Biodegradable polymer nanocomposites provide effective delivery and reduce phosphorus loss during plant growth

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Abstract

Although phosphorus (P) is an essential element, its availability to plants is often limited, and P-containing fertilizers must be supplemented to stimulate plant performance. However, the inefficiencies and negative environmental impacts associated with nutrient delivery strategies have motivated interest in nanotechnology-enabled agriculture. Here, we synthesized biodegradable polymer nanocomposites (PNCs) containing polyhydroxyalkanoate (PHA) and calcium phosphate nanoparticles (Ca-P-NPs) and assessed their efficacy as a P-delivery vehicle, compared to a conventional P source ($CaHPO_4$), using tomato as a test plant. The effectiveness of PHA-Ca-P PNCs was assessed by measuring plant biomass, fruit yield and quality, tissue elemental and chlorophyll content, and enzymatic biomarkers. Phosphorus in the leachate was used as a surrogate for runoff. PHA-Ca-P PNCs biodegraded as a result of microbial activity in the soil, controlling the release of P during the initial stages of plant growth. PHA-Ca-P PNCs supported plant performance comparably to the conventional P source, while significantly reducing the P loss from the soil by over 80%. Given the negative consequences of eutrophication driven by P-rich agricultural runoff, this significant reduction in lost P has broad implications. Our studies also highlight the need to improve the efficiency of P uptake by plants from exogenous P sources. Collectively, these findings demonstrate the significant potential of biodegradable PNCs as a nutrient delivery platform to simultaneously enhance crop productivity and reduce the negative impacts of agriculture on the environment.

Introduction

As climate change threatens the availability of clean water and arable land, global crop production will need to increase by 70% over the next three decades to feed the growing population.[1,2] Currently, of the millions of tons of agrochemicals applied each year, only 10-20% of applied nutrients and pesticides are used by plants.[3] Sustainably providing adequate levels of nitrogen, phosphorus, and potassium (NPK) remains a primary challenge.[2,4-6] P is required for energy metabolism, phospholipid membranes and nucleic acids, photosynthesis, and other biological processes.[7,8] However, dependent on soil physical and chemical conditions, long-term P unavailability often occurs in agricultural systems due to the depletion of P from runoff, leaching, or formation of insoluble phosphates with other soil components.[8, 10-14] Under either condition, the resultant P unavailability affects plant production.[9,10] Consequently, increasing amounts[11] of inorganic P,[12] considered a nonrenewable resource,[12-15] are added to soils as fertilizer.[7] By 2050, global P use is projected at 26,000 Gg/year,[16] but as noted above, delivery efficiency is low.[17,18]

An important factor confounding efficient P delivery is its tendency to be lost from soil as runoff through rain and irrigation events,[19] leading to P accumulation in surface waters, and contributing to eutrophication.[18] Eutrophication is the most common impairment of surface waters in the United States, resulting in excessive algae and aquatic plant growth, and loss of biodiversity.[20,21] Algal blooms often lead to “dead zones,” which are hypoxic areas in surface waters unable to support life.[11,20] These algal blooms pose hazards to environmental and human health, and lead to significant losses of revenue from aquaculture, fisheries, and recreational activities.[7,18] The development of novel and sustainable P delivery platforms that reduce runoff is therefore critical for sustainably enhancing crop yield while simultaneously mitigating negative environmental impacts.

One method for improving P delivery is through nano-enabled agriculture.[1,22-25] Taşkın et al.[26] demonstrated that fertilizing lettuce plants with nanoscale hydroxyapatite resulted in equivalent biomass but greater plant P content, compared to highly soluble H_3PO_4. Lui and Lal[27] reported that nanoscale hydroxyapatite increased the growth and yield of soybean, compared to triple super phosphate ($Ca(H_2PO_4)_2$). Similarly, Mikhak et al.[28] showed that nanoscale zeolite saturated with nanohydroxyapatite significantly increased biomass, tissue P content, and essential oil content from German chamomile (*Matricaria chamomilla* L.) when compared to rock phosphate and triple super phosphate. Although promising, nanoscale P use is limited by the challenges of effectively applying and distributing nanoscale nutrients.[4,29]

The use of biodegradable polymers is an emerging strategy for achieving tunable release and delivery of agrochemicals.[30-32] Biodegradable polymers can be used as coatings for dry or pelleted fertilizers,[33-35] modulating the dissolution of encapsulated nutrients as a function of polymer porosity, soil temperature, and moisture.[30,36] Varying the polymer type provides opportunity for greater temporal control over release to achieve crop-specific nutritional needs. A less common approach is to incorporate nutrients directly into a biodegradable polymer to create a polymer-fertilizer composite.[35] This method relies on polymer biodegradation by soil microbes or biophysical-edaphic factors to release the fertilizer cargo.[37-40] Volova et al.[37] found that urea embedded in a biodegradable polymer provided controlled N release to lettuce, leading to reduced nutrient runoff and greater plant biomass. The ultimate utility of these materials depends on how well they support plant performance; however, there are few available plant-based studies and those that have been published are on short time scales.[37] Many studies only examine nutrient release in well-controlled laboratory settings (e.g. water), without probing the more complex and dynamic interplay that will exist in true agricultural settings, including the effects of soil factors and microbial activity.[29,30,40-43] Moreover, many studies rely on complex and costly polymer synthesis procedures that are not amenable to scaling and commercialization.[39,44]

In this paper, we developed and evaluated a tunable biodegradable polymer nanocomposite (PNC) platform containing calcium phosphate nanoparticles (Ca-P-NPs) and polyhydroxyalkanoate (PHA), a highly biodegradable microbially-synthesized polymer.[45,46] The performance of tomato plants receiving PNCs as a P source was compared to a conventional P source ($CaHPO_4$) through measures of biomass, yield, element content, and various enzyme activity assays. Leached P from the pots was used as a surrogate to assess P loss from soil under planted conditions. The goal of this study was to evaluate the ability of PNCs to support plant performance while mitigating the environmental damage resulting from P losses in agricultural runoff.

