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Efficacy of natural hydroxyapatite nano-particles as a phosphorus fertilizer for soybean

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Hydroxyapatite (HAp) is one of the widely used bio-ceramics in several fields especially in medical and agricultural applications. HAp can be synthetically prepared from bio-wastes not only from the economical point of view but also it is environmentally friend. The present work aimed to prepare and characterize natural HAp nanoparticles from fish back bone (F-HAp) and egg shell (E-HAp) and study their efficiency on cultivation and growth of soybean relative to effects of traditional fertilizer. Characterizations were carried out by using Fourier Transform Infrared spectroscopy (FTIR), transmission electron microscopy (TEM) and X-ray diffraction (XRD). Both F-HAp and E-HAp displayed excellent characteristics in term of particle nano-size, morphology and crystallinity. Present results showed no significant differences in growth rate of using nano-fertilizers produced from F-HAp or E-HAp. The obtained data resulted that growth rate of plants treated by nano-fertilizers was 1.88 times greater than that treated by triple super phosphate. Analysis on macronutrient elements revealed that the concentration of nitrogen (N), phosphorus (P), and potassium (K) in plants treated by nano-fertilizer (F-HAp) were noticeably greater than that in plants treated by commercial one with percentages 1.21, 1.32, and 1.17 respectively. From this study it was found that fish back bone and egg shell as bio-wastes could be used to synthesize high quality HAp with characteristics for agriculture applications as a nano-phosphorus fertilizer. The obtained data suggesting that both F-HAp and E-HAp were able to enhance soybean production in comparison with traditional phosphorus fertilizer, which need more research on greater area of field.

Keywords: Hydroxyapatite, Nanoparticles, Eggshell, Fish bone, Fertilizer, Soybean.

INTRODUCTION

Hydroxyapatite (HAp) is one of the widely used bio-ceramics in different fields especially in medical and agricultural applications. Producing HAp from natural source attends great attention due to their chemical composition and structure from biocompatible and biogenic sources. Natural HAp can be produced from egg shell (Khandelwal and Prakash, 2016), seashell (Vecchio et al., 2007), coral (Abdel-Fattah et al., 2009) in addition to isolation from fish bone (Venkatesan et al., 2011), fish scales (Panda et al., 2014),and bovineor pig bone (Barakat et al., 2009] and also from human teeth (Lü et al., 2007).

A huge amount of eggshells and fish backbones were consumed daily, which are of no use and produce waste. These wastes support microbial growth and lead to environmental pollution. Eggshells are mainly composed of calcium carbonate (94%), calcium phosphate (1%), organic matter (4%) and magnesium carbonate (1%), which makes it preferable for synthesizing CaO (Sasikumar and Vijayaraghavan, 2006, Rivera et al., 1999). Fish sources are harmless, and the wide evolutionary gap between fish and humans suggests a low risk of disease transmission [Hoyer et al., 2012). Also, using of these abundant fish byproducts help in reducing environmental pollution and health hazards.

Several researchers found the importance of nano-hydroxyapatite as a potential fertilizer in improving phosphorous efficiency based on the hypothesis that nano-sized particles can potentially move in the soil and reach the plant roots through the mass flow of soil water to roots created by transpiration [Bala et al., 2014, Liu and Lal, 2014). Due to the importance of P fertilizers to plants especially during early stages of growth for optimum crop production (yield and maturity).So it draws attention in agriculture field as a nano-phosphorus fertilizer for cultivation economic crops such as soybean.

Generally, commercially available P fertilizers such as MAP (mono ammonium phosphate, $NH_3H_2PO_4$), DAP (diammonium phosphate, $(NH_3)_2HPO_4$), or TSP (Triple Superphosphate, $Ca(H_2PO4)_2$) are water soluble phosphate salts, which are easily dissolved in the soil solution and available for plant uptake, and thus, are regarded as high quality fertilizers (Fageria et al., 2009). However, soluble phosphates are very active in the soil and the surplus rise up through overflow in surface water causing eutrophication [Liu and Lal, 2014). Eutrophication of aquatic ecosystems resulted from increasing of nutrients especially phosphorous. The basic mineral nutrients required to maintain plant growth are phosphorous and potassium, which play essential role in improving crop yield and quality (Rhaghothama, 1999, Abel et al., 2002). Also, P is necessary for nucleus formation, cell division, plant growth and photosynthesis. In addition P compounds are implicated in transfer and storage of energy in plants. P moves within plants from older to younger tissues to control plant's cell formation and development of roots, stems and leaves (Ross, 2013).

