71% and 81%, resepctively[16]. Importantly, NMs can alter the endophytic microbial community, subsequently impacting host plant growth and biomass. For example, Yan et al. demonstrated that the number of beneficial host microorganisms in rice roots exposed to 1 mg/kg Ag NPs, including *Frankiales, Rhizobiales*, and *Saccharimonadia*, was significantly increased by more than 120%[17].

The application of nHA can significantly increase soil pH, reduce soil available copper content, and significantly change soil bacterial community structure, increase bacterial abundance and diversity[18]. Given this, we hypothesize that nHA modulates the composition of plant endophytic microbial communities, contributes to the enrichment of beneficial microorganisms, and thereby improves plant growth conditions and biomass.

Rice (*Oryza sativa* L.) is one of the most extensively cultivated monocotyledonous cereal crops globally and is a major source of carbohydrates for approximately 3.5 billion people[19]. Tomato (*Solanum lycopersicum* L.) is a dicotyledonous species with significant economic value; approximately 40 million tons of tomatoes are used for food processing annually[20]. In the present study, the effects of root exposure to different types of nHA (20 nm-nHA, 60 nm-nHA, 1% Cu-nHA, and 10% Cu-nHA) on the growth and development of rice and tomato were studied. Transcriptomic techniques were applied to elucidate differential expression of genes associated with phytohormone signaling, photosynthesis, carbon sequestration, and stress resistance as a function of the different types of nHA. The endophytic microbial communities of two crops as affected by nHA were also investigated by 16S/18S-rRNA sequencing. In addition, physiological endpoints including biomass, nutritional content, and photosynthetic parameters were determined. These findings provide new insights into utilizing of P-based nanoscale agrochemicals to positively alter the plant endophytic microbial communities and subsequently enhance crop yield and quality in a sustainable manner.

Materials and methods

nHA synthesis and characterization

Twenty nm-nHA and 60 nm-nHA were purchased from Shanghai Pantian Powder Material Co., Ltd. A wet chemistry precipitation method was used to prepare 1% and 10% Cu-doped nanoscale hydroxyapatite (Cu-nHA)[21]. Briefly, 1.67 M $Cu(NO_3)_2$ and 1.0 M H_3PO_4 were accurately mixed at a volume ratio of 1:99 and 1:9, respectively, and the prepared mixture was added to an equal volume of 1.67 M $Ca(OH)_2$ solution at a rate of 3 mL min^{-1} to synthesize 1% and 10% Cu-doped nHA. The ammonia water was added to keep pH at 10-11. The mixtures were kept stirring and then filtered to collect the precipitates, which were freeze-dried for 48 hours.

The particle morphology of 20 nm-nHA, 60 nm-nHA, 1% Cu-nHA and 10% Cu-nHA were further characterized by transmission electron microscopy with energy dispersive X-ray spectroscopy (TEM-EDS, Hitachi 7700, Hitachi Incorporation, Japan). The four types of nHA were analyzed by X-ray diffraction (XRD, Rigaku Smartlab, Rigaku Corporation, Japan) on an X-ray diffractometer (2θ range = 10-90°; Counting time: 5°/min). A Fourier transform infrared spectrometer (FTIR; Bruker Tensor 27, Germany) was analyzed using the IR spectra of the nHA types. Full nHA characterization results are provided in Text S1.

Greenhouse experiment

Concentration optimization Tomato (Hezuo 903, Hongqiao Tianlong Seed Industry Limited Company) and rice (Y Liangyou 900, Hunan Yuanchuang Super Dao Technology Co.,Ltd.) seeds were germinated in vermiculite for 3 days under dark conditions, followed by 14 days of growth in a plant growth chamber (temperature 25 °C; light cycle: 16/8 h; Binder KBWF720, Tuttlingen, Germany).

Tomato and rice seedlings were transplanted to soil amended with different concentrations of 20 nm-nHA and 1% Cu-nHA (0, 1, 10, 50 and 100 mg/kg). Five biological replicates were set in each nHA treatment. The treated plants were harvested two weeks later, and the optimal concentration was determined by plant phenotypic images, chlorophyll content, plant height and fresh mass (Figure S3 and S4). Finally, 50 mg/kg 20 nm-nHA and 1% Cu-nHA were selected for the subsequent plant experiment.

Greenhouse experiment To distinguish the effects of particle sizes and types on plant growth, four types of nHA particles were selected, and there were five treatments for each crop, including control (no nHA addition), 20 nm-nHA, 60 nm-nHA, 1% Cu-nHA and 10% Cu-nHA; the concentration of all types of nHAs was set at 50 mg/kg. Additionally, no phosphate treatment was set as an ionic control to mimic the conventional P-fertilizer application. Because the soil P used in the greenhouse experiment was 20,000 mg/kg, it can barely make any difference on plant growth with additional 50 mg phosphate/kg soil input. At harvest, shoot height, root length, fresh mass, and phenotypic images of both crops were then recorded across all treatments. A portion of tissues was freeze-dried for elemental measurement.

Plant nutrient and pigment contents

Approximately 100 mg of plant tissues (shoot or root) or soil samples were digested in a mixture of 3 mL HNO_3 and 0.5 mL H_2O_2 at 105 °C for 40 min. The content of K, Ca, P, Cu, Fe, S, Mg, Mn, and Zn were measured using inductively coupled plasma optical emission spectroscopy (ICP-OES, iCAP 7000, Thermo Scientific, USA). Nutrient element standards (NCS149160) were purchased from NCS (NCS TESTING TECHNOLOGY, Being, China) and tested as the quality assurance/quality control (QA/QC). A known concentration of standard solution was measured every 10 samples.

Additionally, the plant tissues (shoot or root) were ground to a homogeneous powder in liquid nitrogen for the determination of several biochemical endpoints, including the content of chlorophyll, sugars, soluble protein, amino acids, and phytohormones. Details are provided in Method S1.

Determination of photosynthetic parameters

Photosynthetic parameters were assessed with a portable photosynthesis system (CIRAS-4, PP SYSTEMS, USA) from 9:00 a.m. and 11:00 a.m. on the day before harvest. The light intensity of the measuring chamber was maintained at 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and leaves in the same position (avoiding the main vein) across all treatments were chosen for evaluation. Specifically, the leaves were placed in the measuring chamber and data were recorded after the readings became stable.

Chlorophyll fluorescence kinetic imaging

The photosynthetic fluorescence parameters of tomato and rice leaves were quantitatively analyzed by FluorCam 7. After 30 minutes of initial dark acclimation to stabilize the photosynthetic organs in the isolated leaves, the saturation pulse intensity and duration were set for determination. Then, light-induced kinetic curves were produced and evaluated. Details are provided in Method S2.

