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Research article

Fertilizing benefits of biogenic phosphorous nanonutrients on *Solanum lycopersicum* in soils with variable pH

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ABSTRACT

Nanoformulations of Phosphorous (P) have recently been proposed as alternatives to P fertilizers. In this study, the fertilizing efficacies of P-based nanomaterials (NMs), nanohydroxyapatite (nHAP) and nanophosphorus (nP), were examined on *Solanum lycopersicum* (Pusa Rohini, Indian tomato) in growth room pot experiments. These NMs differed in their mode of synthesis, chemical composition, size and shape. Rock-phosphate (RP), phosphoric acid (PA) and di-ammonium phosphate (DAP) were included as bulk materials for comparison. Three varieties of artificial soils were included in the study, neutral (pH 7.2), acidic (pH 4.3) and basic (pH 9.8). The effects of the NMs on germination, plant growth, and P content were assessed at the 15th and 30th days after treatment. The results showed that P-based NMs enhance the overall germination and plant growth by increasing P levels in all types of soils for the tomato plants in comparison to the bulk P sources. Analysis using X-ray fluorescence revealed enhanced P content in the plants indicating the uptake of P-based NMs. Evaluation of H_2O_2, total phenolics and total flavonoids contents after NM treatment suggest that there is no stress caused due to the application of NMs to the plant. The results of this study indicate the beneficial role of P-based NMs as fertilizers at the early stages of plant development, which opens a scope for further investigation of underlying metabolic and molecular pathways and field trials.

1. Introduction

According to reports, nearly 70% of agricultural soil is deficient in phosphorus (P), making it one of the soil's limiting macronutrients [1]. These deficient soils are unsuitable for agricultural crops [2] with plants that are P-deficient having compromised growth than those that are P-sufficient [3]. This compromised plant growth can be linked to the fact that P is essential in a wide range of plant metabolic processes, including the key process involving ATP generation and DNA synthesis [3, 4]. Because P holds metabolic and biochemical importance in a biological system, an insufficient or excessive supply of P can disrupt cellular homoeostasis, causing disruptions in plant metabolic activities, ultimately, decreasing crop yield [4]. As a result, P fertilizers are needed for meeting agricultural demands. In addition to this, even in soil with sufficient P levels, P uptake by plants is limited owing to its slow diffusion and immobilisation due to its interaction with various divalent cations and natural organic matter present in the soil. This frequently results in P unavailability to crops, eventually leading to stunted plant growth [2, 5, 6]. Therefore, P supplements are being applied to the soil regularly to improve crop yield. This frequently results in surface run-off and fertilizer leaching, which is the primary source of eutrophication and environmental hazards [7, 8, 9]. In addition to this, the most common source of P fertilizers, rock-phosphate (RP), has limited availability in nature and is depleting exponentially [10]. To overcome these challenges, an alternative to bulk P-based fertilizers is critically needed for improving crop production and productivity. As a result, it is critical to investigate innovative P fertilizers, not only to improve fertilizer utilization efficiency but also to reduce negative environmental impacts. To address this, due to their unique physicochemical properties that aid in efficient uptake by plants, P-based nanofertilizers may be a solution to the challenges with existing bulk fertilizers [1, 11, 12, 13, 14, 15, 16, 17].

Recently, nanoformulations of hydroxyapatite (nHAP) have started gaining attention to be explored for agricultural applications. Early research has been limited to studies that use nHAPs as a base or carrier to

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deliver other macronutrients (N and K) by its surface modification using most prominently urea [14, 18, 19, 20], zeolite [17], humic acid [21] and cellulose [22]. Another use of nHAP has been restricted to their bioremediation application by immobilizing heavy metals such as lead in agricultural soils [23, 24]. The standalone use of nHAP to supplement P to plants has only been explored in a small number of separate research studies on *Glycine max* (soybean) [25], *Triticum aestivum* (wheat) [1], *Lactuca sativa* (lettuce) [16], *Helianthus annuus* (sunflower) [26], *Brassica oleracea* (broccoli) [15], *Cicer arietinum* (chickpea) [13], *Vicia faba* (faba bean) [11] and *Solanum lycopersicum* (tomato) [27]. However, of these studies, only two research groups have tested the effects of nHAP on two different soil types [1, 26] and only one research group has studied the effects of nanophosphorus (nP) from rock phosphate on *Zea mays* (maize) in a field experiment [12].

Another important aspect to consider when applying these NMs to agriculture fields is a reduction in secondary contamination as well as environmental load, which occurs due to the use of harsh acidic or basic conditions while synthesizing the NMs. A more environmentally friendly approach to the synthesis of P-based NMs can be achieved using biological synthesis routes [28, 29, 30]. The biological synthesis of NMs via the use of different plant extracts [29, 30] and non-pathogenic soil microorganisms [28] is hypothesized to eliminate the need for stringent chemicals and is considered a greener and more sustainable approach.

This study reports the fertilizing effects of biogenic P-based NMs in soils under different pH levels. These biogenic P-based NMs have been demonstrated to have no acute toxicity in plant-growth promoting rhizobacteria [28], *Caenorhabditis elegans* [33] and zebrafish embryos [77, 78]. *Solanum lycopersicum* (Pusa Rohini, Indian tomato variety), a dicot species was chosen for this study. The quick germination and the completely sequenced genome of tomato may facilitate future work on their molecular responses to NMs [31, 32]. The effects of biologically synthesized P-based NMs on tomato seedlings are compared with the results obtained with chemically derived nHAPs, which differ in shape and size from the biogenic nHAP and nP. The OECD TG 208 has been used to define the application of P-based NMs, and the inclusion of suitable bulk controls enabled an overall comparison of fertilizing efficacy of NMs in this study.

2. Materials and methods

2.1. Materials

Nanohydroxyapatite powder (Product no. 677418, size <200 nm, spherical shaped), aluminum-sulphate, di-ammonium phosphate (DAP) and calcium chloride were purchased from Sigma-Aldrich, USA. Phosphoric acid (PA), nitric acid and hydrochloric acid were procured from Merck (Sigma-Aldrich, USA). Needle-shaped nHAP (Product no. 13616, size <200 nm) was procured from Sisco Research Laboratories Pvt. Ltd., India. Soilrite for plant growth was procured from a local vendor (Allied Scientific Sales, New Delhi, India). Seeds of *Solanum lycopersicum* (Pusa Rohini, Indian tomato) were obtained from the Indian Agricultural Research Institute, New Delhi, India.

2.2. Synthesis and characterization of P-based NMs

Biologically generated nHAP and chemically synthesized nHAP were produced in-house using previously published methods [28]. Both of these NMs have also been characterized for physicochemical properties in our previous study using transmission electron microscopy, dynamic light scattering, FTIR and XRD [28, 33]. In brief, the biologically synthesized nHAP were platelet-shaped, size of 35.74 nm, molar Ca/P ratio as 1.58, and hydrodynamic diameter of 325.8 ± 37.1 nm and zeta potential of −31.3 ± 3.5 mV in de-ionized (DI) water. In contrast, the chemically synthesized nHAP were rod-shaped, size of 83.92 ± 26.85 nm, molar Ca/P ratio as 1.79, and hydrodynamic diameter of 756.2 ± 28.8 nm and zeta potential of −45.2 ± 1.7 mV in DI water. Nanophosphorus particles (nP) were kindly provided by the NM synthesis research group from the TERI-Deakin Nanobiotechnology Centre, Gurugram, India, for this study. The nP were tiny dots, size of ~5–10 nm, molar Ca/P ratio as ~3.8, and hydrodynamic diameter of 798.5 ± 23.36 nm and zeta potential of -11.61 ± 0.015 mV in DI water.