Soybeans, *Glycine max*, become important and popular protein so it considers as a one of the most food plants of the world and its growing is important. It is easy to growth annually to produce more protein and oil per unit of land than almost any other crop. They are produced in greatest numbers in the United States and South America, but they are non-primary crop in Egypt. It is one of the highest levels of protein supply for vegetarians, and has created a huge new market (Fageria, 2009).

The present work aimed to prepare and characterize natural HAp nanoparticles from fish back bone (F-HAp) and egg shell (E-HAp) and study their efficiency on cultivation and growth of soybean relative to effects of traditional fertilizer.

MATERIALS AND METHODS

2.1 Sample preparation

Nano hydroxyapatite was processed by extraction from two bio-wastes sources by using different routes.

Fish (Sander lucioperca) bone sample

Sander lucioperca back bones were prepared through three main stages of treatment: cleaning, deproteinization and calcination steps as shown in Fig. 1a. During cleaning step, bones were cleaned by boiling in distilled water for 2 h then washing carefully with running distilled water, to remove the entire meat from the bone. This step was repeated for several times until clear fish bones. In deproteinization step, bones were immersed in (1N) HCl solution with certain solid to liquid (S: L) ratio 1:2 for 1 h at room temperature.

Deproteinizated bones were boiled again with acetone and 1M NaOH for 1h to remove the remaining tissue then dried at 160 °C for 3h to remove moisture. Bones were crushed with a mortar and pestle then grounded in blender. Finally, grounded bones were calcined in electric furnace oven at 1100 °C with 7 h holding time (Venkatesan et al., 2011, Mondal et al., 2012).

-Egg shell sample

In similar manner as in fish bone treatment routes, egg shells were cleaned by boiling in distilled water for 1 h then dried in microwave oven for 30 min as shown in Fig. 1b. The dried egg shell crushed then powdered by using blender. Powdered egg shell was calcined in a muffle furnace at 900° C for 6 h to convert it into calcium oxide according to the equation 1,

$$CaCO_3 \rightarrow CaO + CO_2 \quad (1)$$

1.42 M calcined calcium oxide (CaO) was added to 500 ml of de-ionized water under vigorously stirred for 24 hat 20 ∘C temperature to react and form a suspension of Ca(OH)2,according to equation 2. Temperature was maintained by a thermostat-controlled water bath.

$$CaO + H_2O \longrightarrow Ca(OH)_2 \quad (2)$$

0.852 M of 85% H3PO4 was added drop by drop to $Ca(OH)_2$ solution at a rate of 1.5 ml/min. The reactants were stirred for further 24 h to aid the development stage, under continuous stirring. 0.28 MNH4OH was added to the HAp slurry to stabilize the pH of the super saturation solution to above 9 [Abidi et al., 2013).

$$Ca(OH)_2 + 6H_3PO_4 \longrightarrow Ca_{10}(PO_4)_6(OH)_2 + 18\,H_2O \quad (3)$$