Transcriptomic analysis

According to physiological responses of both tomato and rice seedlings, 20 nm-nHA and 1% Cu-nHA boosted crop growth greater than the other two did. Thus, for transcriptomic analysis, only untreated control, 20 nm-nHA and 1% Cu-nHA treatments were selected. Total RNA was extracted by TRIzol Reagent, and shipped to Novogene Corporation (Beijing, China) for library construction and sequencing with an Illumina Hiseq X-Ten sequencer. The sequencing library was constructed by using the Truseq RNA Sample Prep Kit from Illumina (San Diego, CA). The RNA integrity number value was detected by the Agilent 2100 bioanalyzer. The obtained clean reads were aligned to the tomato and rice reference genome IRGSP-1.0 using HiSat2 software[22]. Gene and transcript expression levels were measured according to RSEM (http://deweylab.github.io/SEM/)[23]. Differentially expressed genes (DEGs) were identified with corrected FDR < 0.05 and |log2 (fold change)| > 1[24]. Gene functions were annotated based on the Gene Ontology (GO)[25] and Encyclopedia of Genes and Genomes (KEGG) pathway databases[26].

Endophytic microbial community analysis

The total DNA of tomato and rice tissues was extracted using DNeasy PowerSoil Pro Kit (QIAGEN, Germany). Three treatments, including Control, 20 nm-nHA, 1%Cu-nHA, with three biological replicates each, were analyzed. The ~450 bp fragment of the V3-V4 region of the 16S rRNA gene was amplified using the 799F and 1107R primer sets for bacterial detection[27], and the ~150 bp fragment of the V7 region of the 18S rRNA gene was amplified using the Euka02F and Euka02R primer sets for fungal detection[28]. The purified PCR products were prepared for library construction following the VAHTS Universal DNA Library Preparation Kit (Illumina V3) (Vazyme Biotech Co., China) program. High-throughput sequencing was performed on the Illumina MiSeq PE150 or PE300 platforms (Illumina, USA).

The raw sequences underwent merging and denoising via the VSEARCH pipeline and QIIME toolbox (version 1.929)[29]. All sequences were clustered into operational taxonomic units (OTU) at 97% nucleotide similarity and annotated bacterial OTUs according to SILVA database (version 138)[30] and fungal OTUs according to PR2 database (version 4.14)[31]. The alpha_diversity.py script in the QIIME toolkit was used to calculate taxonomic diversity indices such as species richness, ACE index, Chao 1 index, and Shannon diversity index. The Kruskal-Wallis test ($p < 0.05$), LDA score > 2.0[32], and linear discriminant analysis (LEfSe) were used to analyze the effects of different nHA on the microbial communities. The effects of different nHA on the interactions of endophytic microbial communities were visually demonstrated by using the R package gg tern to generate ternary plots[33]. Finally, the microbial community composition and variance analysis were conducted using the R platform.

Statistical analysis

The experimental results in each assay are expressed as the mean ± standard error. IBM SPSS (version 25, SPSS, Inc.) was used for one-way analysis of variance (ANOVA) followed by Duncan test ($p < 0.05$). Excel 2019 and Origin Lab 2021 software were used to plot figures.

Results and discussion

nHA effects on physiological responses

Plant growth Both 20 nm-nHA and 1% Cu-nHA exerted significant positive impact on the growth of tomato and rice seedlings when compared to the untreated control (Figure S3 and S4). More specifically, root exposure to different types of nHA positively affected the phenotype and size of tomato and rice seedlings (Figure 1A and B). Compared to the untreated control, the 20 nm-nHA treatment significantly increased the fresh weight and plant height of tomato and rice seedlings by 17.2% and 33.9%, and 29.2% and 17.4%, respectively (Figure 1C and D). The 1% Cu-nHA treatment increased fresh weight and plant height of tomato and rice seedlings by 5.2% and 32.5%, and 18.5% and 5.2%, respectively (Figure 1C and D). Conversely, 60 nm-nHA and 10% Cu-NHA did not significantly promote the growth of either crop. Additionally, the total leaf chlorophyll content of tomato treated with 20 nm-nHA, 60 nm-nHA, 1% Cu-nHA and 10% Cu-nHA was increased by 53.9%, 46%, 55.8% and 51.7%, respectively, relative to the untreated control. The 20 nm-nHA and 1% Cu-nHA treatments significantly increased the total chlorophyll of rice by 19.7% and 29.5% relative to the control, and the carotenoid content also showed a similar trend (Figure 1E and F). Overall, 20 nm-nHA and 1% Cu-nHA exerted the greatest positive impact on tomato and rice growth.