Materials and Methods

Preparation of polymer nanocomposites containing calcium phosphate nanoparticles. Biodegradable PNCs containing calcium pyrophosphate nanoparticles (Ca-P-NPs) were synthesized from PHA and amorphous nanoscale $Ca_2P_2O_7 \cdot H_2O$ (< 150 nm) using solution blending. A schematic of the solution blending process and representative photographs of PNCs can be found in Figure S1. For these proof of concept experiments, solution blending was chosen as a simple route to prepare polymer nanocomposites, one which does not require the use of more complex and expensive melt-mix extruders. PHA was purchased from Metabolix (Woburn, MA) and Goodfellow (Coraopolis, PA); Ca-P-NPs and chloroform were purchased from Sigma (St. Louis, MO) and used without further purification. To create PHA-Ca-P PNCs, chloroform was used as a volatile co-solvent. The appropriate quantity of Ca-P-NPs, either 375, 750, or 1500 mg, corresponding to 5%, 10%, and 20% weight of the polymer, respectively, were added to 200 mL chloroform in an Erlenmeyer flask. The flask was tightly sealed and sonicated using a Branson 1510 ultrasonicator bath (Danbury, CT) at 70 watts for 1 h, after which 7500 mg PHA was added and sonicated for an additional 1 h. The PHA-Ca-P suspension was poured into a glass petri dish and left to dry overnight in a fume hood to form a thin film of PHA-Ca-P PNC. In two of the three tomato experiments, PNCs were vacuum annealed by drying in a Thermo Scientific Lab-Line vacuum oven (Waltham, MA) at 80 °C and -13 psi for 3 days; the rationale for this annealing can be found below. Finally, PNCs were milled into sub-millimeter diameter particles by first freezing in liquid nitrogen and then grinding with a Waring 700S commercial blender (Conair Corporation, Stamford, CT). Pure PHA without Ca-P-NPs, for use as a control, was prepared and ground to sub-millimeter particles in the same way as PHA-Ca-P PNCs.

Characterization. PNCs were characterized using a combination of thermogravimetric analysis (TGA) and inductively coupled plasma optical emission spectroscopy (ICP-OES) to determine the wt% Ca-P-NPs, attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) to assess the chemical bonding present in the composites, and scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (EDS) to probe chemical composition, particularly the presence of residual Cl; complete characterization details can be found in the SI.

Plant growth and leachate analysis. Three separate greenhouse plant growth experiments were conducted using PHA-Ca-P PNCs, pure PHA, or $CaHPO_4$ (model conventional P fertilizer; Sigma) as soil amendments; see individual experiment descriptions for lists of treatments. In each experiment, topsoil (Agway Inc., West Springfield, MA) was mixed 1:1 with silica sand to promote drainage (1.45 bulk density (w/v)). The pH of the soil was 6.8. Because the soil contained initial high levels of available P (> 100 mg/kg soil) as determined through a Morgan test,[47] it was leached daily for 10 days to reduce available P levels to approximately 40 mg/kg. Air-dried soil (2.9 kg for Experiments 1 and 2; 20 kg for Experiment 3) was amended by rotary mixing for 5 min and dispensed into five 10-cm plastic pots for Experiments 1 and 2 (0.58 kg soil/pot, 350 mL/pot) and 7.57 L plastic pots for Experiment 3 (4 kg soil/pot). One 5-week-old tomato plant (cv. Bonnie Best, Totally Tomatoes, Randolph, WI for Experiments 1 and 2; cv. Red Deuce, Harris Seed Co., Rochester, NY for Experiment 3) was transplanted into each of 30 pots (6 treatments, 5 replicates per treatment). Plants were arranged in a randomized block design under high intensity sodium vapor lights at 12 h photoperiods with 25 °C day and 18 °C night. Plants were fertilized one week after planting with a modified Hoagland's solution[42] without P salts (2.0 mM $MgSO_4•7H_2O$; 10.0 mM $Ca(NO_3)_2•4H_2O$; 10.0 mM KNO_3; 2.0 ml of Hoagland's microelements stock solution; 3.0 mM Fe EDTA).[42] Plants in Experiment 1 were fertilized with the no-phosphorus modified Hoagland's solution at a rate of 50 mL/pot only once, plants in Experiment 2 were fertilized with 50 mL/pot at one and two weeks after transplanting, and plants in Experiment 3 were fertilized with 100 mL/pot at one and three weeks after transplanting. At the conclusion of each study, plants were harvested, washed in tap water to remove soil, and fresh and dry biomass were recorded. Soil samples were subjected to acid digestion for ICP-OES analysis.

In each plant experiment, leachate was collected as a surrogate for runoff. For leachate collection, pots were individually set on greenhouse benches in 14.3 x 14.3 cm plastic weigh boats (Fisher Scientific, Inc., Waltham, MA). Pots were irrigated with 100 mL of deionized H_2O and when needed, an extra 50 mL of deionized water was added to produce leachates for collection. The leachates were collected within 10 min, diluted 1:1 in 6% HNO_3, and analyzed by ICP-OES for P and other elements. On days that leachates were not collected, pots that required water were irrigated with sufficient volume (60-80 mL) to moisten soil but not allow leaching.

Three tomato plant experiments were designed to answer different questions and as such, were run with different treatments, time periods, and leachate collection schedules.

<u>Experiment 1.</u> Here, PNCs were not vacuum annealed during synthesis. The six experimental groups were: untreated control, pure PHA (2 g/pot), PHA-Ca-P PNCs with 10% wt Ca-P-NPs (PHA-10%Ca-P, 2 g/pot), Ca-P-NPs corresponding to the amount used in PHA-Ca-P PNCs, and an amount of $CaHPO_4$ corresponding to the P concentration of the PHA-Ca-P PNC treatment; 125 mg P/kg soil. Leachates were collected as described above on the first day only. The experiment was terminated after 8 days due to overt phytotoxicity in experimental groups containing PNCs (Figure S2). To investigate the toxicity, 90 g sterile peat-based potting mix (Jiffy Natural and Organic Seed Starting Mix, Norton, MA) was mixed with 3 g of PHA. There were five replicates for each of the three treatments involved: vacuum annealed PHA, air-dried PHA, and unamended control. The soil was planted with fast-growing lettuce seeds (cv. Black Seeded Simpson, W. Atlee Burpee, Warminster, PA), and placed under two 32 W fluorescent lights and watered as needed. Growth was assessed visually (Figure S3).

<u>Experiment 2.</u> In Experiments 2 and 3, PNCs were vacuum annealed after synthesis to remove residual solvent (chloroform; further explanation below). The six experimental groups were: untreated control, pure PHA (2 g/pot), PHA with 10% wt Ca-P-NPs (PHA-10%Ca-P, 2 g/pot), PHA with 20% wt Ca-P-NPs (PHA-20%Ca-P, 2 g/pot), and two treatments using $CaHPO_4$ in amounts providing P concentrations equivalent to the PHA-Ca-P treatments; 125 and 250 mg P/kg soil, corresponding to the 10% and 20% wt Ca-P-NPs loadings, respectively. The experimental design and greenhouse conditions were identical to Experiment 1. Pots were irrigated and leachate

was collected using the procedure described above, on days 1, 3, 5, 8, 12, and 17. Plants were harvested after 17 d of growth (Figure 1).

Experiment 3. PHA-Ca-P PNC performance was monitored over the full tomato life cycle to assess marketable yield and fruit quality. The same growth conditions were used as above, except that pot size (7.57 L) and soil quantity (4.0 kg soil/pot) were increased. The six experimental treatments were: untreated control, two pure PHA samples with the same amounts of PHA used in the PHA-Ca-P composites with 5% and 10% wt Ca-P loadings (abbreviated as $PHA^{5\%}$ and $PHA^{10\%}$, with 76 and 36 g PHA per pot, respectively), $CaHPO_4$ (4 g/pot), PHA-Ca-P with 5% wt Ca-P-NPs (PHA-5%Ca-P, 80 g/pot), and PHA-Ca-P with 10% wt Ca-P-NPs (PHA-10%Ca-P, 40 g/pot). The amount of P in the $CaHPO_4$ and PHA-Ca-P PNC treatments was equivalent (0.91 g/pot). Pots were irrigated and leachate was collected using the procedure described above, on days 1, 3, 5, 7, 10, 14, and 21, then weekly until harvest at day 155. At day 91, fruit had fully developed in at least one plant from each treatment and the experiment was terminated at 155 days.