2.3. Soil parameters

The procured soil-rite (vermiculite: perlite: peat moss as 1/3:1/3:1/3, *w/w* ratio) was sterilized by autoclaving twice for 30 min at 121 °C and 15 psi. The soil-rite was then supplemented with DI water in a ratio of 1:5 (*w/v*) and checked for pH (Orion pH/ISE meter, Model 710A 203, Thermo Fisher Scientific, USA). To obtain acidic and basic soil, soil-rite was treated with calcium chloride and aluminum sulphate, respectively, until the desired pH was obtained. For basic soil, the pH was adjusted between 9-10 and for acidic soil, the pH was adjusted between 4-5. The pH was measured for the soil samples in a 1: 5 (*w/v*) ratio of soil and DI water.

2.4. Growth room experiment setup

The wild type seeds of tomato were soaked in autoclaved DI water overnight in dark to soften the seed coats. Small test pots (5 cm in diameter) with perforated bottoms (3 holes with ~6 mm diameter) were filled with 25 g of soil-rite maintained at different pH conditions. Five seeds of tomato were placed in each pot per experimental set-up. The pots were placed in plastic trays. Three different concentrations (12.5, 100 and 1000 μg mL^{-1} corresponding to 0.012, 0.098 and 0.98 kg ha^{-1}) of different NMs (nHAP_B, nHAP_C, nHAP_Sigma, nHAP_SRL and nP) and bulk controls (DAP, H_3PO_4 and RP) were prepared in DI water. For fertilization, the application dose for the conventional P materials ranges from 60 to 100 kg ha^{-1} [34,35,36]. The doses selected for bulk sources in this study were similar to those of nano-formulations. This was done to understand the comparative efficacy of using nano-formulations over bulk materials and to test if nano-formulations are needed in lower amounts for fertilizer applications. Of these bulk controls, PA forms a soluble phosphorus control studied previously on *Lactuca sativa* (lettuce) [16] against P-based NMs, DAP has been conventionally used bulk P fertilizer [37, 38, 39] and RP provides a bulk control for nP and has also been used as a common source for deriving P fertilizers [10]. Their suspensions were used for irrigation of the plants. Plants only irrigated with DI water served as solvent controls. The NMs and bulk suspensions were added to the trays to cover the bottoms of pots (2 cm). The seeded pots were kept in the growth room where the appropriate light (14 h, 100 $mE/m^2/s$) and dark cycle (10 h) was maintained at 25 ± 2 °C. The experiment was set up for a period of 15 and 30 days. The analysis was conducted with 3 replicates for each concentration per soil type per end-point duration.

2.5. Effect of nHAP and nP on plant growth endpoints

Assessment of the effects of P-based nanoforms and bulk materials was conducted using the modified OECD test guideline 208, commonly used for the testing of chemicals in terrestrial plants [40]. Briefly, after the 15^{th} and 30^{th} day from the initiation of the experiment, the germination percentage, fresh weight and dry biomass, root and shoot lengths were determined at the designated endpoints for the plants treated with different concentrations of NMs and bulk control samples in the three soil types. The germination percentage was calculated by comparing the number of sprouted seeds to the number of potted seeds. When the radicle reached a length of more than or equal to 1 mm and the plumule was just unfolded, the seed was considered fully germinated [41]. The seedling vigor index (SVI-I) with respect to seedling length was calculated by multiplying germination percentage by root length, and the seedling vigor index with respect to dry weight (SVI-II) was calculated by multiplying germination percentage by biomass [42]. After the test time

(15 and 30 days), plants were harvested carefully and washed under tap water to remove any excess soil. The plants then were gently blotted to remove any external water content and fresh weight was measured. After taking the fresh weight, the plants were kept between filter paper sheets and dried at 65–70 °C in a hot air oven to obtain dry weight (biomass) until consistent values were reached.

2.6. Estimation of P and Ca content in plants

Total P and Ca content in plant samples were calculated using the method described in protocol 365.3 by EPA (USA) [43] and by flame atomic absorption spectroscopy (AAS), respectively. The samples were subjected to acid-digestion using a mix of concentrated nitric acid (HNO_3) and hydrochloric acid (HCl) in the ratio of 1:4. The samples were pre-digested at 60 °C for 15 min and finally heated at a temperature of 120 °C until the samples became brown. After cooling, the volume of digested samples was made to 40 mL using de-ionized water and filtered using Whatman filter paper no. 1 (11 μm pore size at 98% efficiency).

2.7. Plant nutrient status using X-ray fluorescence

The plant nutrient status in terms of elemental distribution in above-ground parts of the plant was evaluated using X-ray Fluorescence (Horiba, Japan) using a protocol from Reidinger et al. (2012) [44]. Briefly, the above-ground parts of the plant were washed under running tap water, then dried at 60 °C for 3 days. The samples were ground to obtain a fine powder. The samples were analyzed by placing them on clear film placed at 0.1 mm from the source in a vacuum.

2.8. Measurement of hydrogen peroxide

Using the previously published methods [45], the H_2O_2 levels in plants were measured after 30 days post-treatment with the different nHAPs and nP (12.5 and 1000 μg mL^{-1}). Briefly, the fresh plants (100 mg), were homogenized in liquid nitrogen. After homogenization, the samples were centrifuged at 10000 g at 4 °C for 15 min. The supernatant was mixed with 10 mM phosphate buffer (pH 7.0) in a ratio of 1:1. 1 mL of 1M potassium iodide was added to the mix and the samples were incubated in dark for 30 min. The absorbance was measured at 390 nm and H_2O_2 was estimated with respect to the volume of extract (V) and fresh weight (FW) using the equation [1]:

$$H_2O_2\left(\frac{\mu mol}{g}\right) = \frac{A390 + 0.004}{3.727 * V * FW} \quad [1]$$

2.9. Estimation of total phenolic content

The total phenolic content was determined by using previously published methods [45, 46, 47]. Briefly, after 30 days post-treatment with the different nHAPs and nP (12.5 and 1000 μg mL^{-1}), the plants were homogenized as described in section 2.9. Methanol (80% *v/v*) was added to the homogenated sample in the ratio of 1:10. The samples were kept at shaking (120 rpm) for 30 min at room temperature. The samples were centrifuged at 10000 *g* at room temperature for 15 min. Folin-Ciocalteu reagent was mixed with phenolic extracts in a 1:1 ratio. After 5 min of incubation, sodium carbonate solution (2% *w/v*) was added to the mix. The reaction mixture was incubated at 25 °C for 1 h. A standard curve was prepared using gallic acid (0.1–2.0 mM). Absorbance was read at 724 nm and the results were presented with reference to the freshweight.