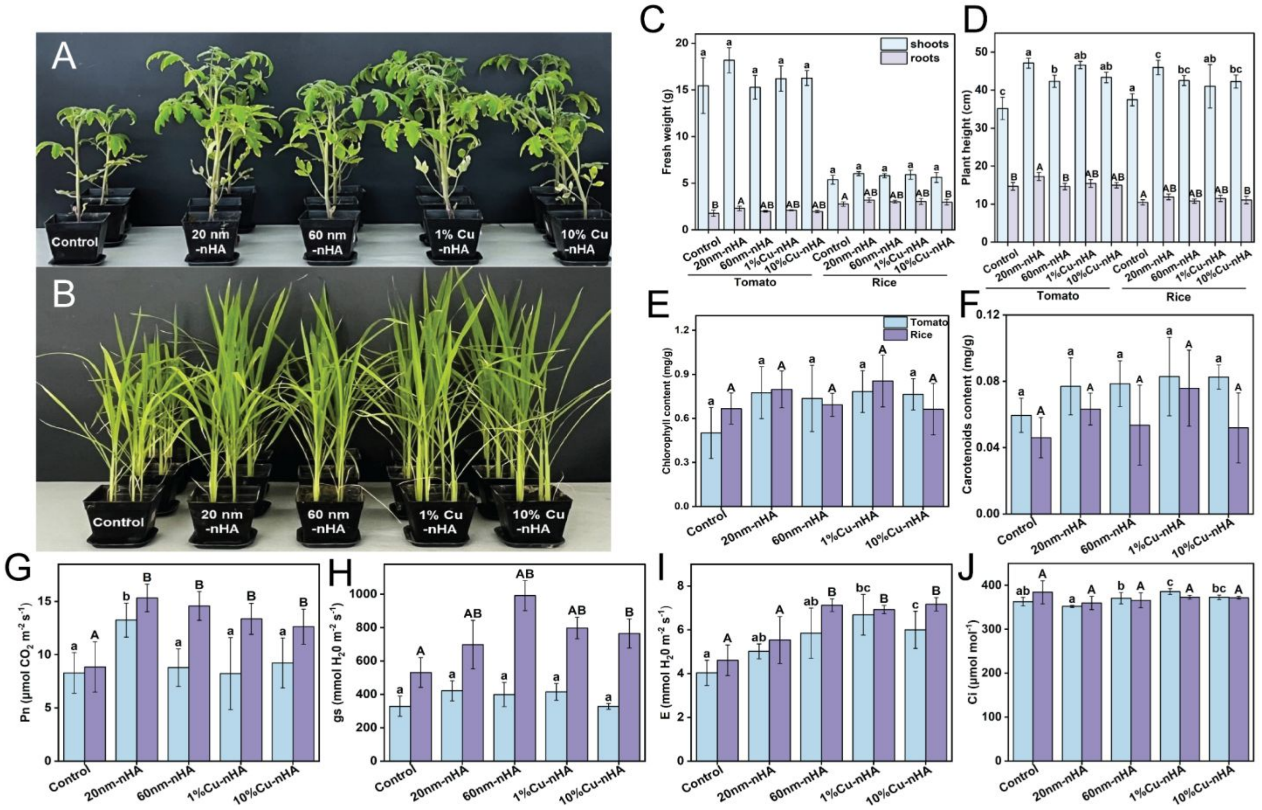

Figure 1. Physiological responses of tomato and rice seedlings upon root exposure to different types of nHA, including 20 nm-nHA, 60 nm-nHA, 1% Cu-nHA, and 10% Cu-nHA. (A) phenotypic images of tomato; (B) phenotypic images of rice; (C, D) fresh weight and plant height; (E) chlorophyll content; (F) carotenoid content; (G) net photosynthetic rate; (H) stomatal conductance; (I) transpiration rate; (J) intercellular CO_2 concentration. The different letters in the figure indicate significant differences ($P < 0.05$) determined by one-way ANOVA, letters in lowercase represent significant differences among shoots and tomato, while uppercase letters represent significant differences in roots and rice.

The positive effects of lower concentrations of nHA on tomato and rice are consistent with previous studies. For example, Rady et al. demonstrated that the addition of 0.05 g/L nano-phosphorus (NP) increased plant growth, photosynthetic efficiency, and total chlorophyll content in *Phaseolus vulgaris* grown under greenhouse conditions[34]. Similarly, nHA transport was negatively correlated with particle size, with smaller particle sizes having a greater promoting effect seedling growth. Additionally, root exposure to nHA increased the content of labile inorganic P and moderately labile inorganic P in soil by 1.52- and 0.73-fold, respectively, and major bacterial taxa such as *Proteobacteria*, *Acidobacteria* and *Gemmatimonadetes* played a major role in maintaining soil properties[35].

Photosynthetic parameters Compared with the untreated control, the net photosynthetic rate (Pn), stomatal conductance (gs) and transpiration rate (E) of tomato and rice seedlings treated with different types of nHA were generally increased (Figure 1G-I). The 20 nm-nHA significantly increased the Pn in tomato and rice by 59.8% and 73.1%, respectively, as compared to the untreated control (Figure 1G); the 60 nm-nHA significantly increased gs in tomato and rice by by 21.3% and 86.6%, respectively (Figure 1H). The E with 1% Cu-nHA was increased to the greatest extent; compared to the untreated control, the tomato and rice E value was increased by 65.6% and 50.3%, respectively (Figure 1I). However, there was no significant change in the intercellular carbon dioxide concentration (Ci) of tomato and rice seedlings with nHA as compared to the control (Figure 1J). Chlorophyll fluorescence parameters can accurately reflect photosynthetic efficiency[33]. Previous studies have confirmed that nanoparticles/nanosheets can enhance photosynthesis by a range of mechanisms, including assisting in photon capture, electron transport and carbon fixation, ultimately leading to increased plant biomass[18].

The effect of different nHA on the photochemical reactions and performance of tomato and rice seedlings was analyzed. There was no significant change in the maximum quantum efficiency of PSII (Fv/Fm) in tomato and rice leaves treated with nHA or Cu-nHA (Figure S5C) as compared to untreated controls. The highest actual quantum efficiency (Φ_{PSII}) was found with 20 nm-nHA, with increases of 31.4% and 98.2% in tomato and rice seedlings, respectively, when compared to the untreated controls (Figure S5D). The non-photochemical quenching (NPQ) within tomato leaves was unaffected by e nHA, while the NPQ values in rice leaves were generally decreased (Figure S5E). Specifically, the NPQ of rice leaves treated with 20 nm-nHA, 60 nm-nHA, 1% Cu-nHA and 10% Cu-nHA was significantly decreased by 48.4%, 48.8%, 88.9%, and 41.7%, respectively, relative to the untreated control.

Chlorophyll fluorescence parameters are indicators that reflect a series of regulatory processes in the photosynthetic process[36]. Previous studies have also demonstrated that foliar application of nHA significantly improved the growth and yield of *Phaseolus*. Upon decreasing the NPQ, the associated chlorophyll fluorescence properties (Fv/Fm and Φ_{PSII}) will increase, and the photosynthetic efficiency will be subsequently increased[34]. For example, the application of 200 mg/L fullerene (C_{60}) NMs improved the photosynthetic efficiency of tobacco mosaic virus-infected *Nicotiana benthamiana*, subsequently increasing the health and growth of the diseased plants [37]. In the current study, the extent of nHA improvement of chlorophyll fluorescence characteristics in tomato and rice was consistently greater than Cu-nHA; in addition, rice seedlings exhibited a better response to nHA application than that did tomato seedlings.

nHA effects on crop elemental content

The addition of nHA increased the Ca and P content of shoots and roots of both species, although the changes in tomato were of a greater magnitude. The Ca content in tomato shoots was increased by 38.8% and 32.4% with 20 nm-nHA and 1% Cu-nHA treatment, respectively (Figure S7A); the Ca content in rice shoots was increased by 21.7% and 13.6% (Figure S7E), respectively, relative to the untreated control. The addition of 20 nm-nHA, 60 nm-nHA, 1% Cu-nHA and 10% Cu-nHA increased the P content in the tomato shoots by 1.2-16.3%; the greatest increase (16.3%) was with 20 nm-nHA treatment. The P content in tomato roots was increased by 7.1-58.6% with nHA treatment, with the greatest value of 58.6% occurring with 1%Cu-nHA; the P content in rice seedlings showed the same trend (Figure S7B and F). Similarly, Rady et al. reported that P contents were increased by 60%-110% upon foliar exposure to 0.05−0.2 g/L P nanoparticles relative to the untreated controls[34]. Additionally, root exposure to nHA had no significant effect on the S content in tomato and rice seedlings as compared to the controls (Figure S7E and H). However, root exposure to different nHA also altered the micronutrient (Cu, Fe, Mn, Zn) content in tomato and rice (Figure S8). Micronutrients play a number of important roles in the photosynthetic system and plant growth[38]. Here, the Fe content in tomato and rice roots as a function of different types of nHA were increased by 33.3%-60.3% and 11.8%-56.7%, respectively, relative to the controls. Fe is essential for many metabolic pathways involved in energy production, energy transfer, and nitrogen reduction[39]. The contents of other trace elements in both crops were largely unaffected as a function of nHA or Cu-nHA. Li et al. reported that the addition of 30 g/kg nHA reduced the Zn and Cu contents in pakchoi when cultivated in heavy metal-contaminated soil[40].

