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The use of phosphorus nanoparticles synthesized by rhizospheric fungus *Aspergillius fumigatus* as a nanofertilizer for flax plant

Rabaa Yaseen[1]* and Basma Hamdy Amin[2]

[1]Fertility and Soil Microbiology Department, Water Resources and Desert Soils Division, Desert Research Center, El-Matariya 11753, Cairo, Egypt.
[2]The Regional Center for Mycology and Biotechnology, Al-Azhar University, Nasr city, 255455, Cairo, Egypt.
Email: rabaa.yassen@yahoo.com

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ABSTRACT

Aims: This study examined the mycosynthesis of phosphorus nanoparticles (PNPs) and its application as a fertilizer for flax plant.
Methodology and results: A total of thirty eight fungal isolates were isolated and screened for their abilities to synthesize PNPs. The fungal isolate was determined and identified as *Aspergillus fumigatus* (NCBI GenBank accession No. MN610566-MN610567). The biosynthesized nanoparticles were characterized by particle size analyzer, UV-visible spectrophotometer, transmission electron microscope (TEM), energy-dispersive X-ray spectroscopy (EDX) and fourier transform infrared spectroscopy (FT-IR). They were found to have an average diameter of 45.1 nm, regular round shape, EDX confirms the 54.63 atom % of phosphorous. The cytotoxicity of produced nanoparticles was performed to determine the safe dose that will be applied in agricultural experiment and was found to be 12.5 µg/mL. Pot experiment was performed to determine the fertilizing impact of mycosynthesized PNPs on flax plant and to equate their influence with granular single super phosphate. Results revealed that growth parameters, phosphorus content and microbial activities in the rhizosphere of flax plants were highly significantly ($p \leq 0.05$) affected by foliar application of PNPs in presence of half dose of super phosphate. The TEM-micrographs of stained ultrastructural leaves showed that the PNPs treated leaves in the presence of half dose of super phosphate had normal cell structure similar to control, while the cell structure of leaves treated with PNPs but did not receive super phosphate were adversely affected.
Conclusion, significance and impact of study: This study clearly indicated that the application of low cost biosynthesised PNPs could save about 50% of recommended dose of phosphorus fertilizer. This study also demonstrates that it is not preferred to use PNPs as a fertilizer alone without adding super phosphate. Hence, this investigation suggests that further studies should be established to detect the safety of this nanofertilizers.

Keywords: Nano-phosphorus, biosynthesis, cytotoxicity, leaf ultrastructure

INTRODUCTION

Flax (*Linum usitatissimum* L.) is an excellent source of palm oil and natural fibers (Kicińska-Jakubowska *et al*., 2012). Flax oil is high in nutritional protein, antioxidant such as omega-3 and omega-6 fatty acids, which play a significant role in maintaining wellbeing, raising immunity and lowering the risk of cancer (Galli and Risé, 2009). Flax is grown in Egypt for a dual purpose, production of oil from seeds and fiber from stalks. There is a gap between the output and consuming of flax plant due to the excessive competition with other financial winter crops. Hence, it is essential to increase the flax yield which could be attained by the improvement of the cultivating practices (Moursi *et al*., 2015).

Phosphorus is one of the essential macronutrients that are important for plant growth (Raghothama, 1999). It is considered as the most significant nutrient of several cellular components and it plays an integral role in various biological functions, including photosynthesis, respiration, energy storage, cell proliferation, membrane synthesis and reliability, enzyme activation and inactivation, redox reactions, cell transporters and cell elongation (Sulieman and Tran, 2015).

Growing population and expanding needs for biomass feedstock would raise the future requirement for phosphorus fertilizers. Generally, commercially available phosphorus fertilizers are either water soluble phosphate salts or solid insoluble phosphate types (e.g. apatite and rock phosphate). The water soluble phosphate forms are more available for plant uptake (Fageria, 2009). However,

*Corresponding author

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such soluble phosphates are often very elastic in the soil and often leaks into surface and groundwater by runoff or leaching. In comparison, solid types of phosphate are rarely transported by drainage or soil erosion. Nevertheless, these solid forms are not as effective as soluble forms in phosphorus nutrition (Fageria, 2009).

The excessive use of phosphorus fertilizers can cause ecological and economic risks where the increased fertilization of phosphorus results in soil and water pollution since soil erosion and runoff water holding great quantities of soluble phosphorus. In addition, the use of mineral phosphate fertilizers has become a costly matter and there is a demand for cost-effective sources (Buckley and Carney, 2013).

The nano-forms of phosphorus could be useful in the nutrient phosphorus supplementary much like the dissolved mineral phosphate fertilizers. In addition, it minimized the secondary contamination hazards and the transportation problem accompanying with solid phosphorus forms. The use of phosphorus nanoparticles (PNPs) fertilizer as an alternative to mineral phosphorus nutrition on agricultural fields may boost agronomic productivity and enhance the efficiency of surface water (Duhan *et al*., 2017).