2.10. Estimation of total flavonoids content

Total flavonoids were estimated using previously published methods [45, 46, 47]. The fresh plant extract was obtained by homogenization as described in section 2.8 after 30 days post-treatment with the different nHAPs and nP (12.5 and 1000 μg mL^{-1}). The supernatant was obtained after centrifugation at 10000 g at room temperature for 15 min. Sodium nitrite (5% *w/v*) was added to the supernatant and the mix was incubated for 5 min at room

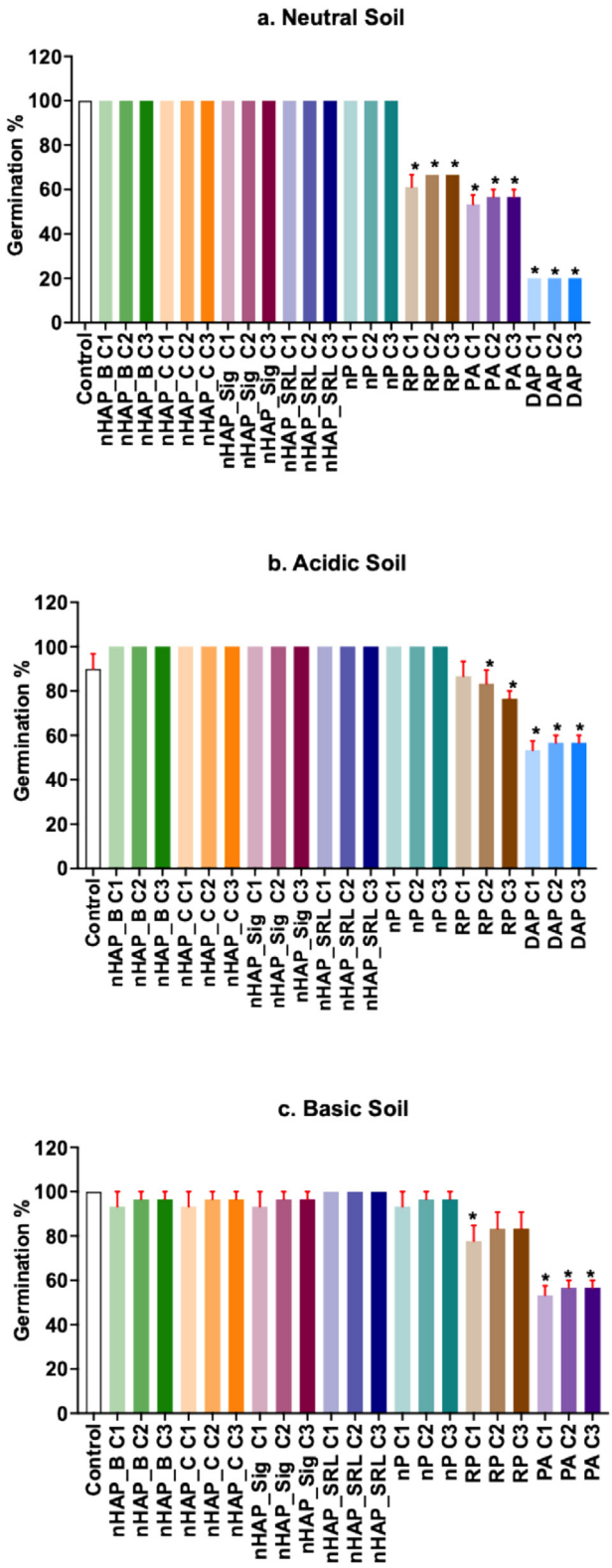

Figure 1. Percentage of germination of *Solanum lycopersicum* seeds in (**a**) neutral (**N**), (**b**) acidic (**A**) and (**c**) basic (**B**) soils treated with 12.5 (C1), 100 (C2) and 1000 (C3) μg.mL^{-1} concentrations of nHAP_B, nHAP_C, nHAP_Sigma, nHAP_SRL, nP, RP, PA and DAP. The values are expressed as mean and S.D. (* denote significance at $p < 0.05$ as compared to untreated control in the particular soil type).

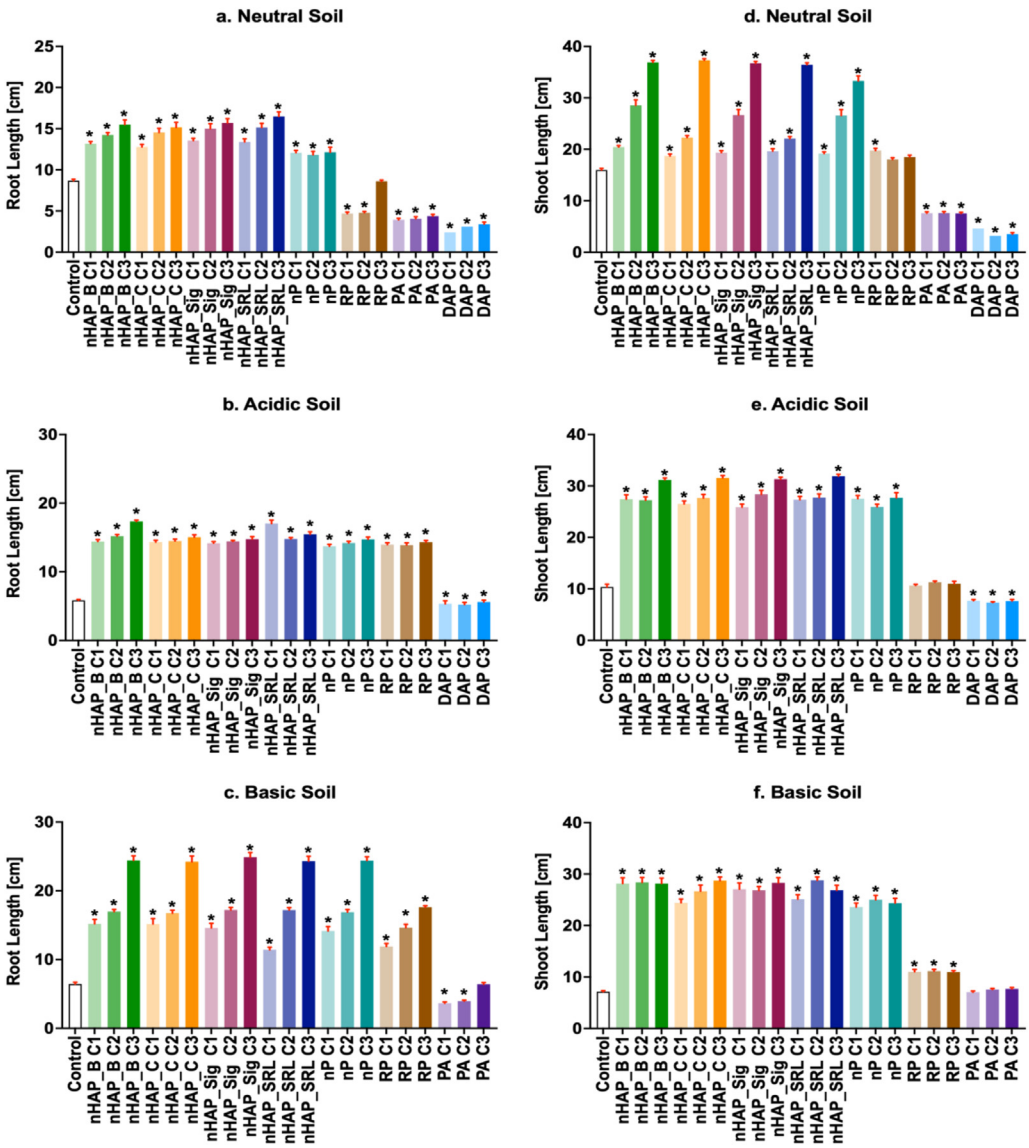

Figure 2. Root and shoot lengths of *Solanum lycopersicum* seedlings grown in neutral (**a** and **d**), acidic (**b** and **e**) and basic (**c** and **f**) soils treated with 12.5, 100 and 1000 μg mL^{-1} concentrations (increasing colour gradient) of nHAP_B, nHAP_C, nHAP_Sigma, nHAP_SRL, nP, RP, PA and DAP. The values are expressed as mean and S.D. (* denotes significance at $p < 0.05$ as compared to untreated control). No. of experimental replicates are 5 per harvest point. No of independent replicates = 3 per harvest point.