nHA effects on phytohormones

The abscisic acid (ABA) content in the 20 nm-nHA treated tomato shoots and roots was decreased by 14.8% and 17.1%, respectively, compared to untreated controls; no differences were evident with 1% Cu-nHA treatment (Figure 2A). In rice roots, exposure to 20 nm-nHA and 1% Cu-nHA increased the ABA content by 50.7% and 43.6%, respectively, relative to the control (Figure 2A). The Auxin (IAA) content in tomato and rice were significantly increased upon root exposure to 20 nm-nHA and 1% Cu-nHA; the greatest impact was in tomato shoots, with increases of 11.5% and 20.6%, respectively, when compared to the untreated control (Figure 2). ABA plays an significant physiological role in plant responses to external environmental stressors, and can interact with other plant hormones (*e.g.*, IAA, ethylene, etc.) to regulate the root growth[37, 41]. For example, root exposure to carboxylated graphene quantum dots (GQDs) significantly increased ABA levels, while simultaneously decreasing IAA content in *Lactuca sativa* L.; the resulted was impaired the growth with a 39% decrease in biomass[42]. Gibberellin A3 (GA3) and Gibberellin A4 (GA4) can maintain the growth and development, increase the yield, and promote the germination and tillering of crops[43]. The GA4 content of tomato shoots was significantly increased by 8.8% and 4.9% with 20 nm-nHA and the 1% Cu-nHA treatment, respectively, compared to the untreated controls; a similar trend was also found in rice shoots (Figure 2). Pereira et al. reported that a chitosan-based nanoparticle system loaded with GA3 increased tomato yield by nearly 4-fold relative to untreated controls[44]. The current findings also demonstrate that pristine and metal-doped nHA increased the levels of GA in both crop species; notably, the performance of 20 nm-nHA at promoting GA levels in both species was greater than that of 1% Cu-nHA. Additional details on other phytohormones (zeatin riboside, dihydrozeatin, brassinosteroids, jasmonic acid, and indole-3-acetic acid) in both crop species as a function of nHA are provided in Text S2.

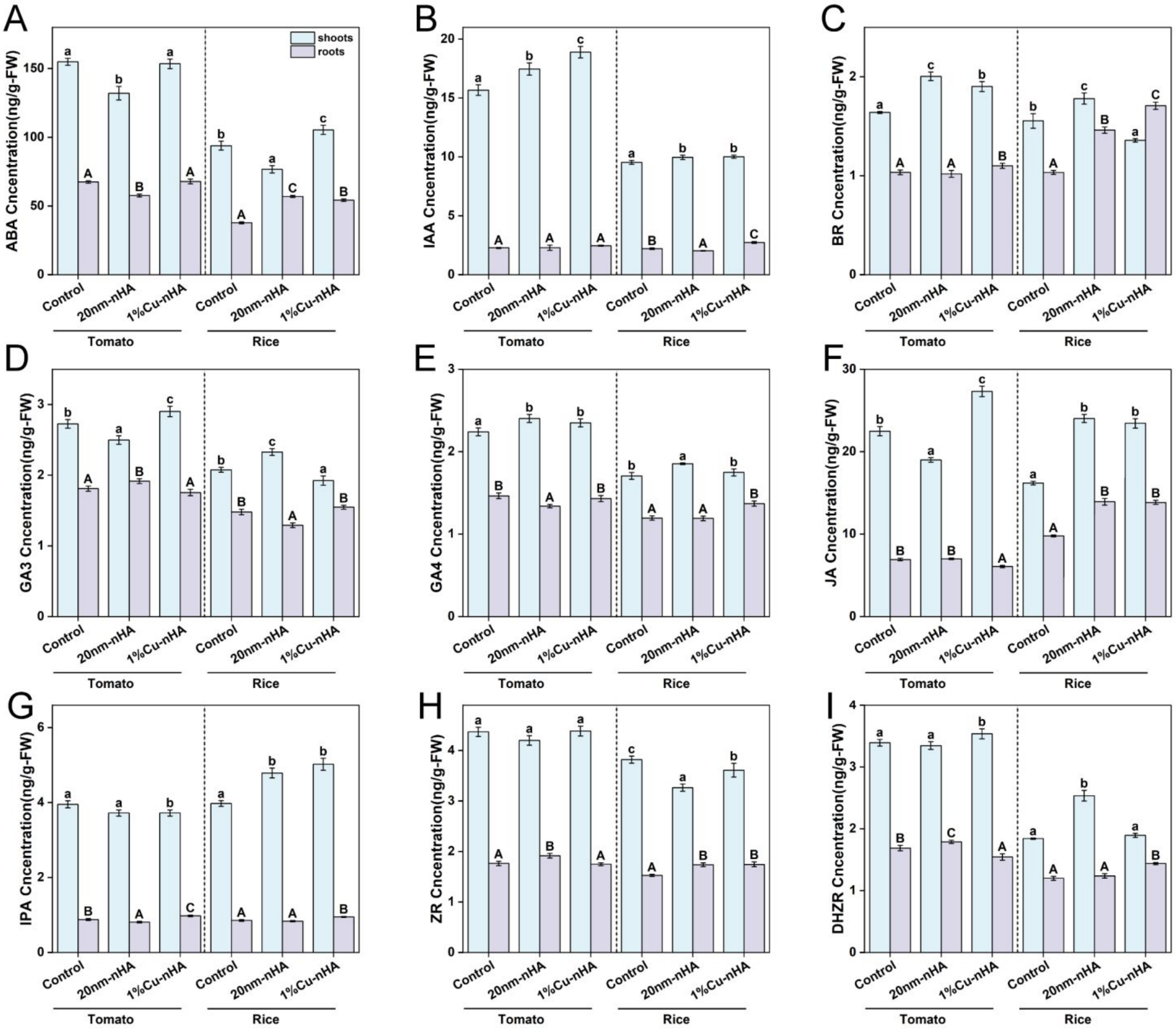

Figure 2. The phytohormone concentration of shoots and roots of tomato and rice plants upon root exposure to different types of nHA, including 20 nm-nHA and 1 % Cu-nHA. (A) ABA concentration; (B) IAA concentration; (C) BR concentration; (D) GA_3 concentration; (E) GA_4 concentration; (F) JA concentration; (G) IPA concentration; (H) ZR concentration; (I) DHZR concentration; The different letters in the figure indicate significant differences ($P < 0.05$) determined by one-way ANOVA, letters in lowercase represent significant differences among shoots, while uppercase letters represent significant differences in roots.