At harvest, the following end points were determined: biomass, elemental composition and total nonstructural carbohydrate (starch, sugars) content of plant tissues (fruit, shoot, root), chlorophyll content of leaves, total protein and enzyme activity (catalase) of roots and leaves, and elemental content of the plant tissues and soil. Methodological details can be found in the SI.

Soil-only biodegradation experiments. Thin films of pure PHA and PHA-Ca-P PNCs containing 20 wt% Ca-P-NPs were prepared for soil-only biodegradation experiments in the same way as above, with 5 mL aliquots of suspension poured into 4.25 cm diameter aluminum weighing dishes for solvent evaporation overnight, followed by 3 days of drying in a vacuum oven. These circular thin films were placed in leached Agway topsoil mixed with sand (see above) that was either autoclaved for 1 h at 121 °C or not autoclaved. Soil was watered as needed with deionized water to maintain moist but not saturated conditions. Thin films were removed from soil each week, rinsed, air-dried overnight, and photographed, after which they were returned to soil.

Statistical Analysis. Statistical differences between treatments were determined by a one-way analysis of variance (ANOVA) with a Student Newman Keuls multiple comparison test. In Experiment 3, the three control treatments (unamended control, $PHA^{5\%}$, $PHA^{10\%}$) were statistically equivalent, and as such, were combined and compared against the P-amended treatments using a Tukey test, Fisher LSD, or Dunn's methods. Differences were considered statistically significant when $p < 0.05$.

Results and Discussion

PHA-Ca-P PNC characterization. ICP-OES results of the Ca-P-NPs wt% in composites nominally containing 5 and 10 wt% Ca-P-NPs were 5.8% and 8.2%, respectively (Table S1). The TGA data (Figure S4) show a large mass loss just below 300 °C attributed to PHA combustion,[48] while the residual mass at 500 °C can be ascribed to inorganic Ca-P NPs.[49] The wt% Ca-P-NPs in the nominal PHA-10%Ca-P PNC was 9% as determined by TGA, comparable to the ICP-OES findings. ATR-FTIR (Figure S5) of pure PHA as compared to PHA-Ca-P PNCs indicate that incorporation of Ca-P-NPs did not change the chemical bonding in PHA. Characteristic absorption bands for PHA[50] in the C-O-C and C-C stretching and CH deformation mode regions (1300-1000 cm^{-1}) overlap with P-O and P=O stretches (1300-1100 cm^{-1}) characteristic of Ca-P (Figure S5).[41,51,52] This overlap and the low concentration of Ca-P-NPs prevent spectral identification of their inclusion in the PNCs.

Plant growth studies

Experiment 1. By day 7, all plants grown in soil amended with PHA were unhealthy and stunted (Figure S2). This deleterious effect from the PHA obscured any potential benefit from the PHA-Ca-P PNCs. Since PHA is widely considered to be a nontoxic biopolymer,[45,53,54] we hypothesized that trace quantities of chloroform used in PNC synthesis remained and caused the toxicity. EDS analysis revealed small quantities (≈ 0.1 %) of chlorine in the air-dried composites (Figure S6). To confirm that residual chloroform was responsible for the stunted growth, fast-growing lettuce seeds were planted in soils amended with PHA generated by solution blending that was either vacuum annealed or dried overnight in a laboratory hood. Lettuce plants in soil amended with vacuum-annealed PHA grew significantly larger than those with air-dried PHA (Figure S3), indicating that residual chloroform was responsible for the phytotoxicity.[55] Chlorine was not detected by EDS in vacuum-annealed composites. As such, all subsequently used PNCs were vacuum annealed for at least 3 days. These results underscore the need for caution and in particular the importance of fully characterizing PNCs made by solution blending and intended for plant growth studies so as to avoid confounding effects from even trace quantities of halogenated organic solvents, like chloroform, that are ubiquitous in synthesis protocols.[56-58] Furthermore, although we were able to remove all traces of residual chloroform through vacuum annealing PNCs, there are nontoxic solvents[59-61] that can be used in solution blending that would be preferable for any commercial-scale synthesis of PNCs for use in sustainable agriculture; exploration of this modification is currently underway.

Experiment 2. Plants with no added P showed severe stunting (Figure 1) and leaves exhibited typical reddish-purple coloration consistent with P deficiency after 17 days of growth (Figure S7); thus, the study was terminated. In contrast, plants grown in soils containing $CaHPO_4$ or PHA-Ca-P PNCs were healthy and visually similar to one another. The average dry mass of shoots and roots from plants treated with PHA-10%Ca-P was 0.80 and 0.22 g, respectively, whereas plants treated with $CaHPO_4$ had average dry shoot and root masses of 1.15 and 0.29 g, respectively (Figure S8). In comparison, the dry weight of the shoots and roots from PHA control treatments was 0.08 and 0.05 g, respectively.

The concentration of P leached from the soils over 17 days is shown in Figure 1. In soils amended with $CaHPO_4$ salts equivalent to 10 and 20 wt% Ca-P-NPs, the P concentration in the leachate was 4 and 1.6 mg/L at day 1, respectively, while leachates from all of the other soils contained < 0.3 mg/L P. In all soils, the amount of leached P decreased over time. However, over the course of the 17-day experiment, soils amended with $CaHPO_4$ leached nearly an order of magnitude more P than those amended with PHA-Ca-P PNCs. Notably, soils amended with PHA-Ca-P PNCs leached only slightly more P as compared to the background levels of P leached from the PHA or unamended controls.