temperature. Further, aluminum chloride (10% *w/v*) was added to the mix and incubated for 10 min at room temperature. This was followed by the addition of 1M NaOH. The reaction mix was vortexed briefly before measuring the absorbance at 510 nm. The total flavonoids were calculated using the formula described in equation [2].

$$\text{Total flavonoids} = \frac{A510 - 0.008}{0.25 * V * FW} \quad [2]$$

2.11. Statistical analyses

GraphpadPrism (v 9.1) was used to statistically analyze all experimental data. To choose between ANOVA and the Kruskal-Wallis test, the data were checked for normal distribution. The Shapiro-Wilk test was used to determine the normality of each dataset. To compare the group means, the normally distributed data were subjected to two way-ANOVA. The ANOVA tests checked to see if the variance was significant overall, but they did not specify where the significant differences were. Following this, a Tukey post hoc multiple comparison test was used to identify significant differences in mean values. The mean and standard deviation were used to express all of the results (S.D.). The significance level was set at values less than 0.05 for all p values.

3. Results and discussion

3.1. Effects of the NMs on tomato seed germination

The germination rate of tomato seeds was recorded after the emergence of the radicle from the seeds placed in soil-rite, and irrigated with DI water or NM treatment solutions (12.5, 100 and 1000 μg mL^{-1}) from all the experimental pots and has been shown in Figure 1. The effects on seed germination were found to be different among the various soil types and the NM concentrations applied. The germination remained unaffected in neutral soil (Figure 1a.), where all the seeds germinated after treatment with NMs. For acidic soil (Figure 1b.) the overall germination percentages increased by a factor of 1.1 on adding NMs as compared to the untreated control (90 ± 16.73). For basic soil (Figure 1c.), the overall germination percentages were comparable to untreated control. Overall the NMs were more efficient than bulk controls and did not show significant inhibition on germination as compared to the bulk sources under different soil conditions and doses. The decrease in germination by the bulk control may occur due to two possibilities: (i) restricted P in the soil and (ii) release of other potential toxicants like free ammonia from DAP [48] and heavy metal ions from RP [49]. This may potentially interfere

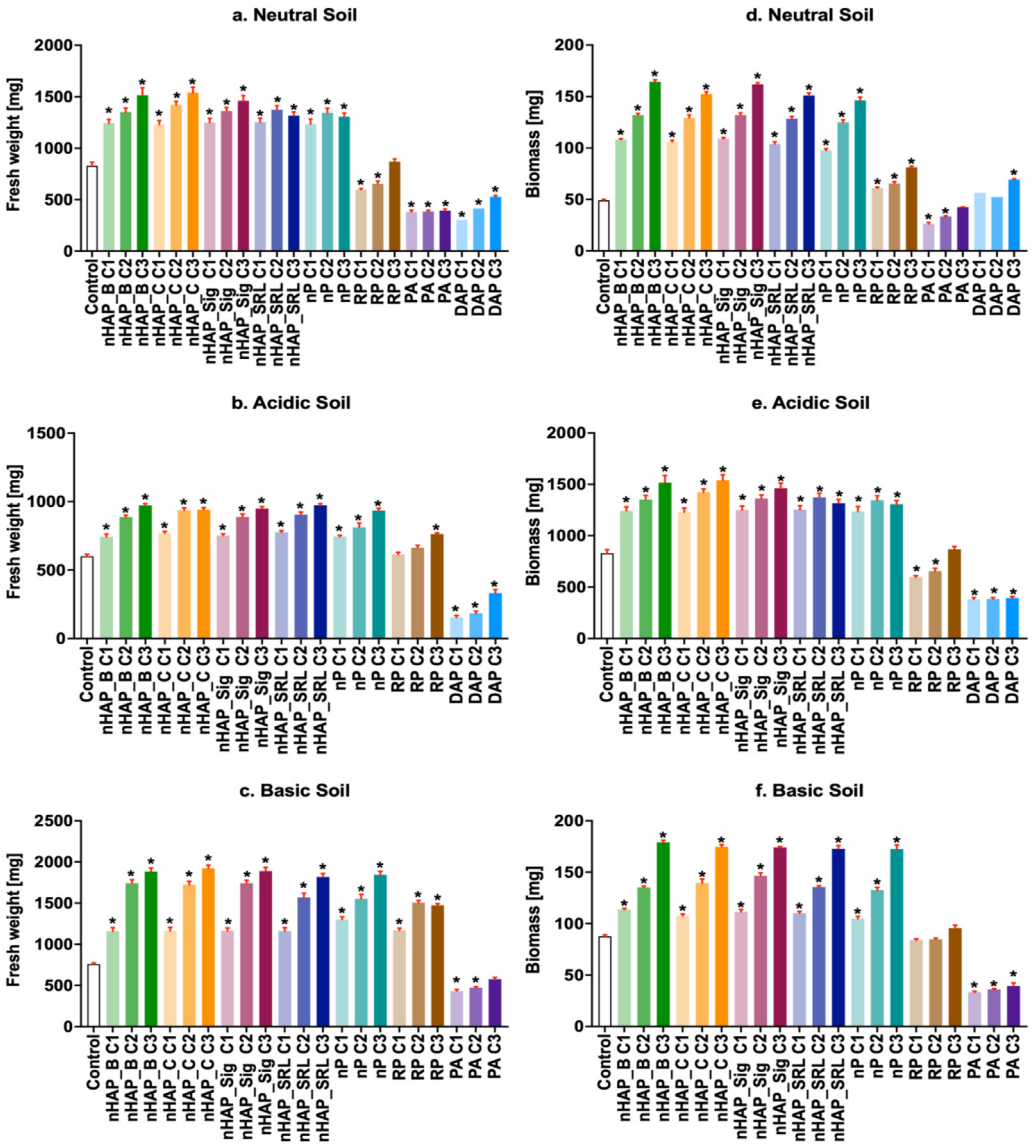

Figure 3. Fresh weights and biomass of seedlings grown in neutral (**a** and **d**), acidic (**b** and **e**) and basic (**c** and **f**) soils treated with 12.5, 100 and 1000 μg mL^{-1} concentrations (increasing colour gradient) of nHAP_B, nHAP_C, nHAP_Sigma, nHAP_SRL, nP, RP, PA and DAP. The values are expressed as mean and S.D. (* denotes significance at $p < 0.05$ as compared to untreated control). No. of experimental replicates = 5. No of independent replicates = 3 per harvest point.