Micro-biota such as fungi, yeasts, actinomyces and bacteria having good natural properties for metal ions nanoparticles biosynthesis (Bansal *et al*., 2014). It is true that the small size and high surface area to volume ratio in nanoparticles can be beneficial in general but using these justifications without understanding the mechanisms of interaction between nanoparticles and crops, may be in the long term undermine the potential of nanotechnology in agriculture. Therefore, the novelty of the present investigation lies in describing the relevant reported green synthesis of PNPs using soil fungi as ecofriendly environmentally sustainable and low-cost nano-producers. The evaluation of PNPs as nanofertilizer for flax plant instead or in combination with mineral phosphate fertilizers has been conducted in this study.

MATERIALS AND METHODS

Isolation of soil microbes

Rhizospheric soil samples were taken from the North West and the South West of EL-Minya and the South East of El-Monkhafdd area, Giza, Egypt. The samples were kept in plastic bags at 4 °C until being used. For fungal isolation, serial dilutions were made and plated on potato dextrose (PDA) medium (20% potatoes extract, 2% dextrose and 2% agar). Single fungal colonies were picked up and maintained on PDA slants.

Screening of fungal isolates for synthesis of phosphorus nanoparticles

The fungal isolates were grown-up in 250 mL Erlenmeyer flask containing 150 mL yeast extract-malt extract broth medium (Kieser *et al*., 2000), composed of 1% glucose, 1% sucrose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract and pH 6.8. After five days of incubation, the mycelium of fungal isolates was harvested by filtration using 0.45 µm size membrane filter, washed three times with double distilled sterile water to eliminate all remnants of broth medium and dried at 50 °C. For nano-phosphorus production, 1 g of each dried fungal biomass was inoculated into 150 mL Erlenmeyer flasks containing 50 mL of 1 mM tricalcium phosphate ($Ca_3(PO_4)_2$) and then incubated at 30 °C for 72 h under shaking (150 rpm). The particles were purified by filtration using a sterile Millipore 0.22 µm syringe filter followed by dialysis and centrifugation and were used for further characterizations. Fungal isolate which exhibited lowest diameter of phosphate nanoparticle was chosen and identified.

Molecular identification of fungal isolate

The genomic deoxyribonucleic acid (DNA) of the selected fungus was extracted using cetyl trimethyl ammonium bromide (CTAB) extraction technique described by Stirling (2003) and amplified by using 18S and 28S primers according to the method of White *et al*. (1990). The amplified polymerase chain reaction (PCR) product was sequencing, and the ribosomal ribonucleic acid (rRNA) sequence was submitted to GenBank of National Centre for Biotechnological Information (NCBI). Phylogenetic tree was built using Close Neighbor-Interchange (CNI) algorithm employing maximal parsimony analysis tool. The stability of trees from the above-mentioned cluster study was evaluated using MEGA4's bootstrap software in sets of 1000 resamplings (Tamura *et al*., 2007).

Characterization of phosphorous nanoparticles (PNPs)

Particle size analysis

The size measurement and particle size scattering of PNPs were monitored using dynamic light scattering (DLS) (Beckman DelsaNano C series, USA) by measuring the rate of variations in the intensity of laser light scattered by particles as they path through solvent.

Ultraviolet–visible (UV–Vis) spectroscopy analysis

Primary characterization of PNPs was done through visual examination for color change of salt solution. The UV–Vis spectra of the mycosynthesized PNPs were recorded at room temperature using spectrophotometer (Jenway, Model 6715 UV/VIS). The confirmation of phosphorus nanoparticles synthesis was conducted at the central laboratory, Desert Research Center. The UV-Vis spectra of PNPs and phosphorus ion (from tricalcium phosphate salt) were measured at 190 to 800 nm.

Transmission electron microscope (TEM)

The shape and size of PNPs were evaluated using transmission electron microscope (TEM), as outlined by

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Singhal *et al.* (2011). This analysis was done by drop coating technique; a drop of solution was put on the carbon-coated copper grids and dried at ambient temperature. Electron micrographs were obtained using JEOL GEM-1010 transmission electron microscope at 70 kV. This analysis was carried out at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

Energy dispersive X-ray analysis (EDX)

The elemental composition and purity percent of PNPs was evaluated by using EDX. The EDX microanalysis was done at the RCMB, Al-Azhar University by using X-ray micro-analyzer (Oxford 6587 INCA) connected to scanning electron microscope (JEOL JSM-5500 LV) at 20 kV. The EDX spectrum observed on the film's surface in spot profile made from one of the heavily populated area of phosphorous nanomaterial.

Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectroscopy was used to analyze the functional groups of dried fungal mat and freeze-dried powder of the synthesized PNPs (Nicolet avatar 230 spectrometers). Measurements of sample were recorded at a resolution of $4cm^{-1}$ in the range of 500-4000 cm^{-1}.