The significantly reduced P loss observed from the PHA-Ca-P PNCs as compared to the $CaHPO_4$ treatments can be ascribed to the slow release of P as the PHA undergoes microbial biodegradation. This is seen in Figure S9, which compares the biodegradation of pure PHA and PHA-20%Ca-P added to autoclaved (left hand side of each panel) and non-autoclaved soil (right hand side of each panel) after one week. PHA and PHA-Ca-P PNCs exposed to autoclaved soil showed no visible or overt signs of biodegradation, while PHA and PHA-Ca-P PNCs exposed to soil with an active microbial population were significantly fragmented and decreased in size as a consequence of biodegradation after one week. As PHA biodegradation proceeds, embedded Ca-P-NPs will become exposed and subsequently released from the polymer matrix, rendering P available for plant uptake.[37,62] The rate of PHA biodegradation is determined by soil microbial activity and the chemical and physical properties of PHA.[63-65] Microbial biodegradation of PHA into water-soluble organic acids proceeds via extracellular PHA depolymerases produced by a wide range of microorganisms in both aerobic (as in this study) and anaerobic environments.[66-68] In these studies, we used thin films to visualize the effects of biodegradation, since direct measurements of PHA biodegradation rates in our experiments were precluded by an inability to quantitatively recover the finely milled PHA-Ca-P PNCs powders (Figure S1) from the soil. The kinetics of the biodegradation process are illustrated in Figure 2 which shows photographs of how the size and structure of pure PHA or PHA-20%Ca-P thin films evolved over the course of 5 weeks of incubation time in non-autoclaved soil. Figure 2 reveals a systematic decrease in the size of both PHA and PHA-20%Ca-P, such that after the fifth week, both polymers had completely biodegraded and were not recoverable. This indicates that controlled P release from the Ca-P-NPs embedded in the PHA is likely to have occurred over the course of the first month or so of the tomato plant experiments. This is consistent with the literature which reveals that the timescale needed for complete PHA biodegradation in soils ranges from 1-12 months.[63-65]

Experiment 3

Plant growth effects. The dry weight of shoots and roots of plants across the different treatments after 155 days of tomato plant growth are shown in Figure 3. Root and shoot biomasses follow a similar trend. The greatest dry shoot masses were observed in plants receiving $CaHPO_4$ and PHA-10%Ca-P PNC; these were the only treatments significantly different from the control ($p < 0.05$).

$CaHPO_4$ and PHA-10%Ca-P PNC treatments also yielded the highest root masses, which were 72% and 84% greater than the control, respectively. Notably, the mean weight of shoots and roots in plants grown with $CaHPO_4$ and PHA-10%Ca-P PNC were approximately double those of the PHA and controls. The PHA-5%Ca-P treatment exhibited biomass values intermediate to those of the PHA-10%Ca-P and controls. Figure 3 demonstrates that PHA-10%Ca-P was equivalent to $CaHPO_4$ in supporting plant growth. PHA alone provided no enhancement of plant growth, with values being statistically equivalent to the unamended controls.

The P content of roots and shoots showed trends somewhat similar to the biomass data (Figure 3). There was a significant difference ($p < 0.05$) in shoot P content between treatments with added P, including $CaHPO_4$ (4.719 g/kg), PHA-10%Ca-P (3.420 g/kg), and PHA-5%Ca-P (1.933 g/kg), and those without, including $PHA^{5\%}$ (0.491 g/kg), $PHA^{10\%}$ (0.537 g/kg), and the control (0.566 g/kg). The concentration of P in both roots and shoots was highest in $CaHPO_4$, followed by PHA-10%Ca-P and PHA-5%Ca-P PNCs. There were no significant differences among treatments without added P (PHA, control). It should be noted that in previous reports,[27,69] plants receiving nanoscale hydroxyapatite had greater biomass compared to conventional fertilizers. One possibility is that nanoscale hydroxyapatite is more easily assimilated by plants than Ca-P-NPs, or that the higher water solubility[70,71] of the conventional fertilizer $Ca(H_2PO_4)_2$,[27,69] as compared to $CaHPO_4$, led to increased P runoff and a reduced fraction of plant-available P.

The total chlorophyll content of leaves from harvested plants was statistically equivalent across treatments (Figure S10), suggesting that changes in the P level resulting from the $CaHPO_4$ or the PHA-Ca-P PNC treatment did not impact photosynthetic activity in the plants. This finding is important, given that the photosynthetic competence of plants is a function of their total chlorophyll content.[72] P deficiency can compromise plant productivity by altering electron transport to photosystem I, resulting in reduced overall photosynthetic efficiency.[73] Total protein content (Figure S11) across the different treatments was measured, which is an indication of overall metabolism, including enzyme activity and protein synthesis, of which P is an integral constituent.[74] Interestingly, the PHA-Ca-P PNC treatments resulted in 2-3 times higher root total protein compared to $CaHPO_4$. This could potentially be a consequence of enhanced microbial activity due to PHA biodegradation.[10,37,75] Sawan et al.[76] observed significant increases in cotton seed protein as a result of higher P application rates, although this could cause increased P leaching into water bodies; notably, that phenomenon was avoided with the PNC platform. Lastly, tissue catalase levels were not impacted by treatment (Figure S12). Catalase converts H_2O_2 to H_2O and O_2 in response to biotic and abiotic stresses, which mitigates the effect of reactive oxygen species and other free radicals.[77] The lack of change in catalase activity suggests that free radical production was unaffected by the source of the P supply.

Fruit effects. Given the importance of nanoscale treatments to yield, a number of fruit-based parameters were evaluated. There were no statistically significant differences in the fruit number or fruit mass between controls and treatments with added P (Figure 4). It should be noted that the soil used in this study contained 239 mg/kg of total P, which provided sufficient available P to support minimal physiological processes like flowering and fruit set. In contrast to fruit number and weight, the fruit P content was significantly higher ($p < 0.05$) in plants grown with added P; fruit from the $CaHPO_4$, PHA-5%Ca-P, and PHA-10%Ca-P treatments had P contents that were 129%, 77%, and 91% greater than the untreated control, respectively (Figure 4). Fruit P content was approximately 20% higher in plants treated with $CaHPO_4$ than those treated with PHA-Ca-P composites.

No significant difference was observed in the fruit lycopene content across all treatments (Figure S13). Lycopene is a naturally occurring red pigment present in tomato fruits and is an antioxidant that scavenges free radicals and protects cells against oxidative damage. Since lycopene is synthesized in plants via the mevalonic acid and methylerythritol phosphate enzymatic pathways, the lack of change in lycopene content suggests that PNCs had no impact on these important biochemical pathways. In addition, the fruit total sugar content was measured (Figure S14); sugars are critical for determining the nutritional value of plant tissues.[78] As carbohydrates are the most abundant organic macromolecules in plants and require P for their production via photosynthesis, the photosynthesis rate has been correlated with the amount of sugars produced in plants.[79] Here, increased fruit sugar content was observed in plants exposed to PHA-5%Ca-P, although no effect was observed with PHA-10%Ca-P. The reason for the higher sugar content with the PHA-5%Ca-P treatment is unclear, but regardless, sugar content was clearly not negatively impacted by PNC treatments.