with the seed germination process by reducing tissue respiration rate in soil [48] and by decreasing the activities of enzymes related to germination such as α-amylase and protease [50]. The process of seed germination can be considered as a multistep starting with the enhanced water uptake by the quiescent dry seed and ending with the elongation of the embryonic axis [51, 52]. The addition of P-based NMs while planting the seeds might promote the water uptake, cell division, elongation of embryonic axis or other intermediate processes for enhanced germination as these NMs can act as a source for P and Ca both required as nutrients by plants. Seed germination provides an outlook for further plant development, improvement and good economic yield. Several previous studies have reported similar enhanced germination effects of NMs on tomato seeds [53, 54, 55, 56, 57, 58]. In one study, the germination was enhanced only up to 20 ppm of treatment with Cu_2O NMs in a hydroponic system and any concentration above 20 ppm up to 160 ppm decreased the germination rate [53]. Similarly, in a study with ZnO NMs, the seed germination increased from 0-100 $mg.L^{-1}$ of applied concentrations of ZnO NMs and decreased in a dose-dependent manner from 200-1600 $mg.L^{-1}$ in an artificial hydroponic system [56]. In another study with carbon nanofibers, the germination was enhanced under salt stress by seed priming with carbon nanofibers and placing the seeds on artificial growth substratum [54], whereas, in another study on carbon-based NMs, it was found that in a hydroponic system, seed germination was enhanced by multiwalled carbon nanotubes and no effects on germination were observed with fullerol NMs [55]. Similarly, 8 $mg.L^{-1}$ of nano-SiO_2 [57] and 10 $mg.L^{-1}$ of CeO_2 [58] have been shown to increase germination in the artificial substratum. Different from these studies, our study uniquely reports enhanced germination in soils with limiting P conditions due to acidic and basic pH. It has been reported previously that the use of nHAP in presence of nanoclinoptilolite has resulted in the slow release of P in calcareous soil having alkaline pH [59]. In other previous studies, it has also been reported that nHAP has the slower release of P in the case of an acidic environment [1, 26]. It is, therefore, suggested that by the use of P-based NMs, there is higher uptake and slow release of P. The representative images for germination events are shown in **ESI** (Figure 1).

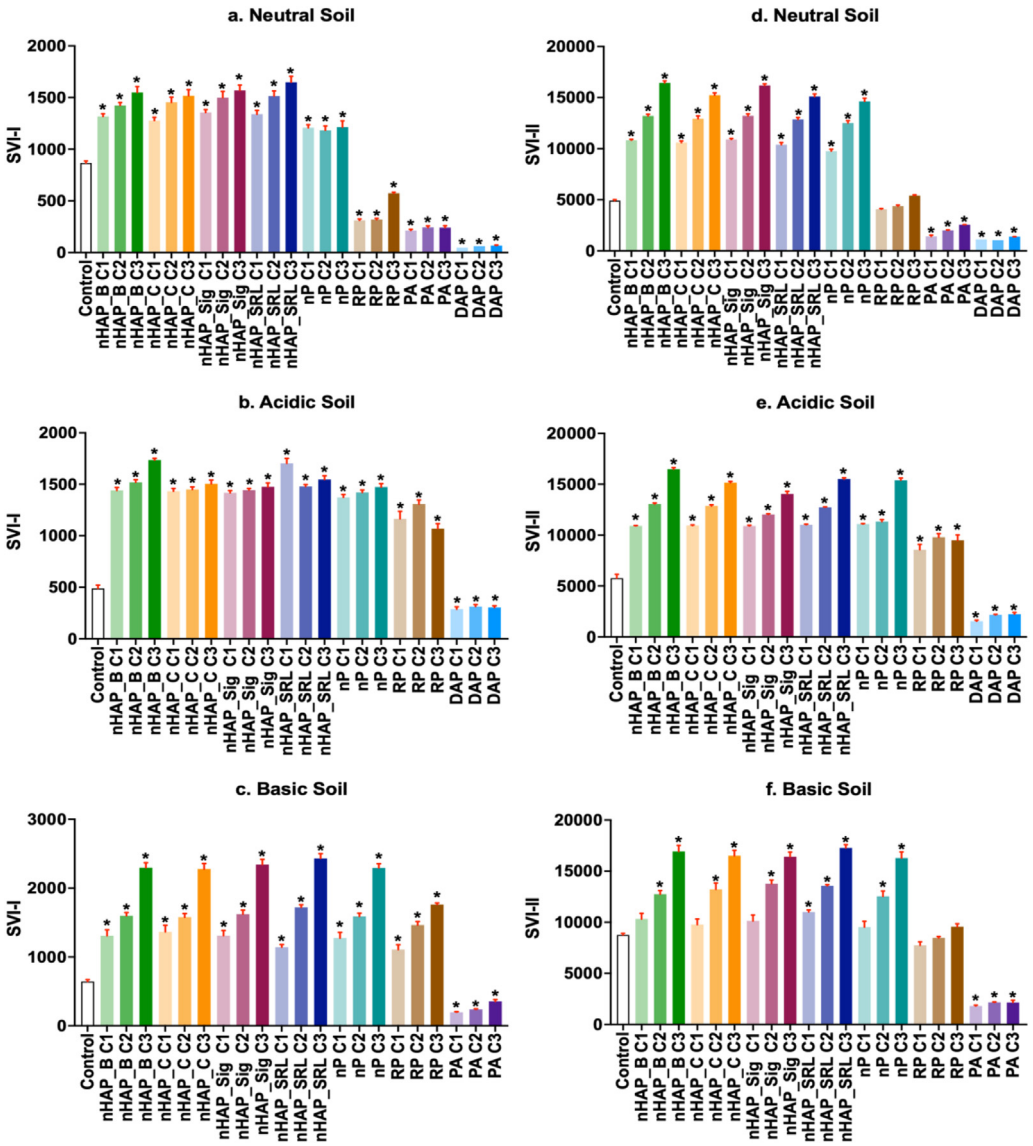

Figure 4. Seedling vigor indices (SVI-I and SVI-II) in neutral (**a** and **d**), acidic (**b** and **e**) and basic (**c** and **f**) soils treated with 12.5, 100 and 1000 μg mL^{-1} concentrations (increasing colour gradient) of nHAP_B, nHAP_C, nHAP_Sigma, nHAP_SRL, nP, RP, PA and DAP. The values are expressed as mean and S.D. (* denote significance at $p < 0.05$ as compared to untreated control). No. of experimental replicates = 5 per harvest point. No. of independent replicates = 3 per harvest point.

3.2. Effect of nHAPs and nP on seedling growth in terms of root and shoot development in tomato

A significant ($p < 0.0001$) increase in overall plant lengths was observed after the 30th day of growth after treatment with NMs in comparison to untreated controls as well as bulk sources (Figure 2). This was true for all soil types, neutral, acidic and basic soils. The representative images for growth events are shown in **ESI** (Figure 1). Interestingly, the lowest NM concentration was also observed to increase the shoot length at both the endpoints, 15th and 30th day, as compared to untreated plants and bulk controls. A prominent concentration-dependent effect on shoot length was observed in neutral soil, where for both the 15th (**ESI** Figure 2.) and 30th days, the shoot lengths increased with the applied concentration of NMs as compared to untreated and bulk controls. Similar to our observations, increases in plant length has been previously reported for tomato seedlings when treated with carboxymethylcellulose (CMC) stabilized nHAP [27], ZnO NMs [60], Cu_2O NMs [53], carbon NMs [55] and CeO_2 [58]. Additionally, nHAP has been reported to enhance the plant lengths under neutral pH conditions in carrot [61], chick-pea [62], faba bean [11] and sunflower [26].