Cytotoxicity of synthesized phosphate nanoparticles

Normal human lung fibroblast cell line (WI-38 cells) was obtained from VACSERA Tissue Culture Unit and used for cytotoxicity assay using the method described by Mosmann (1983). Dulbecco's modified Eagle's medium, complemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 50 µg/mL gentamycin and HEPES buffer (hydroxyethyl piperazine-ethanesulfonic acid), were used for cultivation of mammalian cells. The cells were incubated at 37 °C in a ventilated climate and were sub-cultured twice a week. The cells were inoculated into 96-well plate containing 100 µL of growth medium at a cell concentration of 1×10^4 cells per well. After 24 h of cell inoculation, fresh medium containing different concentrations of the PNPs (0, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL) was added. Serial repeat dilutions of the PNPs is applied to convergent cell monolayers dispensed into 96-well flat-bottomed microtiter plates (Falcon, NJ, USA) and incubated at 37 °C in a CO_2 incubator for 48 h. For each concentration of the test sample, three wells were applied. Control cells were incubated without PNPs and with or without dimethyl sulfoxide. After incubation period, the crystal violet solution (1%) was added to each well for 30 min then, the plates were rinsed by distilled water. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly. The cell viability was determined by measuring absorbance at 595 mm using microplate reader (SunRise, TECAN, Inc, USA) as formula below:

$$\text{Cell Viability } (\%) = \frac{\text{ODt}}{\text{ODc}} \times 100$$

Where, ODt is the mean optical density of wells treated with PNPs and ODc is the mean optical density of untreated cells.

Greenhouse experiment

An open greenhouse pot experiment was performed to determine the impact of PNPs on flax plant growth. Flax seeds cultivar Sakha 1 were obtained from Field Crops Research Institute, Agricultural Research Center, Giza, Egypt. Soil was collected from Ismailia governorate, Egypt, having pH 7.27, EC 1.61 dS/m, clay texture and its available P content was 5.7 µg/g. Four fertilizing treatments with five replicates were used in this study. These treatments include T1: foliar spraying with PNPs (200 ppm/pot), T2: foliar spraying with PNPs plus half dose of super phosphate (1 g/pot) and T3: addition of full dose of super phosphate (2 g/pot) and control (without any treatments). Mineral fertilizers doses of nitrogen and potassium were added for all treatments (1.5 g/pot ammonium nitrate for nitrogen fertilizer, 0.5 g/pot potassium sulp hate for potassium fertilizer). A total of 50 mL PNPs solutions with a phosphorus concentration of 12.5 µg/mL was sprayed twice a week to each pot in set 1 and 2. The foliar application of PNP fertilizer started after 25 days from sowing. Thinning of seedling was carried out after 21 days from sowing; only five seedlings were left in each pot.

The flax plants were harvested after two months, and phosphorus concentration was determined in plant tissues (acid digests) according to Watanabe and Olsen (1965). The total microbial counts, phosphate dissolving bacteria, nitrogen fixers, actinomyces and fungal counts in the rhizospheric soil were evaluated by plating on nutrient agar, modified Bunt and Rovira medium, nitrogen free semisolid medium, starch nitrate agar medium and Czabek's Dox agar medium (Atlas and Parks, 1993), respectively. In rhizospheric soil samples, the activity of dehydrogenase enzyme was determined according to Klein *et al.* (1971) approach, 3% triphenyltetrazolium chloride (0.2 mL) and 1% glucose (0.5 mL) were added to 1 g of each soil sample and incubated at 28 °C for 24 h. After incubation, 5 mL of acetone was added and incubated at 28 °C for 1 h. The produced triphenyl formazan was estimated by spectrophotometer at 485 nm.

Electron microscopy ultrastructure examination

For TEM examination, the control leaf samples, and all treatments were placed in 3% glutaraldehyde, soaked in phosphate buffer, and post-fixed in potassium permanganate solution for 5 min at room temperature. The leaf samples were dehydrated in an ethanol series ranged from 10% to 90% for 15 min and in concentrated ethanol for 30 min. Samples were penetrated into a graduated sequence of epoxy resin and acetone, before

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eventually in pure resin. Extremely thin fragments were fixed on copper grids. Fragments were then double stained in uranyl acetate first then by lead citrate. Stained fragments were examined with TEM (JEOL-JEM 1010) at 70 kV at the RCMB, Al-Azhar University.

Statistical analysis

SPSS 21.1 software program (SPSS, 2014) was used to calculate the least significant difference (LSD) between treatments. Duncan's multiple range tests, at $p \leq 0.05$, was used to compare the means of variance.