Transcriptomic profile as affected by nHA

The percentage of reads alignment region and the distribution of expressed genes in each sample are provided in Figure S9. The principal component analysis (PCA) plots suggest that 20 nm-nHA and 1% Cu-nHA in tomato and rice are clearly separated from the untreated control, indicating a distinct transcriptomic profile in tomato and rice as a function of treatment. Gene Ontology (GO) was used to divide all identified DEGs into three main categories; detailed descriptions are shown in Text S3 and Figure 3, S11−14.

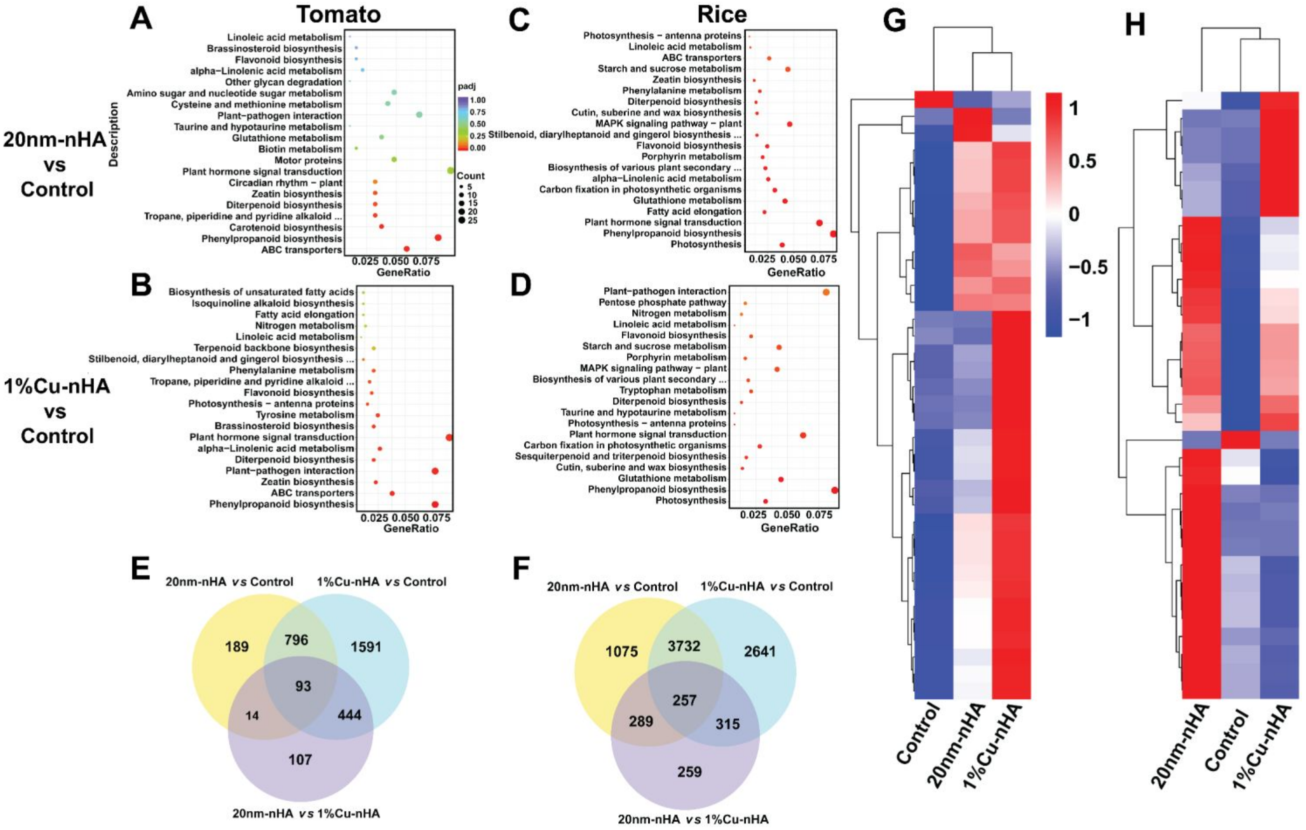

Figure 3. The transcriptome profiles of tomato roots and rice roots in each group. The top 20 KEGG pathways with significant enrichment of DEGs in the leaves of tomato and rice roots (A-D) were exposed to 20 nm-nHA and 1 % Cu-nHA. (E) Venn diagram of DMs in tomato roots. (F) Venn diagram of DMs in rice roots. Cluster heatmap of DEGs in tomato roots (G) and rice roots (H).

The addition of 20 nm-nHA and 1% Cu-nHA upregulated genes involved in the starch and sucrose metabolism in tomato shoots (Figure S15A). Importantly, sugar is the primary cellular energy source and can act as signaling molecule in plants[45]. Specifically, trehalose 6-phosphate synthase converts sucrose into downstream trehalose, contributing to osmotic balance. The expression of trehalose 6-phosphate synthase genes 1 (*TPS1*) and trehalose 6-phosphate synthase genes 2 (*TPS2*) in tomato shoots treated with 20 nm-nHA and 1% Cu-nHA were upregulated by 1.6-2.8 and 0.8−1.9

fold, respectively (Figure S15A) compared to untreated controls. Previous study has shown that in *Arabidopsis thaliana*, TPS1 catalyzes the synthesis of the sucrose signaling metabolite Tre6P, which is essential for embryogenesis and normal postembryonic growth and development[46]. In addition, α-amylase is currently the most well-studied amylase, which can help plants to better utilize starch reserves at different growth stages[47].The expression of the α-amylase encoded gene *AGXA* in the tomato shoots was upregulated by 1.3- and 2.5-fold with 20 nm-nHA and 1% Cu- nHA, respectively, relative to the untreated control.

The addition of 20 nm-nHA and 1% Cu-nHA upregulated genes involved in the carbon fixation in the rice shoots (Figure S15B). For example, in the crassulacean acid metabolism pathway, the expression of malate dehydrogenase (*CMD4*) in rice shoots with 20 nm-nHA and 1% Cu- nHA was 1.8- and 2.8-fold that of the untreated control, indicating that select nHA particles accelerated the reversible conversion between malate and oxaloacetate for subsequent energy metabolism. In the Calvin cycle, the expression of genes related to ribulose-1-5 bisphosphate (RuBP) regeneration, such as *FBP*, *TKA* and *GAPA1*, were also upregulated to varying degrees, and the 20 nm-nHA and 1% Cu-nHA promoted carbohydrate production as the product of the Calvin cycle. Generally, 1% Cu-nHA upregulated the expression of genes involved in carbon sequestration and promoted the absorption of light and CO_2 in seedlings to a greater extent than did 20 nm-nHA; this may be the reason that gs and E with 1%Cu-nHA were higher than that in the 20 nm-nHA treatment (Figure 1H and I).