3.3. Effect of NMs on fresh weight and biomass of tomato plants

Treatment of the plants with nHAP and nP resulted in an increase in fresh weight and biomass at both 15 and 30 day endpoints at all NM concentrations tested (12.5, 100 and 1000 μg mL^{-1}). In the case of neutral soil, the fresh weight of the plants increased by ~1.9–2.3 times after treatment with the lowest concentration (12.5 μg mL^{-1}) of NMs when compared to the control. In acidic soil, the fresh weights of tomato plants were ~1.1–1.3 times higher from the lowest NM concentration treatments compared to the control. Similarly, in basic soil, the fresh weights of tomato plants were ~1.7 times higher after exposure to the lowest NM doses when compared to the controls. The biomass of the tomato plants also showed similar trends to those observed in the fresh weights after treatment with nHAP and nP (Figure 3). For neutral soil, exposure of the tomato plant to the lowest dose of the NMs caused a

Table 1. P and Ca content (ppm) of *Solanum lycopersicum* plants harvested from neutral, acidic and basic soils treated with 12.5, 100 and 1000 μg mL^{-1} concentrations of nHAP_B, nHAP_C, nHAP_Sigma, nHAP_SRL and nP. The values are expressed as mean and S.D. (# denote significance at $p < 0.05$ as compared to untreated control. No. of experimental replicates = 2, no of independent replicates = 3 per harvest point. C1, C2 and C3 are the concentrations 12.5, 100 and 1000 μg mL^{-1} respectively.

Treatment	Neutral soil		Acidic soil		Basic soil	
	P-Content	Ca - Content	P-Content	Ca - Content	P-Content	Ca - Content
Control	32.94 ± 2.87	204.62 ± 8.36	12.67 ± 0.95	30.43 ± 3.30	22.02 ± 1.11	181.18 ± 3.44
nHAP_B C1	152.72 ± 6.36#	131.28 ± 1.87#	52.00 ± 5.54#	14.73 ± 0.66#	107.17 ± 0.93#	139.02 ± 1.92#
nHAP_B C2	168.35 ± 3.19#	206.10 ± 7.45	60.49 ± 3.36#	23.45 ± 2.61#	117.71 ± 2.71#	192.67 ± 2.73#
nHAP_B C3	183.28 ± 7.68#	258.30 ± 3.22#	83.12 ± 2.44#	55.60 ± 3.10#	124.70 ± 2.07#	264.03 ± 11.64#
nHAP_C C1	131.09 ± 3.14#	109.48 ± 5.33#	42.80 ± 2.12#	12.35 ± 1.45#	105.38 ± 3.87#	107.57 ± 5.01#
nHAP_C C2	150.68 ± 3.20#	149.22 ± 2.52#	49.55 ± 1.78#	15.55 ± 0.86#	111.26 ± 1.05#	151.35 ± 2.23#
nHAP_C C3	181.10 ± 3.08#	204.50 ± 5.00	81.11 ± 5.11#	46.35 ± 3.34#	123.44 ± 3.80#	191.37 ± 1.75#
nHAP_Sigma C1	146.64 ± 2.13#	141.53 ± 3.71#	47.02 ± 5.07#	15.58 ± 0.77#	124.16 ± 3.10#	140.85 ± 3.81#
nHAP_Sigma C2	171.05 ± 4.18#	203.20 ± 5.75	56.20 ± 1.35#	26.77 ± 2.01	129.61 ± 2.12#	211.48 ± 2.46#
nHAP_Sigma C3	204.08 ± 4.43#	259.22 ± 2.82#	87.53 ± 4.49#	53.05 ± 2.13#	136.49 ± 1.85#	266.12 ± 9.71#
nHAP_SRL C1	124.07 ± 4.09#	109.43 ± 5.29#	36.33 ± 3.01#	13.80 ± 1.70#	106.52 ± 5.31#	106.33 ± 5.35#
nHAP_SRL C2	142.08 ± 2.31#	149.37 ± 2.74#	42.23 ± 1.96#	15.93 ± 0.93#	108.40 ± 3.80#	150.40 ± 3.29#
nHAP_SRL C3	172.03 ± 3.70#	203.87 ± 7.80	78.85 ± 0.90#	44.63 ± 3.01#	116.30 ± 0.86#	191.30 ± 1.53
nP C1	45.34 ± 2.11#	261.32 ± 2.95#	12.53 ± 0.64	27.70 ± 1.94	40.26 ± 2.66#	260.67 ± 8.97#
nP C2	50.79 ± 1.98#	384.57 ± 6.56#	14.83 ± 0.64	44.52 ± 3.74#	38.24 ± 1.48#	386.90 ± 4.66#
nP C3	60.07 ± 6.18#	512.82 ± 2.99#	13.48 ± 1.55	113.67 ± 2.53#	42.58 ± 1.59#	485.48 ± 5.29#

~1.9–2.2 times increase in the biomass of plants when compared to the control. In acidic soil, the NMs caused the plant biomass to increase by ~1.5–1.6 times compared to the control. Interestingly, each of the NM exposures had the greatest effect on tomato plant biomass in the basic soil and caused a dose-dependent increase compared to the control. At the highest NM doses (1000 μg mL^{-1}) the tomato plant biomass in basic soil was ~2 times higher than the control plants. Similar trends were also observed at the 15th day end-point (**ESI** Figure 3.). With these results, it can be suggested that the increase in biomass is likely due to the increased P availability from the NM treatment. As discussed in the previous section 3.1, the potential increase in water uptake efficiency by the seeds due to NM treatment, may result in higher absorption of water and nutrients and correspond to an increase in translocation of vital components to the plants. This may be one of the factors contributing to the enhanced plant biomass. As reported by Yoon et al. (2019), another potential reason could be the possibility of enhanced photosynthesis on the application of NMs which would eventually contribute to higher biomass as compared to untreated plants [63]. In their study, it is reported that the zero-valent iron oxide nanoparticles increase the biomass in *Arabidopsis thaliana* via enhanced photosynthesis [63]. Similar to the results reported in the present study, the application of P-based NMs has been shown to increase plant biomass for maize [12], chickpea [62], soybean [25] and lettuce [16, 61].

3.4. Effect of nHAP and nP on seedling vigor indices of tomato

The root dependent SVI (referred to as SVI-I) and biomass dependent SVI (referred to as SVI-II) are presented in Figure 4. In the neutral soil conditions, the SVI-I and II were increased in tomato plants after 30 days of exposure to each of the NMs at all tested concentrations of 12.5, 100 and 1000 μg mL^{-1}. The bulk P sources have contributed to lower SVIs as compared to NMs. This could be due to their restricted availability from soil to seeds and can be related to their effects on germination. In acidic soil, exposure of the tomato plants to each of the NMs resulted in a ~2.3–3.7 increase in SVI-I compared to the untreated control plants. Similar trends and increases in SVI-I and II after NM exposure were also observed in tomato plants growing in basic soil. Observations on the 15th day were similar to the 30th day results (**ESI** Figure 4). These enhanced SVIs can be related to enhanced plant length and biomass. Similar to the results reported here, enhanced SVI-I were reported for bitter almond under saline conditions by the application of urea-hydroxyapatite nanohybrid as compared to only urea and ammonium sulphate [64]. Interestingly, besides the soil application, NMs have shown beneficial effects on SVI by a different approach of seed priming. As an example, very recently, chitosan-based NMs containing silica [65] and copper and salicylic acid [66] have been shown to enhance SVI in maize. It has been observed that the enhanced SVI is due to the increased production of α-amylase and protease [65]. The increased SVI due to the application of chitosan NMs has also been linked to stress tolerance of the plants [65, 67, 68] and thus, can also be related to the improved plant growth in P-limiting conditions of acidic and basic soils in this study after application of P-based NMs to tomato seeds via soil application.