2.2 Characterization

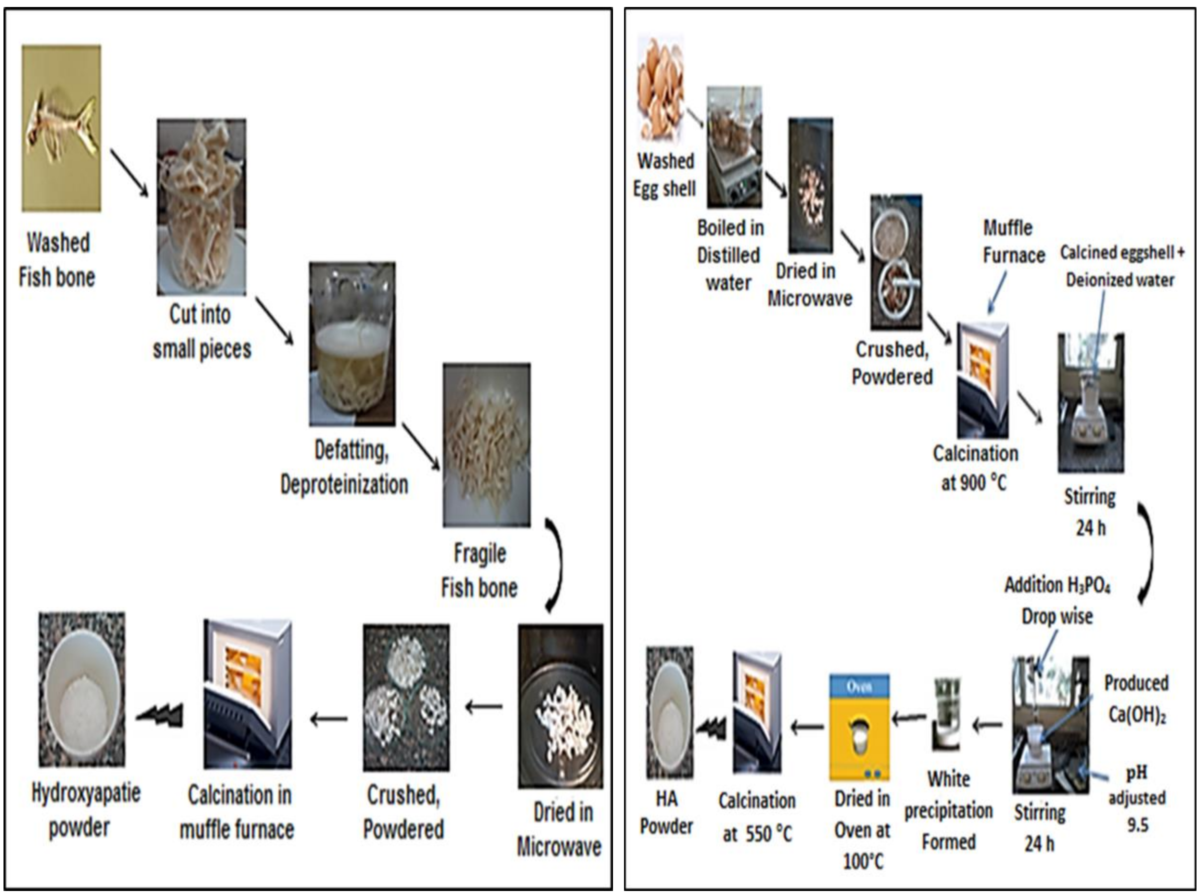
Structural compositions of synthesized powder were studiedby using Fourier Transform Infrared spectrophotometry(FTIR, Nicolet 6700 at 4 cm^{-1} resolution).Spectra were measured in the 4000-400 cm^{-1} region with samples dispersed in KBr pellets. The crystallinity and phase of synthesized powders were confirmed by X- ray diffraction (XRD) using D/Max-rAdiffractometer (Rigaku, Tokyo, Japan) with Cu radiation. Diffraction scans were recorded in the 10-60° angular range with scan range 1°/min.

Mean crystallite size (D) of the particles was calculated from XRD line broadening measurement using the Scherrer equation.

$$D = 0.89\,\lambda / \beta \cos\theta$$

Where λ is the wavelength of X-ray (0.154 nm), β is FWHM (Full width at half maximum) in radians, θ is the diffraction angle and D is the grain diameter.

Average sizes and Morphologies of powder samples were observed through transmission electron microscopy (TEM) analyses.



(a) (b)

Figure 1.Flow chart of steps for isolation of Hydroxyapatite from (a) Fish bone, (b) Eggshell.

2.3 Cultivation of soybean

Cultivation of soybean was performed in greenhouse in Faculty of Agriculture, Ain Shams University. Twenty soybean seeds were sowed in each 3-gallon plastic containers (plant pots) filled with clay soil in January, 2017. Four fertilizing schemes (treatments) were used in this study and each treatment applied to 3 containers (3 replicates). These treatments included tap water without fertilizer denoted W, synthetic fertilizer with regular P (triple super phosphate) denoted S, and synthetic fertilizer with nHAp from eggshell denoted E and from Fish bone denoted F.