RESULTS AND DISCUSSION

Isolation and screening of soil fungi for biosynthesis of PNPs

In this study, 38 fungal isolates were isolated from 25 soil samples and screened for their abilities to synthesize PNPs by incubating fungal biomass with tricalcium phosphate solution (1 mM). The colour of aqueous solution of phosphorus ions was changed from colorless to bale yellow (Figure 1). The particle size of PNPs was measured by using particle size analyzer (Table 1). Among tested isolates, 11 isolates could synthesize PNPs with average size ranged from 94.7 to 45.1 nm. Isolate numbered as SEM34 was chosen for further studies due to its effectiveness in PNPs production with mean diameter of 45.1 nm as shown in Table 1.

Tarafdar *et al*. (2012) attributed the nanoparticles formation by fungi due to the catalytic action of their extracellular enzymes, which aids in the reduction of the metal salt and transform it into nanoscale diameter. Generation of extracellular enzymes by fungus helps in gaining pure nanoparticles. Several authors have informed that nicotinamide adenine dinucleotide (NADH) dependent nitrate reductase enzyme has an essential role in metal ions transformation into metal nanoparticles. For example, Li *et al.* (2012) and Guilger-Casagrande *et al*. (2019) stated that the NADH-dependent reductase protein present in samples of biogenic silver nanoparticles, is responsible for formation of silver nanoparticles.

Figure 1: Change of tricalcium phosphate $Ca_3(PO_4)_2$ solution color from colorless (left conical flask) to yellowish (right conical flask).

Identification of fungal isolate

The fungal isolate numbered SEM34, which could synthesis phosphorus nanoparticles was identified based on its molecular parameter. The sequence was submitted to NCBI (accession no. MN610566-MN610567). The 18S and 28S rRNA nucleotide sequence revealed that it belonged to *Aspergillius fumigatus* with 99% blast identity. Phylogenetic tree of the isolated fungus and closely related strains indicated that the fungus isolate was grouped with *Aspergillius fumigatus* with 100% bootstrap value (Figure 2).

Characterization of PNPs dynamic light scattering (DLS) analysis

Particle size of myco-synthesized PNPs (SEM34) was measured using particle size analyzer (Figure 3). Histogram displays levels in particle size in 10-15 nm and 40-100 nm with an average size of 45.1 nm.

UV-visible spectroscopy analysis

The production of PNPs in aqueous solution was confirmed using UV-Visible spectroscopy. PNPs showed a broad peak in the UV-visible spectrum and centered at

Table 1: Particle size of phosphorus subjected to different fungal isolates.

Isolate no.	Mean diameter (nm)	Isolate no.	Mean diameter (nm)
SWM1	94.0	SEM20	50.0
SWM2	90.9	SEM21	108.0
SWM3	93.3	SEM22	201.0
SWM4	88.9	SEM23	731.9
SEM5	256.0	SEM24	290.6
SEM6	3085.0	SEM25	251.6
SEM7	308.0	SWM26	56.9
SEM8	1382	SWM27	934.4
SEM9	107.9	SWM28	208.0
SWM10	161.1	SWM29	293.4
SWM11	745.2	SWM30	522.0
SWM12	813.2	SWM31	75.0
SEM13	637.4	SWM32	722.0
SEM14	694.9	SWM33	330.0
SWM15	308.0	SEM34	45.1
SWM16	2298.7	SEM35	290.6
SWM17	6443.3	SEM36	251.6
SWM18	451.0	SWM37	68.5
SWM19	94.7	SWM38	54.4

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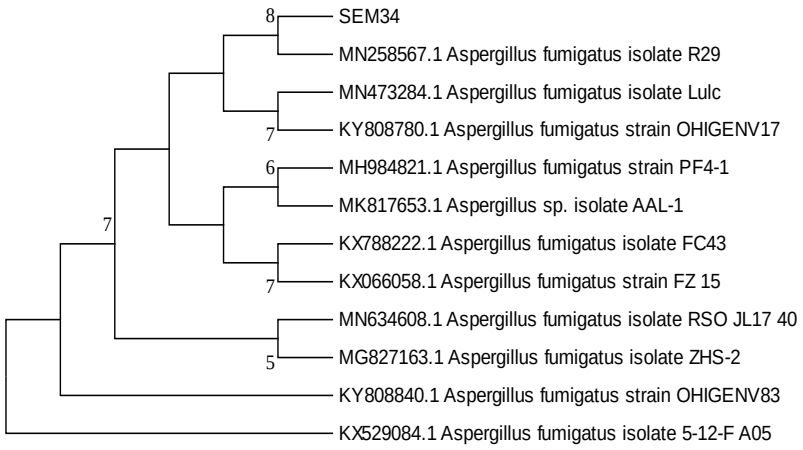

Figure 2: Neighbour joining phylogenetic tree of partial 18S and 28S rRNA sequence of selected fungal isolate (SEM34).