3.5. P and Ca content in plants after treatment with NMs

The contribution of P and Ca from the NMs to plants is shown in Table 1. On the 30th day, the control plants in neutral soil had lower concentrations of P compared to the nHAP_B, nHAP_C, nHAP_Sigma, nHAP_SRL and nP. The P content was increased as compared to control in acidic and basic soil. There was a dose-dependent increase in P content in all soil types for the P-based NMs. The Ca concentrations also showed a dose-dependent increase for nHAPs and nP in all neutral, acidic and basic soil types. These increases were significant ($p < 0.05$) for nHAPs in all soil types and nP at all concentrations for neutral and basic soil. In the case of acidic soil the changes were significant ($p < 0.05$) for 100 and 1000 μg mL^{-1} for Ca content. A similar trend in fold changes was observed on the 15th day with respect to P and Ca contents (**ESI** Table 1). The increased P and Ca content can be attributed to enhanced uptake of P-based NMs in plants from the soil due to their small sizes as compared to the bulk P sources. Such increases in the P-content on the application of nHAPs has been previously shown under neutral pH conditions in wheat [1], sunflower [26], broccoli [15], tomato [27]. Besides nHAPs, increased P-content was also observed by the application of Nano-KH_2PO_4 in rice [69]. Xiong, et al. (2018) have attributed this increase in plant length in different soils (ultisols and vertisols) due to increased P-concentration in plants after application of nHAP [26]. Similar conclusions can be drawn from the present study as both plant lengths and growth, and P contents are increased on the application of P-based NMs in all soil conditions. The results reported in this study show that nanoforms of P-based NMs have better performance over bulk P sources in all the soil types suggesting that these P-based NMs can overcome the challenge of limited and fixed P in acidic or basic soil in terms of plant availability.

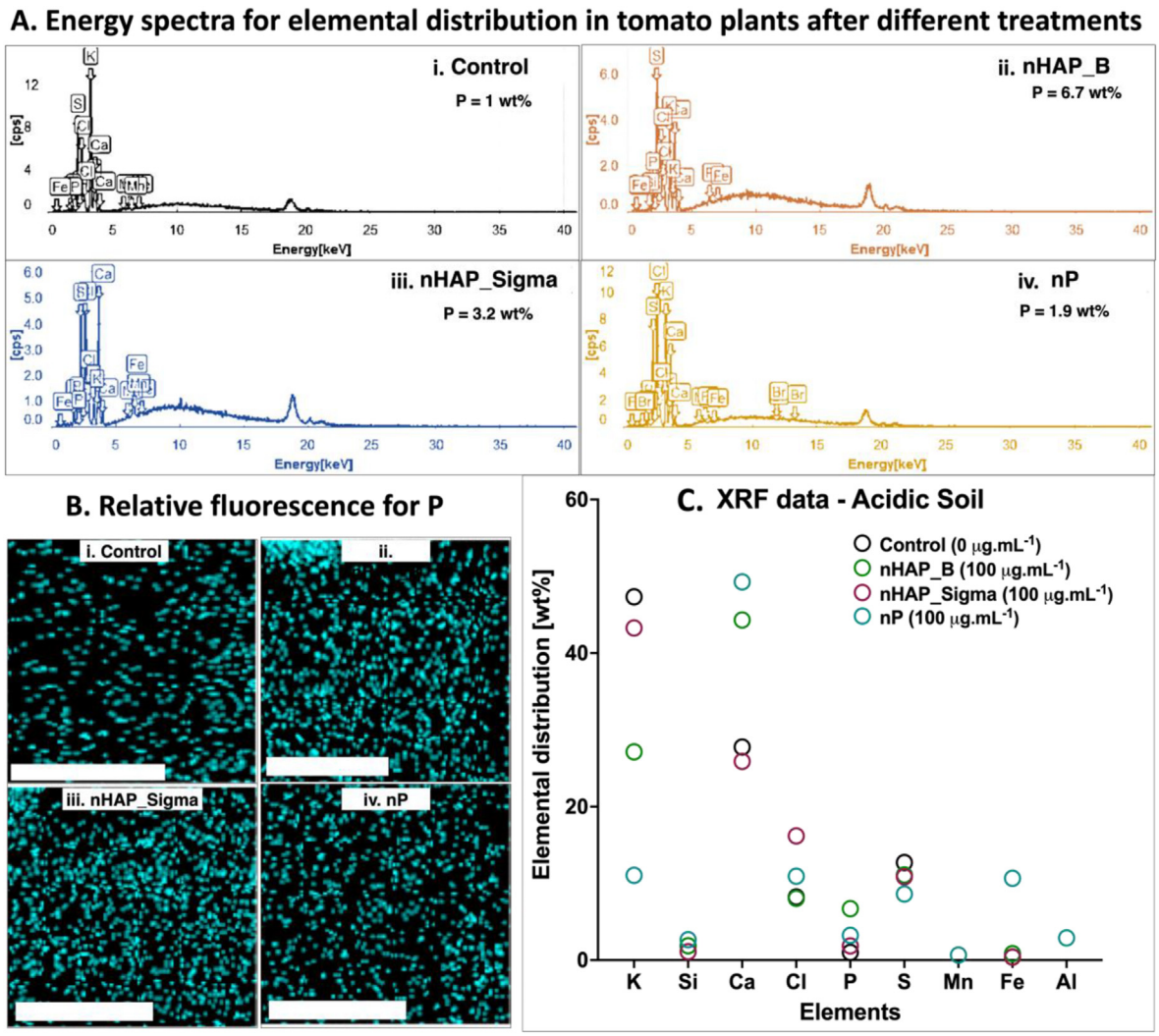

Figure 5. XRF analysis of plant samples under low calcareous conditions treated with 100 μg mL^{-1} of nHAP_B, nHAP_Sigma and nP. **A.** Shows the XRF energy spectra for different elements and amount of P (wt %) in the plants for i. untreated plants, ii. nHAP_B, iii. nHAP_Sigma and iv. nP. **B.** Shows relative fluorescence for P inside the plants for i. untreated plants, ii. nHAP_B, iii. nHAP_Sigma and iv. nP (scale: 0.5 mm). **C.** Shows the elemental distribution in the plants.

3.6. Plant nutrient status using X-ray fluorescence

Phosphorous uptake into the tomato plants was confirmed by XRF analysis (Figure 5.). The elemental distribution and P content (Figure 5A.) in the case of acidic soil were 1, 6.7, 1.9 and 3.2 % wt for the untreated control and plants treated with nHAP_B, nHAP_Sigma and nP respectively. The treatment with P-based NMs increased the P, Ca and K levels in the plants (Figure 5B.). The increased P in plants after treatment with P-based NMs is qualitatively represented in Figure 5C and demonstrates the highest P levels in plants exposed to nHAP_B. The highest Ca levels were observed in plants exposed to nP. These results support the data obtained for P and Ca contents using analytical techniques. A comparison between the fold changes in P content with reference to untreated control as determined by XRF and analytical techniques is shown in **ESI** Figure 5. Similarly, by using XRF, some previous studies have reported the increased Ti concentrations in cucumbers after exposure to TiO_2 NMs [70], increased Ce and Zn in corn exposed to CeO_2 and ZnO NMs [71], increased La levels in stems and leaves of *Pfaffia glomerate* (Spreng) after exposure to La_2O_3 NMs [72] and increased Si and P in *Deschampsia caespitosa* (L.) Beauv. following exposure to Si and P NMs [44]. The enhanced concentration and elemental distribution of P suggest the possible uptake of the P-based NMs by the tomato plants.