In the root, the additions of 20 nm-nHA and 1% Cu-nHA positively impacted plant hormone signal transduction in both crops (Figure 4A and B). Specifically, auxin plays a crucial role in various cellular and developmental responses throughout the plant life cycle[48]. Plants can rapidly sense and respond to changes in auxin levels through the activity of a number of auxin response genes. The expression of ent-kaurenoic acid monooxygenase (KAO) in the tomato roots with 20 nm-nHA and 1% Cu- nHA was 1.1- and 7.7-fold greater than the untreated control, and the expression of KAO in rice root was 4.3- and 2.1-fold of the control, respectively. Similarly, Yan et al. reported that upon exposure to 40 mg/L Ag NPs, the levels of GA receptor related genes in rice seeds were increased by 1.8−2.4-fold relative to the control, thus accelerating seed germination and salt tolerance[14]. In addition, 20 nm-nHA and 1% Cu-nHA upregulated the expression of auxin acid (*Aux*) and the auxin response factor (*ARF*) in the tomato roots by 0.8−1.7- and 1.8−1.5-fold, respectively. The expression of *Aux* and *ARF* in rice roots were 0.2-2.9-fold and 0.1-3.3-fold of the control, respectively. Similarly, Wang et al. observed that 1 mg/L manganese ferrite nanoparticles upregulated the expression of ARFS by 5.12-fold in tobacco cell suspensions, which stimulated cell differentiation and growth[49]. Thus, root exposure to 20 nm-nHA and 1% Cu-nHA clearly initiated the AUX/IAA pathway, thereby enhancing the IAA response signal and promoting root development of both crops.

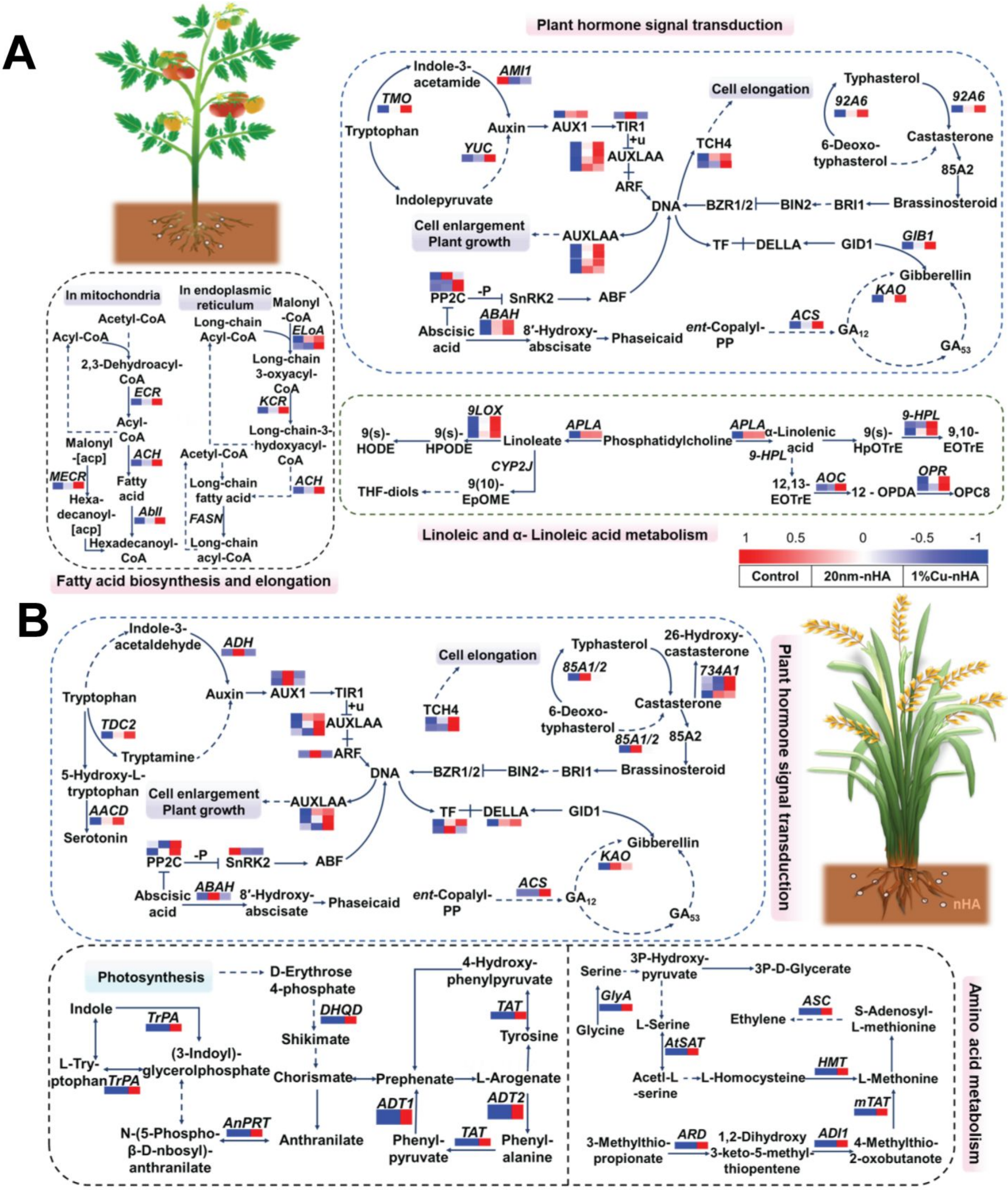

Figure 4. The KEGG pathway network of DEGs in tomato (A) and rice roots (B) as impacted by 20 nm-nHA and 1% Cu-nHA. The heatmap of the expression of DEGs was added; the log2 value of the level of gene expression shares the color key inserted below the figure. The colored boxes from left to right in each row stand for untreated control, 20 nm-nHA and 1% Cu-nHA, respectively. Words in italics: Annotated function related enzyme synthesis in KEGG pathway.