Phosphorus fertilizer was applied at sowing and after17 and 40days as superphosphate (S) and fish nHAp (F) and egg nHAp(E) at the rate of 150kg /feddan.

Nitrogen fertilizer was applied as ammonium nitrate at the rate of 30kg/feddan in three equal doses at sowing and after17, 36 and 50 days from sowing.

Potassium fertilizer was applied as potassium sulphate at the rate of 50kg/feddan in three equal doses at sowing and after15, 36and 50 days from sowing. Irrigation was two times in a week in pot experiment.

2.4 Plant Laboratory analyses

E laboratory analyses were applied to characterize the chemical and total nutrient conditions. Leaves samples dried at 65°C then grounded. Adding10ml H_2SO_4 (98%) and $H_2O_2$to 0.5 g from dried grounded leaves until it become colorless then completing the volume to 50ml.

Chemical compositions were determined as follows:

-Total nitrogen was determined by micro Kjeldahl distillation method (VELP. Scientific, model UDK 127) using 5% boric acid and 40% NaOH.

-Total phosphorous was determined using spectrophotometer (Perkin Elmer Cb320002) at 840nm.

-Total potassium using flame photometer (Jenway PFF7).

Least significance difference (LSD) test used to separate significantly differing treatment means.

RESULTS AND DISCUSSION

3.1 Structural Analyses

Fig. 2a represents all characteristic absorption peaks of HAp. A major peak of PO_4^{3-} v_3 asymmetric stretching vibration band was centered as strong broad band at1100-960 cm^{-1} in both samples [Rocha et al., 2005]. In addition, detection of sharp band at 576.3 cm^{-1} represents to v_4 symmetric P-O stretching vibration of the PO_4^{3-} group [Varma and Babu, 2005]. Small peaks of OH^- groups were observed at 3573 and 633 cm^{-1} in both egg shell and fish bone samples. Analyzing XRD diffraction patterns, Fig. 2b confirms the structural compositions, phase and purity of extracted powder. XRD patterns recorded sharp peaks at 2θ=31.7°, 32.3° and 32.9° (JSCD of HAp 76-0694) were attributed to hydroxyapatite suggested that there producibility of hydroxyapatite crystallization with crystal size=10.98 nm for fish and 9.57 nm for egg shell.

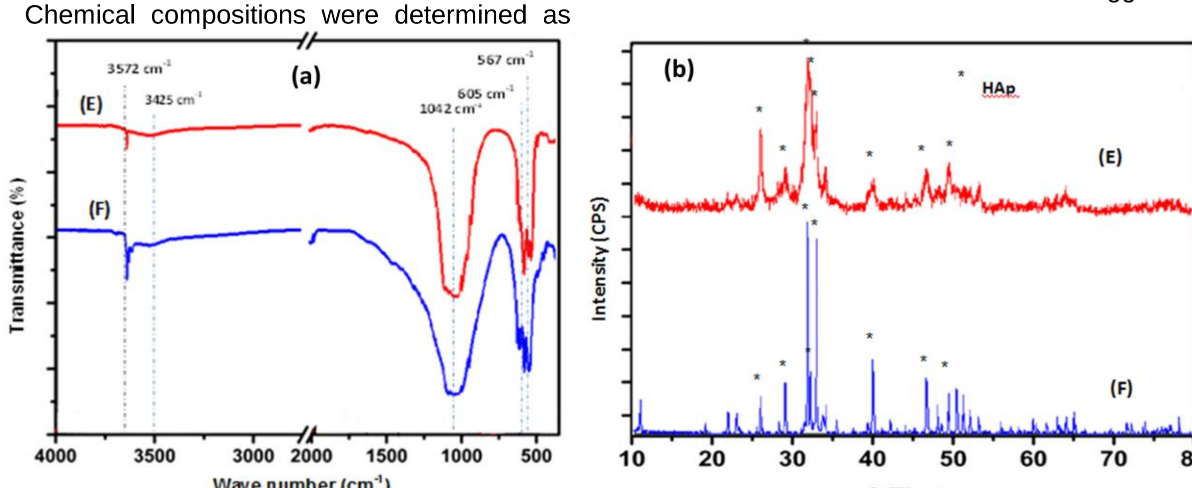


Figure 2. (a) FTIR spectra and (b)XRD patterns of HAp powder extracted from fish backbone (F) and from Egg shell (E)

It is well known that increasing temperature lead to increase peak's and decrease its width (Koutsopoulos, 2002) The intensities of the raw fish bone and eggshell were found to be dispersed by x- ray radiation with higher intensities and sharp peaks. Calcination of both samples at higher temperatures especially F-HAp, the subsequent peaks were highly intense and sharp, indicating the removal of organic portion (Glimcher, 1959).