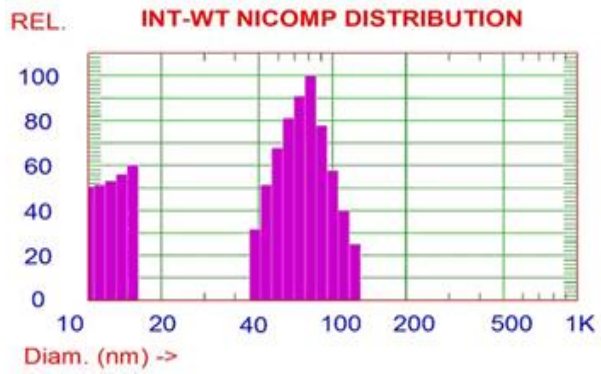

Figure 3: Particle size of PNPs synthesized by *A. fumigatus*.

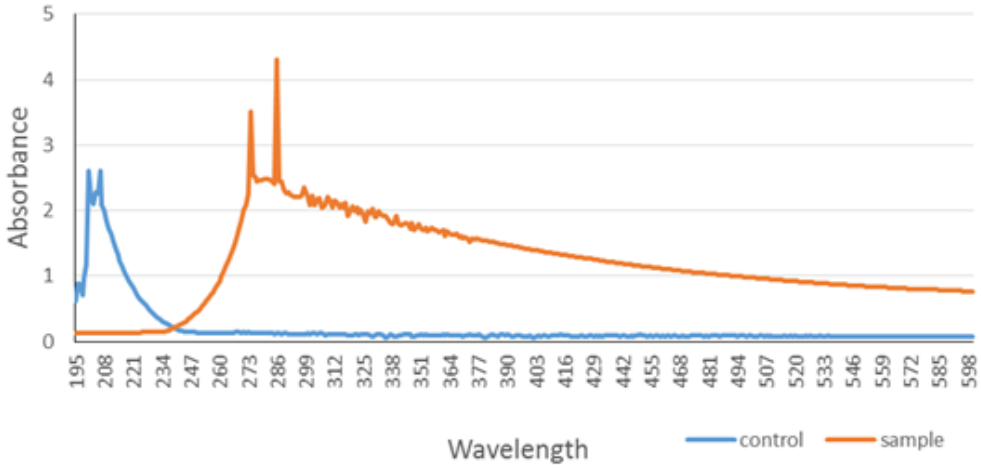

Figure 4: UV-visible spectra of phosphorus ion, (acted as control, shown in blue line) and phosphorus nanoparticles synthesized by *A. fumigatus* (orange line).

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274 nm and 286 nm whereas the absorbance of phosphorus ion occurs at 201 and 206 nm (Figure 4). Hou *et al.* (2013) recorded the absorption of nano-calcium phosphate produced by chemical process at 230-260 nm, while Pokale *et al.* (2014) illustrated the absorption of nano-calcium phosphate particles at 276 nm. The alteration in UV-visible spectra absorbance may be attributed to the biomolecules that present in the fungal cells such as enzymes and cellular proteins.

Microscopic characterization by transmission electron microscope (TEM)

TEM analysis was used in determine the size and shape of bio-synthesized PNPs. The micrograph in Figure 5 showed nearly spherical nanoparticles with mean diameter size 46.26 nm. The mean diameter was taken from 10 nanoparticles, the minimum diameter was 29.27 nm and the maximum diameter was 61.39 nm.

Energy dispersive X-ray analysis (EDX)

The descriptive and analytical status of the elements involved in the development of PNPs was studied by using X-ray imaging. The energy and intensity distribution of X-ray signals produced on a specimen by a focused electron beam were measured to perform the EDX micro-analysis. Results shown in Figure 6 illustrated the registered EDX spectrum in the spot-profile mode. It is obvious from the EDX analysis, that PNPs produced by *A. fumigatus* had the weight percentage of phosphorus as 54.63%.

FT-IR analysis

FT-IR studies were performed to confirm the potential interactions between the phosphorus salts and the capping proteins, which could account for the reduction of phosphorus ions with consequent stabilization of PNPs. The FTIR spectra reported the dried fungal mat and the freeze-dried powder of PNPs were illustrated in Figure 7. The FTIR spectra of *A. fumigatus* and PNPs is characterized by the protein amide bands. In particular, the bands appeared at 3372 cm^{-1} and 3448 cm^{-1} were referred to primary and secondary amine stretching vibrations, respectively, while their corresponding bending vibrations were observed at 1635 cm^{-1}. The bands appeared at the range from 1380 cm^{-1} to 1029 cm^{-1} can be attributed to the aromatic and aliphatic amines C–N stretching vibrations, respectively (Ojeda and Dittrich, 2012). The overall FTIR pattern confirms that capping proteins are present in the PNPs. The free amine and carbonyl groups of fungal proteins could possibly be responsible for the formation and stabilization of PNPs (Vigneshwaran *et al.*, 2007). It has been previously reported that proteins can attach to nanoparticles either through free amine groups or through the electrostatic attraction of negative charges of carboxyl groups in enzymes present in mycelia's cell wall (Mandal *et al.*, 2005; Vigneshwaran *et al.*, 2007) and thus stabilize the PNPs.