Soil Effects. The extent of P leached from soils over 155 days of plant growth was significantly greater for the $CaHPO_4$ treatment than the PHA-Ca-P PNCs. Figure 5 shows the cumulative P leached from soils as measured by concentration (a) and mass (b); these data represent an average of all pots within each treatment group. For both measures, the leachate trends are $CaHPO_4$ >> PHA-10%Ca-P > PHA-5%Ca-P ≈ PHA ≈ control. P was leached at an approximately constant rate during the initial 20 days, with decreasing quantities thereafter, in each treatment. As expected, the untreated control and the PHA treatments demonstrated similar leachate results. Over 155 days, these three treatments released between 2.7-3.4 mg P, only slightly less than the 4.4 mg P released by the PHA-5%Ca-P PNC, which itself was approximately half of the 9.0 mg released by the PHA-10%Ca-P PNC. In contrast, the $CaHPO_4$ released almost 50 mg P. Compared to the background levels of P released from the control and PHA, the $CaHPO_4$ released over 7 times more than P the PHA-10%CaP. Considered alongside plant and fruit data, this clearly demonstrates that these P-containing PNCs can support plant growth to an extent comparable to conventional P-containing compounds while dramatically decreasing the magnitude of P loss from soil. It should be noted that other conventional sources of P commonly used in agriculture (e.g. triple super phosphate (TSP) or diammonium phosphate (DAP)) are actually more water soluble than $CaHPO_4$,[70,71] presumably leading to even higher quantities of P loss than observed in this study.

Figure 5(c) shows post-harvest total soil P concentrations for each of the six treatments. Soil P concentrations were greatest in treatments with added P ($CaHPO_4$ and PHA-Ca-P PNCs); the final soil P concentrations in PHA-5%Ca-P, PHA-10%Ca-P, and $CaHPO_4$ were 143%, 91%, and 112% higher than the control, respectively (significant at $p < 0.05$). It is both notable and interesting that in spite of the significantly greater amounts of P lost from the soil in the $CaHPO_4$ treatment relative to the PHA-Ca-P PNCs, the total amount of P present in the soil is statistically equivalent across the three treatments. Since 0.91 g P was added to soils amended with the $CaHPO_4$ and PHA-Ca-P PNC treatments, it is evident that the overwhelming majority of added P is retained in the soil, underscoring the generally observed inefficiency of P uptake from inorganic sources of this nutrient. This assertion is supported by the data in Figure 5(d) which shows the mass of P in the plant (root and shoot) and the fruit calculated from the data in Figures 3 and 4, along with the data in Figure 5(b) on the mass of P leached. Analysis of Figures 5(b) and 5(d) reveals that although the efficiency of P uptake into the plant and fruit is approximately 60% higher for the $CaHPO_4$

treatment compared to the PHA-10%Ca-P PNC, the magnitude of P loss from the soil with $CaHPO_4$ is over 700% higher. In comparing the two PHA-Ca-P PNC treatments with the PHA controls, the increase in total P uptake into the plant and fruit is nearly double for the PHA-10%Ca-P versus the PHA-5%Ca-P. Since the total P present in both treatments is the same, this suggests that a PHA-15%Ca-P treatment would deliver P uptake comparable to the $CaHPO_4$ treatment with P loss still dramatically reduced.

Several worthwhile questions remain regarding the fraction of P retained in the soil. For instance, it is possible that the retained soil P in the PNC treatments is present in different and more bioavailable forms than the $CaHPO_4$ treatments. It is well known that P forms insoluble complexes with other soil elements, such as Mg, Al, Fe, and Zn,[14] and that P in soils can adsorb to soil particles,[21,80,81] both of which render the nutrient unavailable to plants. As such, it is possible that the time-dependent reactions of P in the soil may differ when initially present in the nanocomposite form. Additionally, up to 80% of soil P is classified as organic,[82] meaning that it is covalently bound to biological macromolecules within soil organisms. Although organic P is unavailable to plants, over time it is mineralized and solubilized into plant-available, ortho forms through microbial activity.[75,83-85] By initially trapping P within a polymer matrix, it is conceivable that significantly less added P would be sequestered through the above processes when using PNCs as compared to conventional fertilizers. Furthermore, there is a possibility that PNCs have not completely biodegraded at harvest and some amount of P is still contained within the composite to be eventually released. If the retained P in the PNC treatments is indeed present in a more available form, this could lessen the quantity of P-containing fertilizer needed in subsequent cropping events, providing further environmental benefit. Several of these questions are topics of current investigation.

The single greatest advantage of using PHA-Ca-P PNCs compared to conventional fertilizer is the reduced quantity of P loss from the soil. It is important to note that although the quantity of the P lost in these greenhouse studies is proportionally small (Figure 5), given the enormous worldwide use of P-containing fertilizers, a fractional decrease in lost P of over 80% will translate into significant reduction in negative environmental impacts associated with P-mediated eutrophication. It is important to acknowledge that although the majority of P losses worldwide are presumed to be due to erosion of soil particles bound to particulate phosphorus,[21] P loss via leaching can occur, especially on flat land that is not subjected to typical water-driven erosive processes found on sloped terrain.[86] Additionally, many strategies to reduce agricultural erosion[87] have led to greater P losses through leaching, such as tile drain systems, no-till practices, and cover cropping.[88,89]

Beyond the environmental benefits of reduced P loss from soil, the use of PNCs as agrochemical delivery platforms has other advantages. By using polymers that biodegrade at different rates, PNCs could be designed to provide tunable temporal control of P release to meet the life cycle requirements of different crops or the environmental conditions of specific soils or climates. Since P contained in PNCs remains trapped in the polymer matrix until biodegradation, P is presumably unavailable for sorption to soil particles and will not be lost to erosion that occurs in floods and seasons of heavy rains.[21,80,81,90] PNCs could also be prepared to contain multiple nutrients in addition to P or to contain synergistic components, like boron, that have been shown to increase nutrient uptake efficiency.[91] Additionally, other studies have shown that biodegradable polymers such as PHA support strong and diverse soil microbial communities,[37] which could subsequently impact the availability of typically unavailable fractions of P.[10,75]

Despite their many benefits, PNCs will not be a commercially viable method of agrochemical delivery until they can become cost competitive with conventional approaches. Our PHA-Ca-P PNCs, produced on a small scale with laboratory-grade reagents, cost approximately 400 times more than conventional P-fertilizers such as TSP. Notably, this calculation accounts only for synthesis; a full life cycle analysis that incorporates added environmental benefits would most certainly narrow that economic gap. Moreover, commercial-scale synthetic methods suitable for scale up, like melt mixing, would reduce the cost of producing PNCs as well as eliminate the need for costly and potentially toxic organic solvents. Using less expensive biodegradable polymers, such as starch or even biowaste, or a less expensive source of P, such as nanoscale hydroxyapatite, would also drastically lower the cost of PNCs and potentially improve the efficiency of P delivery.[27,69] Indeed, it is anticipated that other P-containing PNCs could be developed that will not only reduce P runoff but also improve crop yields compared to traditional P-fertilizers, further improving their cost benefit. Additionally, as global polymer markets shift in favor of biodegradable products, it is reasonable to assume that the price of PHA and other biodegradable polymers will decrease in the future.[62] Using larger, micron-sized particles would likely also lower the cost, although it is unclear how the inclusion of larger particles would impact the kinetics of P release from composites. As research increasingly indicates that P, not N, is the primary driving force behind eutrophication,[92-95] policies are being implemented worldwide to manage and limit agricultural P use.[96-100] This, in combination with the dwindling supply of nonrenewable mineral P as rock phosphate,[12,101] necessitates new and innovative ways to manage the global P supply, and the PNC platform evaluated in this work demonstrates significant potential for having a positive impact on the sustainability of agriculture in the future.