Amino acid metabolism is closely related to carbohydrate metabolism, protein synthesis requirements, and secondary metabolism[50]. The addition of 20 nm-nHA and 1% Cu-nHA upregulated genes involved in amino acid metabolism in rice roots. Specifically, the expression of arogenate dehydratase 1 (*ADT1*) and arogenate dehydratase 2 (*ADT2*) in rice roots treated with 20 nm-nHA and 1% Cu-nHA was 0.1−2.7- and 0.1−2.6-fold that of the untreated control, respectively (Figure S15A). *ADT2* contributed the most to anthocyanin synthesis in *Arabidopsis*, followed by *ADT1*. Anthocyanin plays an important role in plant stress resistance, and anthocyanin levels are positively correlated with the amount of *ADT* transcripts induced by sucrose[51]. Changes in amino acid levels can determine the availability and demand for nitrogen in crop species[50]. Importantly, 20 nm-nHA, the 1% Cu-nHA up-regulated the expression of genes involved in amino acid metabolism, thereby promoting nitrogen uptake into the rice root; while the amino acid metabolism in tomato was not changed much as affected by 20-nm nHA and 1% Cu-nHA.

Endophytic microbial community as affected by nHA

The rarefaction curves of upon exposure to different types of nHA were showed in Figure S16. PCA of bacterial and fungal communities is provided in Figure S17. Endophytes can regulate and promote the absorption of nutrients, thus promoting plant growth and development[52, 53]. The ACE and Shannon indices of endophyte communities in tomato and rice roots across all treatments did not change significantly (Figure 5A, B, E and F). At the phylum level, the dominant endophytic bacterial groups in the shoots and roots of tomato and rice were similar. Specifically, the top three endophytic bacteria were *Proteobacteria*, *Bacteroidota*, and *Actinobacteriota* (Figure S18A-D), accounting for 90% of the total relative abundance in the shoots and roots of both crops. The relative abundance of *Proteobacteria* was the highest; specifically, the relative abundance of *Proteobacteria* in tomato roots treated with nHA were increased by 3.2-5.5% compared controls, while those in the rice root were decreased by 3.2-8.4%.

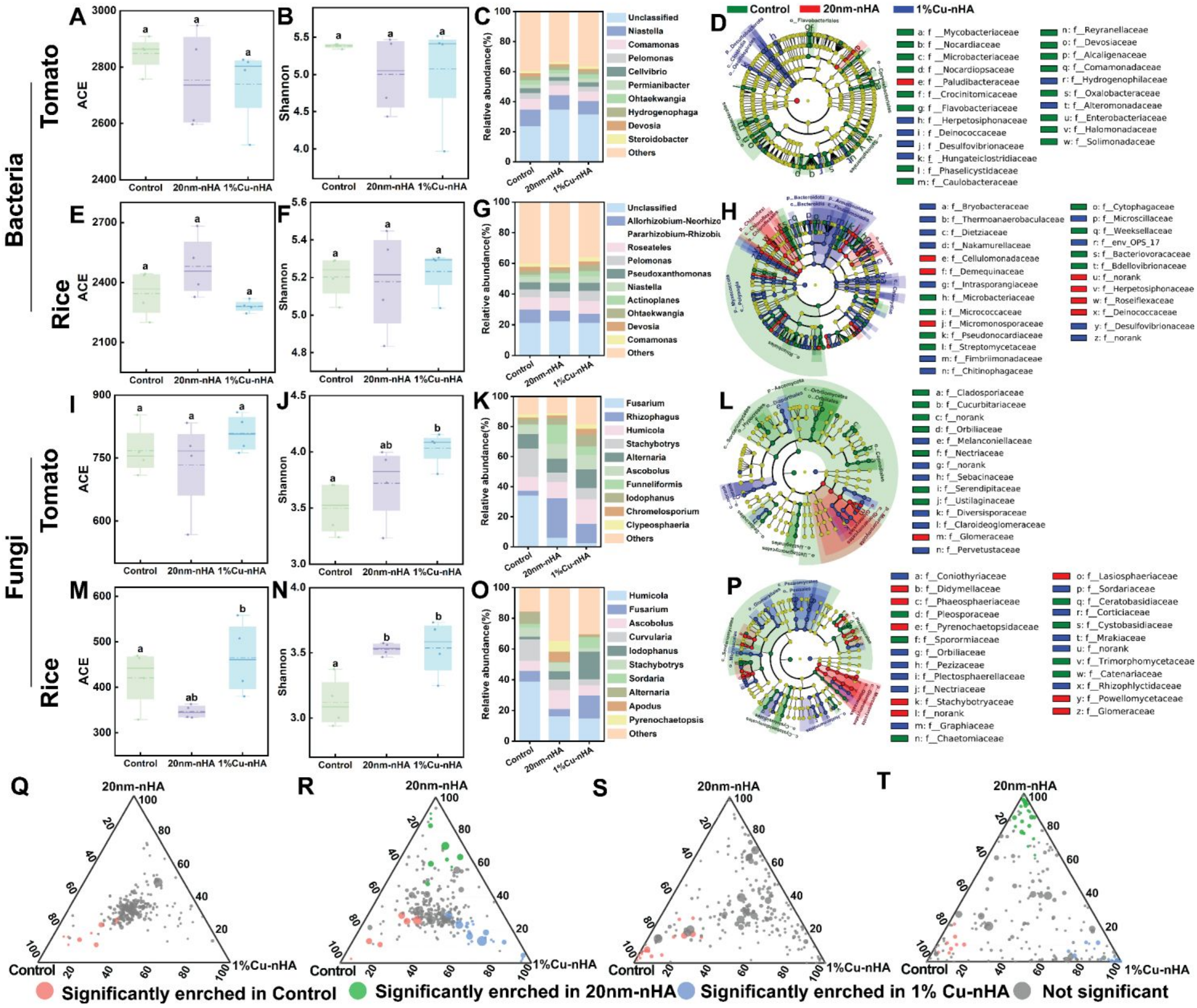

Figure 5. Community of endophytic bacteria in shoots of tomato and rice plants as affected by different nHA (20 nm-nHA and 1 % Cu-nHA). (A, B) ACE and Shannon's index of the endophytic bacterial community in tomato roots; (C, G) the relative abundance of endophytic bacteria at the genus level in tomato and rice roots; (D, H) cladograms of LEfSe showing bacterial indicator taxa treated with different nHA in tomato and rice roots; (E, F) ACE and Shannon's index of the endophytic bacterial community in rice roots; (I, J) ACE and Shannon's index of the endophytic fungal community in tomato roots; (K, O) the relative abundance of endophytic fungus at the genus level in tomato and rice roots; (L, P) cladograms of LEfSe showing fungal indicator taxa treated with different nHA in tomato and rice roots; (M, N) ACE and Shannon's index of the endophytic fungal community in rice roots; (Q-T) ternary plot of fungi from left to right describes the bacterial communities in tomato root, bacterial communities in rice root, fungal communities in tomato root and fungal communities in rice root as affected by different nHA. Each circle in the ternary plot represents an OTU detected in the data set, the circle size indicates its relative abundance.