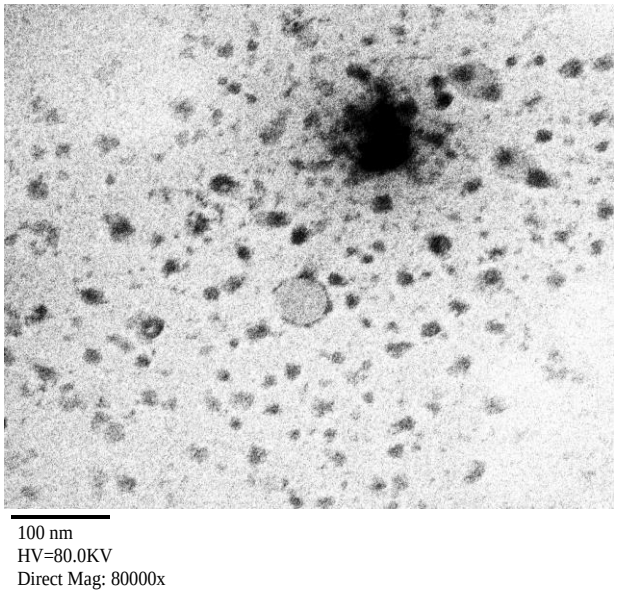

Figure 5: TEM micrograph of PNPs biosynthesized by *A. fumigatus* (black dots). Scale bar = 100 nm.

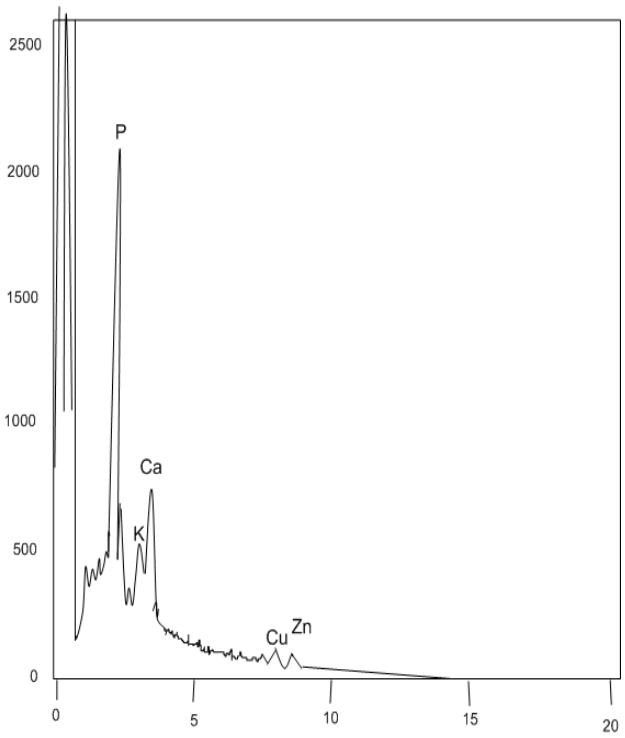

Figure 6: EDX spectrum of PNPs. The weight percentage of phosphorus was 54.63% and calcium was 18.37%.

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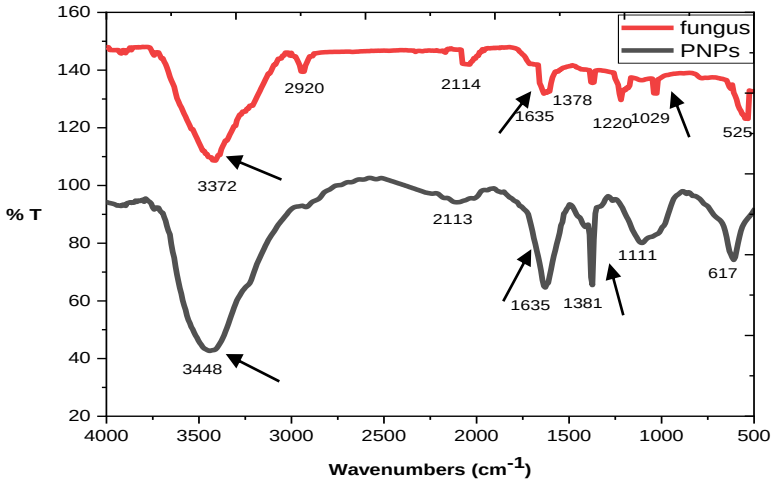

Figure 7: FTIR spectra of dried fungal mat (red line) and bio-synthesized phosphorus nanoparticles (black line).