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Supporting Information. Overview of PNC synthesis and details of characterization (TGA, ATR-FTIR, EDS, ICP-OES). Photographs of soil-only experiments, and lettuce and tomato plants from Experiments 1 and 2. Details for acid digestion, plant harvest and collection, and chlorophyll, protein, catalase, lycopene, and fruit sugar measurements.

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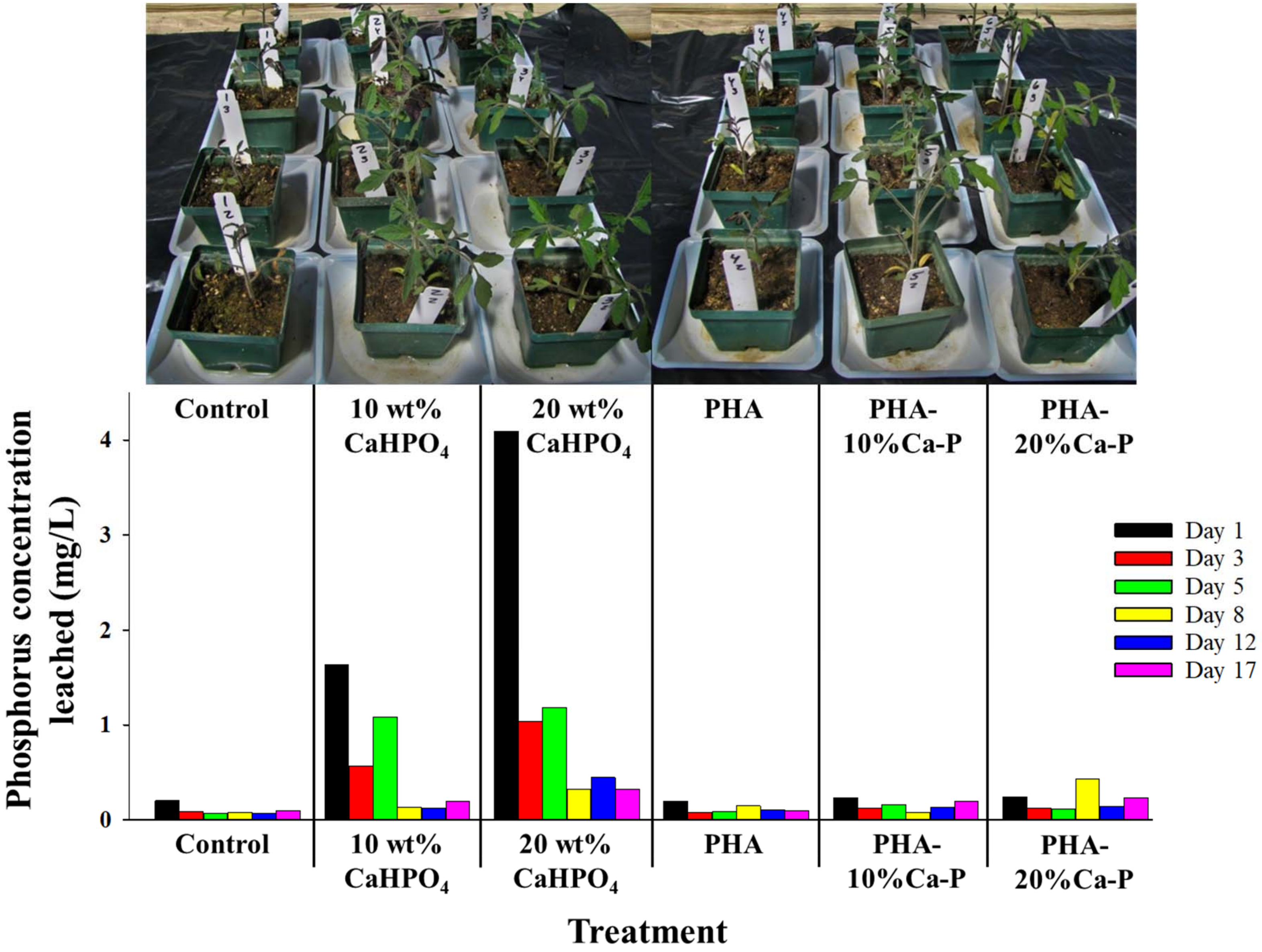

Figure 1. Tomato growth observed in Experiment 2 after 17 days in six different soil treatments. Top (from left to right): Unamended soil (control), $CaHPO_4$ salt with a phosphorus mass equivalent to PHA-10%Ca-P, $CaHPO_4$ salt with phosphorus mass equivalent to PHA-20%Ca-P, pure PHA, PHA-10%Ca-P, and PHA-20%Ca-P (see text for full details). Bottom: Average phosphorus concentration in leachate collected from each treatment on selected days during tomato plant growth.