The dominant fungal phyla were *Ascomycota*, *Basidiomycota,* and *Mucoromycota* (Figure S18E-H), accounting for 90% of the total relative abundance in tomato and rice seedlings. The relative abundance of *Mucoromycota* in 20 nm-nHA and 1% Cu-nHA treated tomato roots was increased by 7.2- and 3.8-fold, respectively; in rice roots, the increases were 1.7- and 0.9-fold, respectively, relative to the corresponding control. *Mucoromycota* can release P and N to their host plants, subsequently improving the utilization of these macronutrients[54, 55]. Field et al. demonstrated that *Mucoromycota* fungi can form nutritionally mutualistic relationship with host plants and participate in the bidirectional exchange of plant-fixed C for mineral nutrients[56, 57]. The increased abundance of *Mucoromycota* suggests that 20 nm-nHA and 1% Cu-nHA improved P and N utilization, as well as the absorption of mineral nutrients, in tomato and rice seedlings.

The abundance of *Pseudomonas* and *Roseateles* in rice roots in the both nHA treatments was increased by 13-21.7% and 5.2-6.9%, respectively, as compared to the control (Figure 5C and G). Chi et al. reported that inoculation of *Pseudomonas* improved photosynthesis and Cd accumulation in *Sedum alfredii*, and that potential modulation of the expression of the photosynthesis- and carbon fixation-related genes seemed likely[58]. Additionally, *Roseateles* can produce chlorophyll and carotenoids under aerobic conditions, subsequently actively regulating the synthesis of leaf chlorophyll to promote plant photosynthesis[59]. The endophytic fungal communities in tomato and rice roots were significantly changed with 20 nm-nHA and 1% Cu-nHA (Figure 5K and O). For example, the abundance of *Fusarium* in tomato roots treated with 20 nm-nHA and 1% Cu-nHA was decreased by 4.5- and 11.8-fold relative to the untreated control. Similarly, the abundance of *Humicola* in tomato roots was increased by 1.4- and 1.6-fold, while its abundance in rice roots was increased by 20.3% and 10.2% with 20 nm-nHA and 1% Cu-nHA, respectively, when compared to the control. Decreases in the abundance of *Fusarium* suggest that the additions of both types of nHA exhibited the potential to enhance plant resistance to soil-borne plant pathogenic fungi[59]; importantly, Cu-doping seemed to more effectively inhibit the abundance of *Fusarium*. Fungi such as *Penicillium* can produce 1-aminocyclopropane-1-carboxylate deaminase, which contributes to root elongation and promotes plant growth[60]. The application of 20 nm-nHA and 1% Cu-nHA to tomato and rice roots enriched a wide variety of beneficial endophytic colonizing fungi, producing direct (such as increasing nutrient and photosynthetic pigment production) and indirect benefits (such as improving resistance to abiotic and biotic stresses), thereby promoting plant health.

LEfSe was conducted to identify key biomarkers driving taxonomic differences in endophytic fungal and bacterial communities across treatments. LEfSe analysis shows that the addition of both types of nHA increased the abundance of plant-associated beneficial microorganisms as compared to the control (Figure 5 and S19). For example, the abundance of *Glomeromycotina* and *Funneliformis* were increased in tomato roots with 1% Cu-nHA. *Arbuscular mycorrhizal* (AM) fungi belong to the *Glomeromycotina* phylogeny and form a mutually beneficial symbiotic relationship with the roots of more than 80% of terrestrial plant species, providing the host plant with water and nutrients beyond the rhizosphere, especially phosphorus, which often has low availability in soil[61]. Zhao et al. reported that *Funneliformis* was the dominant AM fungal group in the root systems of three soybean varieties, and that *Funneliformis* inoculation could improve soil protease and polyphenol oxidase activities[62]. The abundance of *Blastocladiomycota* in tomato and rice roots was increased by 89.6% and 31.3% with 1% Cu-nHA relative to the untreated control. *Blastocladiomycota* in soil environments can decompose insoluble macromolecules such as proteins, starch, and cellulose in dead fungi and plant tissues, thereby increasing nutrient availability[63]. Ternary plots were used to highlight the significant enrichment of OTUs between the control and nHA treatments and to determine the effects of different nHA on the microbial communities in tomato and rice roots (Figure 5Q-T; Figure S19Q-T). The 1% Cu-nHA treatment exhibited the greater number of enriched OTUs of fungi in rice roots than the control and 20 nm-nHA treatment, suggesting that 1% Cu-nHA might selectively enrich beneficial OTUs; however, the application of 20 nm-nHA and 1% Cu-nHA had no differential effect on OTUs enrichment in tomato roots.

Conclusion

Due to the low use efficiency, increasing amounts of commercial P-based fertilizers have been applied annually to meet the demands for crops. However, the low delivery/utilization efficiency of P-based fertilizers wastes tremendous amounts of resources for production and the resulting overuse exacerbates the eutrophication of water bodies and associated environmental damage. Our findings suggest that the use of nHA as an alternative to conventional P-based fertilizers can significantly increase agricultural yields and reduce the use doses of agrochemicals. Importantly, 20 nm-nHA and 1% Cu-nHA can recruit beneficial microbes and positively alter the endophytic microbial community in crops, thereby promoting plant growth, nutrient uptake, and stress resistance. Collectively, the application of nanoscale agrochemicals has the potential to reduce the use of agricultural products and environmental pollution in agricultural fields. In addition, metal-doped nHA with micronutrients offers an additional strategy to enhance crop health and inhibit pathogenic microorganisms. This work adds to the growing body of evidence demonstrating how nanoscale agrochemical amendments can be tuned for maximum benefit and highlights the significant potential of these materials for precision nutrient delivery as a key component of sustainable agriculture in the 21st century.

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Author Contributions

Mengchen Tian: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing–original draft. **Zihao Zhao**: Data curation, Methodology, Validation. **Qingqing Li**: Resources, Project administration. **Zeyu Cai**: Data curation, Formal analysis, Methodology, Software, Validation, Visualization. **Weili Jia**: Resources, Software. **Xinxin Xu**: Investigation. **Anqi Liang**: Data curation. **Shuai Wang**: Validation. **Jason C. White**: Conceptualization, Resources, Supervision, Writing–review and editing. **Chuanxin Ma**: Conceptualization, Funding acquisition, Resources, Methodology, Project administration, Supervision, Writing ‑ review and editing. **Baoshan Xing**: Methodology, Resources, Writing – review and editing.

Data availability

All relevant data are provided in this article.

Conflict of Interest Statement

None.

Supplementary Information

There are 2 Text, 2 Method, 22 Figures and 2 SI Tables in the supplementary information (SI) file.

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Data Availability Statement

All relevant data are provided in this article.