Evaluation of cytotoxic effect of synthesized phosphorus nanoparticles

Cytotoxicity effect of biosynthesized PNPs was investigated using human lung fibroblast cells to determine the cytotoxic concentration which inhibits 50% of viable cells (CC50). The results revealed that the PNPs had no cytotoxic effect at 12.5 µg/mL toward human lung fibroblast (Table 2). Identical findings were obtained by Peetsch *et al.* (2013) who examined the cytotoxic effect of calcium phosphate nanoparticles, doped and undoped with silver, toward mammalian cells. They observed that nanoparticles made of silver doped calcium phosphate had a toxic impact at concentrations of 1-3 µg/mL. Though undoped nanoparticles of calcium phosphate had no harmful effects at the same particle concentration on human cells. Many studies demonstrated the shape, size and concentration depend the effects of nanoparticles, for example, Zhao *et al.* (2013) revealed that needle-shaped and plate-shaped hydroxyapatite nanoparticles caused significant cell death compared with sphere-shaped and rod-shaped. Also, Alkhatib *et al.* (2019) displayed a strong impact on the toxicity of iron oxide nanoparticles by size and intensity.

Table 2: Cytotoxicity effect of biosynthesized phosphorus nanoparticles towards human lung fibroblast cells.

Sample concentration (µg/mL)	Viability* (%)
100	89.46 ± 1.28
50	94.78 ± 0.34
25	99.52 ± 0.06
12.5	100
6.25	100
3.125	100
0	100

*Each reported value is the mean of triplicate samples (n=3).

The impact of phosphorus nanoparticles on flax plant growth

A pot experiment was performed to evaluate the fertilizing impact of PNPs on flax plant growth compared with super phosphate fertilizer. The flax plant growth is influenced by the different treatments (T1: PNPs only, T2: PNPs plus half dose of super phosphate fertilizer and T3: full dose of super phosphate fertilizer) and the results were shown in Table 3. The growth parameters and phosphorus concentration in flax plants were determined, and results revealed that all measured parameters were higher in plants treated with PNPs. The fresh and dry weights of the plants treated with PNPs were more or less similar compared to super phosphate fertilizer. Growth parameters were highly significantly (at $p \leq 0.05$) affected by T2 followed by T1 and T3. The fresh and dry matter productions were increased by 28.6% and 121.9% in T2 over T3, respectively. There was no significant difference (at $p \leq 0.05$) in plant height between all treatments. T2 also showed significant increase (at $p \leq 0.05$) in phosphorus content in flax compared with T1 and T3. The increase in growth factors can be contributed to the potential benefits of nanoparticles that have high surface areas and high reactivity which simplify nanofertilizer absorption by the plants (Shang *et al*., 2019). Preceding results are in accordance with those obtained by Rajonee *et al*. (2017) and Dhansil *et al*. (2018).

The results in Table 4 clearly indicated that T1 and T2 significantly increased the populations of phosphate dissolving bacteria and actinomycetes. Dehydrogenase activity in soil was enhanced with the application of T2 followed by T1. There is no significant effect between different treatments on total microbial counts, nitrogen fixers and fungi. These results are in harmony with those obtained by Manjunatha *et al*. (2018) who noticed no significant effect of studied nanoparticles (zinc oxide,

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Table 3: Growth parameters and total phosphorus concentration in flax plant (60 days old) as affected by PNPs and P fertilizers.

Treatments	Fresh shoot weight (g/pot)	Dry shoot weight (g/pot)	Plant height (cm)	Phosphorus concentration (%)
Control	8.10[b]	0.91[b]	17.0[a]	0.10[c]
PNPs + no super phosphate	8.80[ab]	1.15[b]	19.0[a]	0.16[b]
PNPs + half dose of super phosphate	10.80[a]	2.13[a]	20.0[a]	0.211[a]
Full dose of super phosphate	8.40[b]	0.96[b]	17.60[a]	0.149[b]
*LSD at 0.05	2.30	0.89	2.50	0.03

* LSD: least significant difference, n = 5

Table 4: Microbial populations and activity in flax plant rhizosphere as affected by PNPs and P fertilizers.

Treatments	Microbial counts (log CFU/g dry soil)					Dehydrogenase (µmol TF/g dry soil)
	TMC	PDB	NF	Actinomyces	Fungi	
Control	6.68[a]	2.91[b]	3.70[a]	3.40[b]	3.51[a]	13.4[b]
PNPs + no super phosphate	6.86[a]	3.67[a]	3.63[a]	3.97[a]	3.63[a]	15.1[ab]
PNPs + half dose of super phosphate	6.85[a]	3.79[a]	3.82[a]	3.88[a]	4.04[a]	19.1[a]
Full dose of super phosphate	6.71[a]	0.00[c]	3.56[a]	3.60[b]	4.05[a]	13.7[b]
LSD at 0.05	0.48	0.58	0.65	0.23	0.99	4.90

Each reported value is the mean of five replicates of the treated plants.
TMC: total microbial count; PDB: phosphate dissolving bacteria; NF: nitrogen fixers; TF: triphenyl formazan; CFU: colony forming unit.