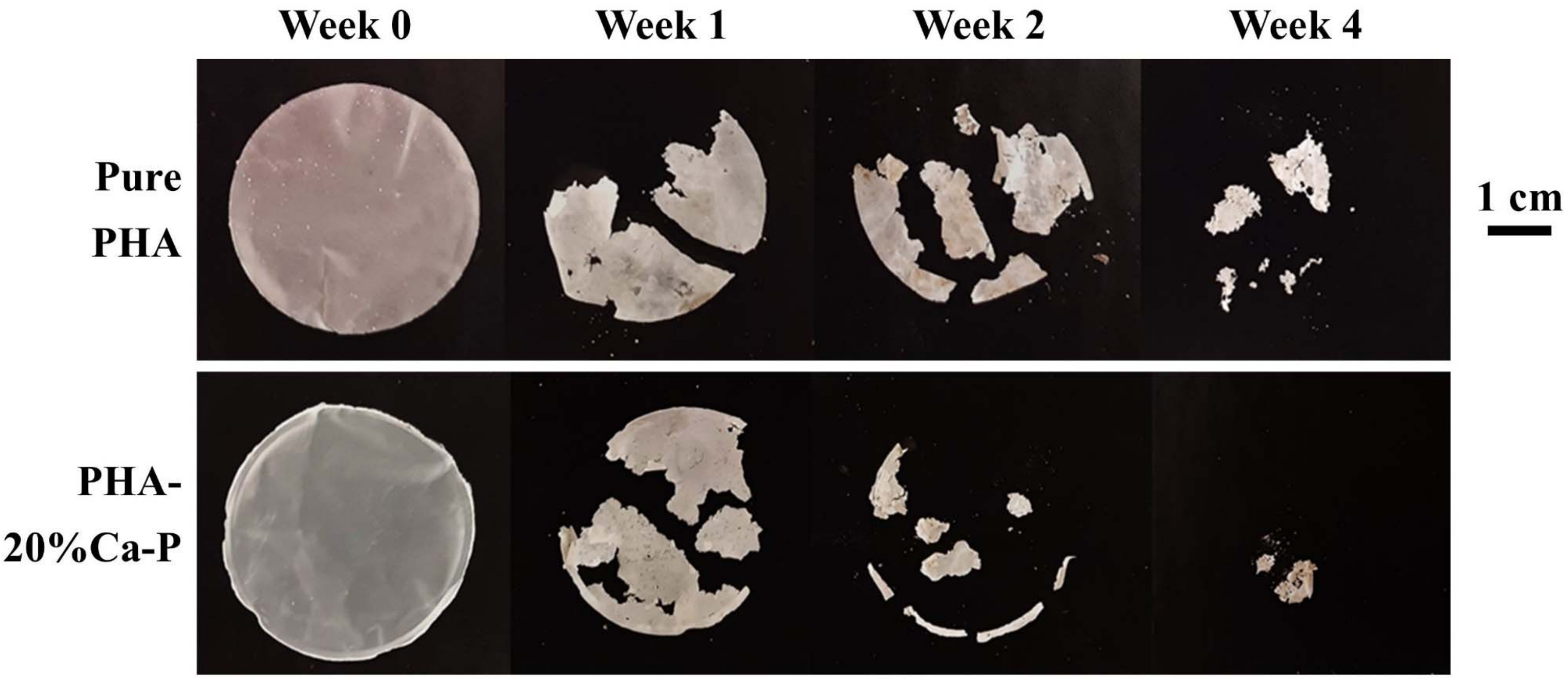

Figure 2. Biodegradation of PNCs containing pure PHA (top) or PHA-20%Ca-P (bottom) over four weeks in soil.

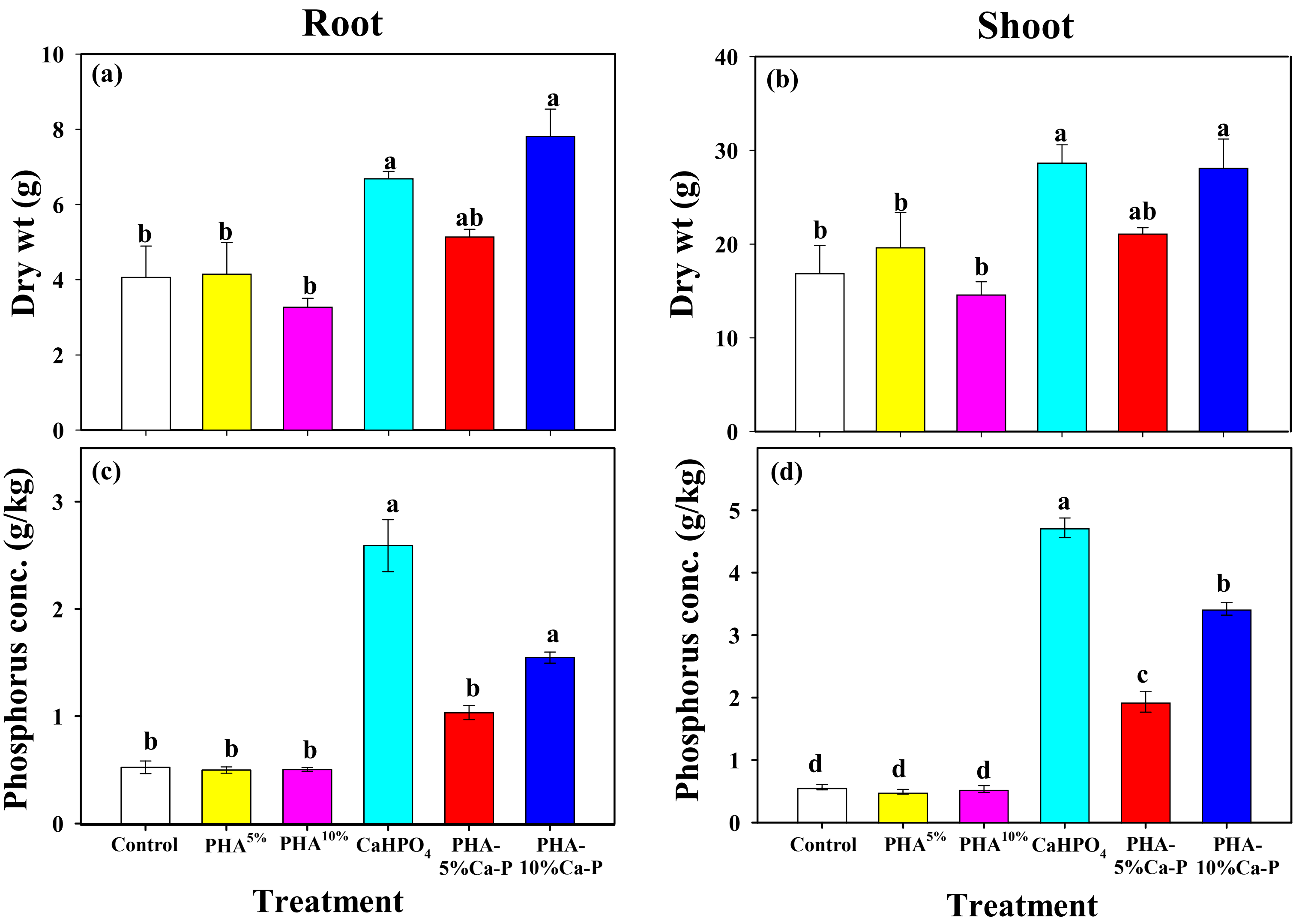

Figure 3. The effect of six different treatments, including polymer nanocomposites (PNCs) on the dry weight (g) of tomato root (a) and shoot (b) and P content (g/kg) of tomato root (c) and shoot (d). The PNCs consisted of polyhydroxyalkanoate (PHA) solution blended with either 5 or 10% wt calcium phosphate (Ca-P) nanoparticles. Controls included $CaHPO_4$ added to deliver P equivalent to the PHA-Ca-P PNCs, as well as PHA added in mass quantities equal to the PHA in the PHA-5%Ca-P and PHA-10%Ca-P PNC treatments. An unamended control treatment was also included. Within a figure panel, bars with different letters are significantly different by a one-way ANOVA followed by a Student Newman Keuls multiple comparison test ($p < 0.05$). In cases where the three controls were statistically equivalent, these three groups were composited to a single control treatment for statistical analysis. In those cases, a Holm-Sidak multiple comparison test was used. Error bars represent the standard deviation of 4 replicates.