3.7. Effect of treatment with nHAPs and nP on stress indicators

Total H_2O_2, phenolics and flavonoids in fresh plant extracts after 30 days post-treatment with lowest (C1 = 12.5 μg mL^{-1}) and highest (C3 = 1000 μg mL^{-1}) doses of nHAPs and nP are presented in Figure 6. As compared to the untreated control, none of the NM treatments increased the H_2O_2 content in the plant extracts (Figure 6a.). The total phenolics (Figure 6b.) and flavonoids (Figure 6c.) contents were also comparable to the untreated control. This indicates that the plants do not experience stress when NMs are applied. There have been no significant reports on stress indicators in plants exposed to P-based NMs using biochemical attributes. No enhancement in H_2O_2 content indicates that there is no oxidative stress due to the exposure to nHAPs and nP. Phenolics and flavonoids are plant secondary metabolites that have radical scavenging activities against reactive oxygen species [73]. Their increase indicated potential stress in the plants. Unlike the study reported here, exposure of basil to green synthesized zinc and copper has resulted in increased phenolics and flavonoids levels [74]. In another study, exposure to copper sulphate nanoparticles was shown to increase the phenolics and flavonoids content in *Verbena bipinnatifida* Nutt [75] and silver NMs have shown increased oxidative stress and increase in phenolics and flavonoids levels in potato [76]. The data in this study for total H_2O_2,

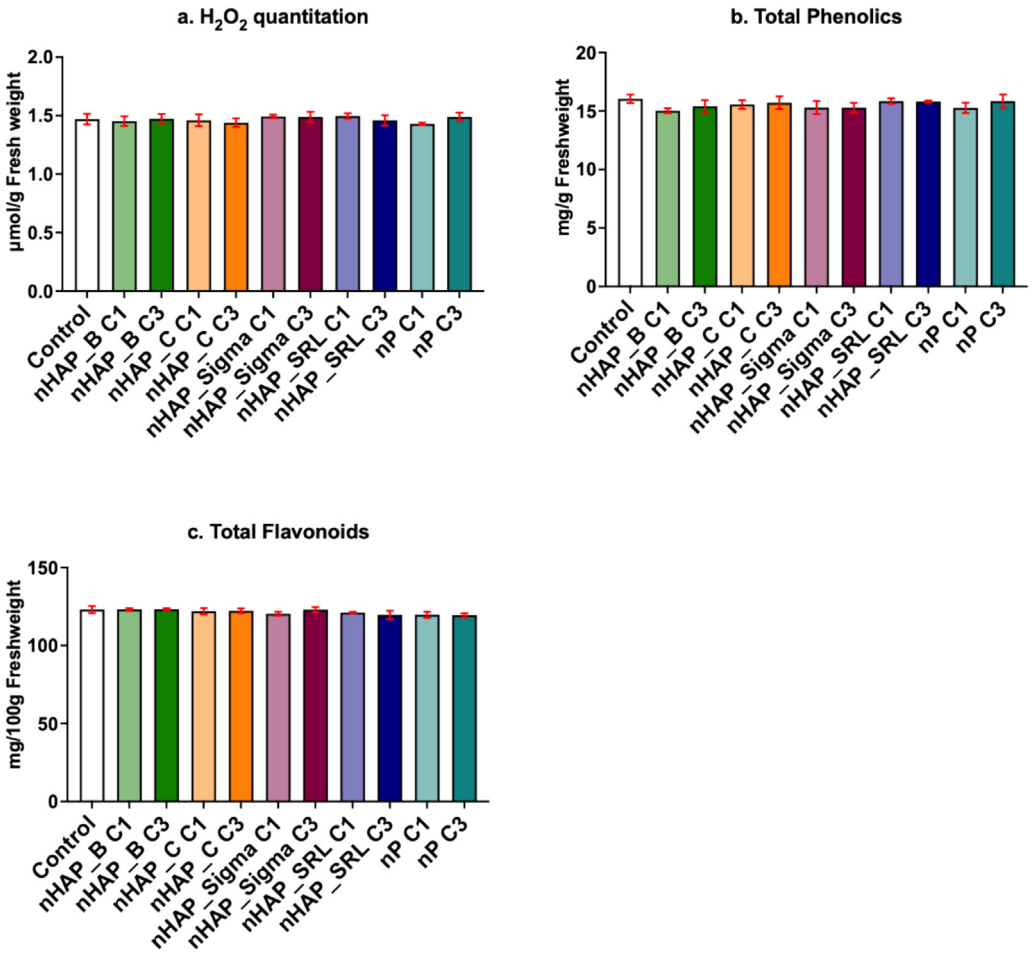

Figure 6. Estimation of total a. H_2O_2, b. phenolics and c. flavonoids in fresh plant extracts after 30 days post treatment with lowest (C1 = 12.5 µg mL^{-1}) and highest (C3 = 1000 µg mL^{-1}) doses of nHAP_B, nHAP_C, nHAP_Sigma, nHAP_SRL and nP.

phenolics and flavonoids contents support the plant growth data obtained in previous sections.

4. Conclusions and future research recommendations

This study investigated the fertilizing effects of various types of P-based NMs differing in shape, size and synthesis route using OECD TG 208 on *Solanum lycopersicum* (Pusa Rohini, Indian tomato). Soil application of nHAPs and nP were used as phosphorus nanonutrients. Our results have indicated that treatment with P-based NMs under acidic and basic soil conditions benefit the overall germination, biomass, and plant length in a growth room pot experiment. Increased P content from AAS and XRF analysis confirms P uptake to the plants. This suggested that due to the smaller size and therefore, higher surface area to volume ratio, P-based NMs may have higher uptake in the plants resulting in improved fertilizing efficacy at the same doses compared to the treatment with bulk P sources. The results also suggest that P-based NMs do not induce any stress to the plants. These results support that biogenic P-based NMs can serve as an "environmentally friendly" alternative to existing P-based NMs with chemical origins. The results from this study have, therefore, highlighted germination and growth promotion benefits of using P-based NMs as fertilizers in a variety of soil types that possess variations in pH and would address the challenges experienced using conventional bulk P-fertilizers that have low availability in acidic and basic soil. Further analysis of the uptake, translocation, tissue distribution, metabolism and transformation of these types of biogenic P- based NMs in plants is required in the future. The positive results from this study also highlight the importance of testing these P-based NMs in real-time field conditions with varied crop types and temperature zones to further validate the germination and growth promotion effects of the NMs.

Declarations

Author contribution statement

Ayushi Priyam: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Natasha Yadav: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Pallavolu M. Reddy: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Luis O.B. Afonso: Analyzed and interpreted the data.

Aaron G. Schultz: Analyzed and interpreted the data; Wrote the paper.

Pushplata Prasad Singh: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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