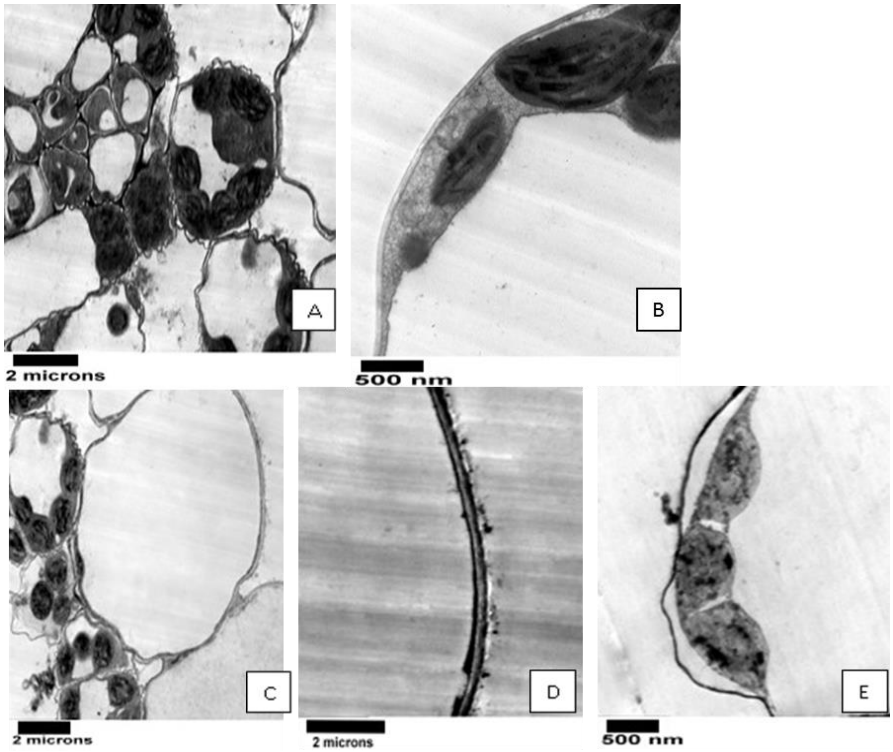

Figure 8: The ultrastructure of flax plant leaves observed using TEM. A: control plant (without any treatment); B: full dose of super phosphate; C and D: PNPs with half dose of super phosphate and E: PNPs only. Scale bars: A, C, D = 2000 nm and B, F = 500 nm.

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titanium oxide, copper oxide and silver) on soil microbial populations during the field study. Raliya and Tarafdar (2013) displayed an improvement in rhizospheric microbial community through addition of zinc oxide (ZnO) nanoparticles. The better response of PNPs might be because it serves as cofactor for many enzymes and its essentiality for many biological processes (Vance, 2011).

Ultrastructure of PNPs treated flax plant leaves

The stained ultrastructure of flax plant leaves was examined with TEM. The TEM-micrographs showed the leaves of flax plant that treated with PNPs in presence of half dose of super phosphate (Figure 8C), were similar to the control plants (Figure 8A) and also to plants treated with full dose of super phosphate (Figure 8B). The cell wall and cell membrane were intact with ideal structures of thylakoids that arranged in tight stacks grana with the appearance of PNPs (arrow) affinity to the cell membrane (Figure 8D). In contrast, the leaves of PNPs treated flax plant that did not receive super phosphate showed irregular cell wall and decrease in thickness of the cell membrane with undistributed thylakoids with accumulation of PNPs in and out of the plant structure (Figure 8E). These findings are in alignment with those reported by Jiang *et al*. (2007) and Rahman *et al*. (2018) who showed that P addition has reduced the effects of metal toxicity on plant cells. However, the toxicity of the nanoparticles and their harmful effects on plant development has not been completely illuminated. Nanoparticles may have a hormone-like nature thus the nanoparticle at small doses have a positive effect, but at large doses they are very dangerous to plants (Vargas-Hernandez *et al.*, 2017).

CONCLUSION

This study dealt mainly with the fungal mediated synthesis of PNPs to be used as phosphorus nutrition to enhance the flax plant growth. The PNPs produced by *A. fumigatus* were round in shape with mean diameter of 45.1 nm, their phosphorus weight percentage was 54.63% and had no cytotoxic effect at 12.5 µg/mL against human lung fibroblast cells. Growth parameters, phosphorus content and microbial activity in the rhizosphere of flax plants were highly significantly ($p \leq 0.05$) affected by foliar application of PNPs in presence of half dose of super phosphate. This study clearly indicated that the application of low cost biosynthesized PNPs could save about 50% of recommended dose of phosphorus fertilizer and also demonstrate that it is not preferred to use PNPs alone without adding mineral phosphate fertilizer (super phosphate). As a result, further studies should be established to detect the safety of nano-fertilizers.

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