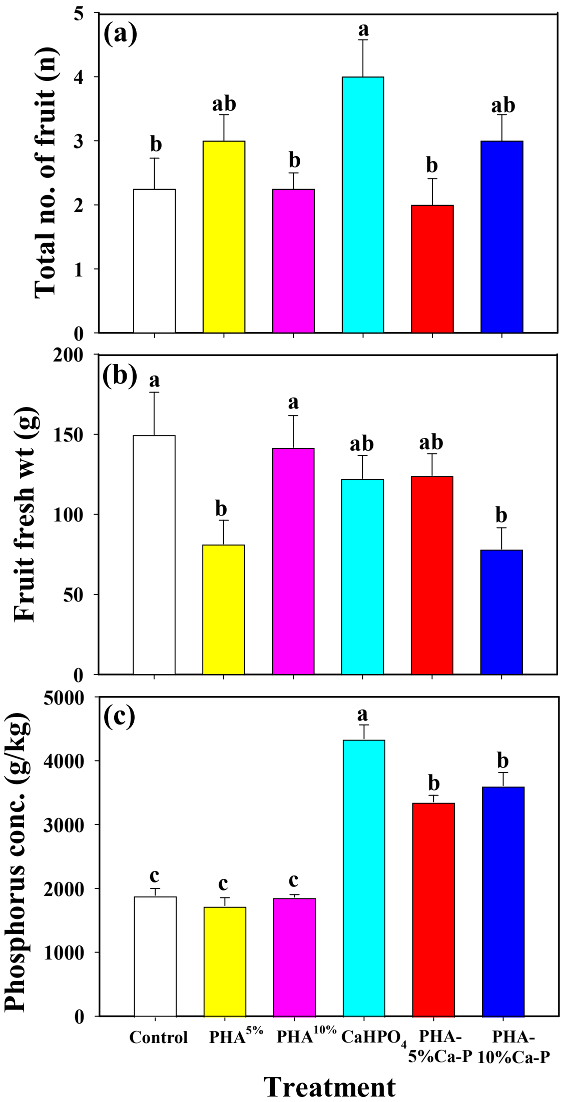

Figure 4. The effect of six different treatments, including polymer nanocomposites (PNCs) on tomato fruit number (a), fruit fresh biomass (b), and fruit P content (c). The PNCs consisted of polyhydroxyalkanoate (PHA) solution blended with either 5 or 10% wt calcium phosphate (Ca-P) nanoparticles. Controls included $CaHPO_4$ added to deliver P equivalent to the PHA-Ca-P PNCs, as well as PHA added in mass quantities equal to the PHA in the PHA-5%Ca-P and PHA-10%Ca-P PNC treatments. An unamended control treatment was also included. Within a figure panel, bars with different letters are significantly different by a one-way ANOVA followed by a Student Newman Keuls multiple comparison test ($p < 0.05$). In cases where the three controls were statistically equivalent, these three groups were composited to a single control treatment for statistical analysis. In those cases, a Holm-Sidak multiple comparison test was used. Error bars represent the standard deviation of 4 replicates.

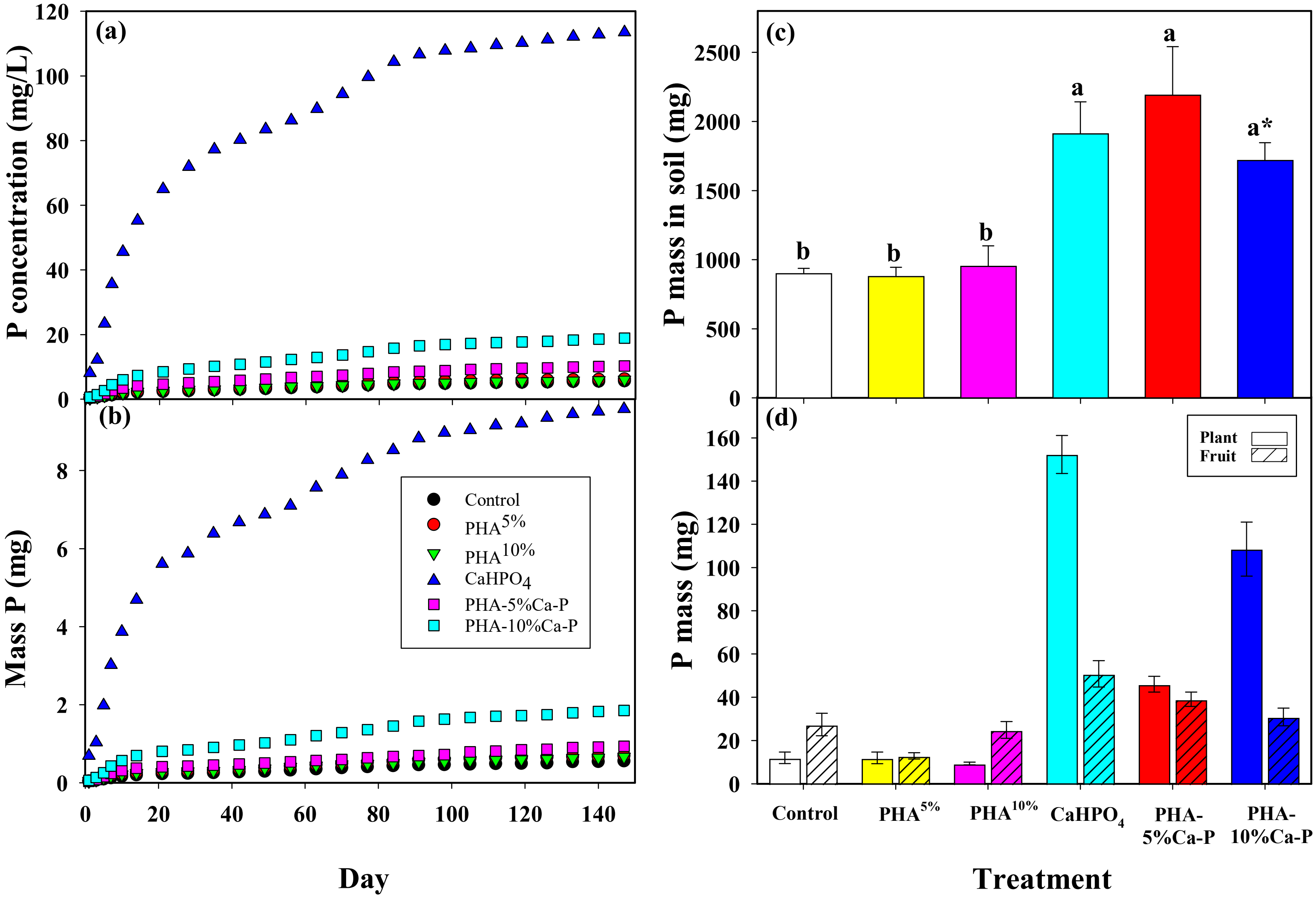

Figure 5. Average phosphorus concentration (a) and mass (b) in leachate collected from each treatment on selected days during tomato plant growth. For each treatment, following harvest, (c) shows the mass of phosphorus residual in the soil, while (d) shows the mass of phosphorus in the tomato plant (defined as the shoot plus root) and the fruit. Within a figure panel, bars with different letters are significantly different by a one-way ANOVA followed by a Student Newman Keuls multiple comparison test ($p < 0.05$). In cases where the three controls were statistically equivalent, these three groups were composited to a single control treatment for statistical analysis. In these cases, a Holm-Sidak multiple comparison test was used. Error bars represent the standard deviation of 4 replicates.