Although calcination has no effect on the HA phase stability, it was found that the crystallinity of the powders is highly affected by the calcination temperature. Increasing the calcination temperature resulted in higher crystallinity of the powders (Teh et al., 2014). The results confirmed that both synthesized powder samples from fish bone and egg shell are one phase "pure HAp".

3.2 TEM Analysis

Fig. 3 a, b shows TEM pictures of synthesized HAp derived from fish bone (F) and egg shell (E). In Fig. 3a several shapes, rounded particles and plates, were observed with non-homogeneous particle size distribution, ranged from 8.76 to 80.00 nm. While egg shell HAp (Fig. 3b) showed nearly homogenous shape and particle size distribution, ranged from 22.50 to 46.70 nm.

The morphologies of calcined powder showed the coarsening of powder particles as the calcination temperature was increased(Teh et al., 2014).

3.3 Greenhouse test

Soybean was selected in this greenhouse study to evaluate the fertilizing effect of nHAp on a life cycle basis and compare with that of a regular P fertilizer TSP (Triple Superphosphate, $Ca(H_2PO4)_2$) (Liu and Lal, 2014). As can be observed on Table 1, after 7 weeks from sowing the average number of leaves in case of eggshell nHAp (E) and fish nHAp (F) are nearly similar but larger than triple superphosphate (S) and water without fertilizer (W). The greatest average number of pods and the highest length of shoot system were observed in case of using (F) while the shortest length of shoot recorded with (W). Both (E) sample and (S) sample are equal in the average length of root system followed by (F) and (W). The longest average length of leave was observed in case of (E) sample followed by (F), (S), and (W). The average width of leaves in case of (F) is the greatest, than (E), (S), and (W) samples. Obtained data resulted that growth rate of plants treated by nano-fertilizers was 1.88 times greater than that treated by triple super phosphate.

These increases in growth parameters may be attributed to the beneficial effects of nanoparticles which have high reactivity because of more specific surface area, more density of reactive areas or increased reactivity of these areas on the particle surfaces. These features simplify the absorption of fertilizers that was produced in nano scale. Moreover, nHAp led to stimulate the growth of the root zone allowing the plant to get nutrients and water to distant areas of metabolic activity(Soliman et al., 2016, Uarrota, 2010).

3.4 Effect of nHAp on macronutrient elements

The importance of adequate tissue p concentrations during early-season growth has been reported in many different crop species. The results indicate major differences in growth and p status in response of the four treatments to P supply.

Mean values representing the effect of applied treatments on N, P, and K concentration in the soybean plant 7 weeks from sowing under different treatments are presented in Table 2 and Fig. 4.

E treatments showed positive effect on N and K concentration. Mean values of %N and %K were 1.54 and 1.8%, respectively. The effect of E and F treatments on increasing P availability and concentration P was significantly increased in plant tissues were 0.312 and 0.404% compared to water without fertilizer treatment, respectively. Mineral nutrients in the form of nano-fertilizers can contribute to plant nutrition in two ways. The first is to use nanostructured elements incorporated in a carrier complex that may or may not be a nanomaterial, as is the case of nanoparticles of essential elements incorporated by absorption or adsorption in a matrix such as chitosan, polyacrylic acid, clay (Mohammad et al., 2017). The second is to use the element per Se in a nanostructured form (suspension or encapsulated), Both types of nano-fertilizers contributions have certain advantages, such as greater solubility and rapid absorption or less leaching, compared with traditional fertilizers. The first method is preferred because it provides greater control over the speed and timing of release of the nutrient element. For this reason, fertilizers efficiency is maintained at a low value, especially for nutrients applied in relatively high quantities such as N and P.

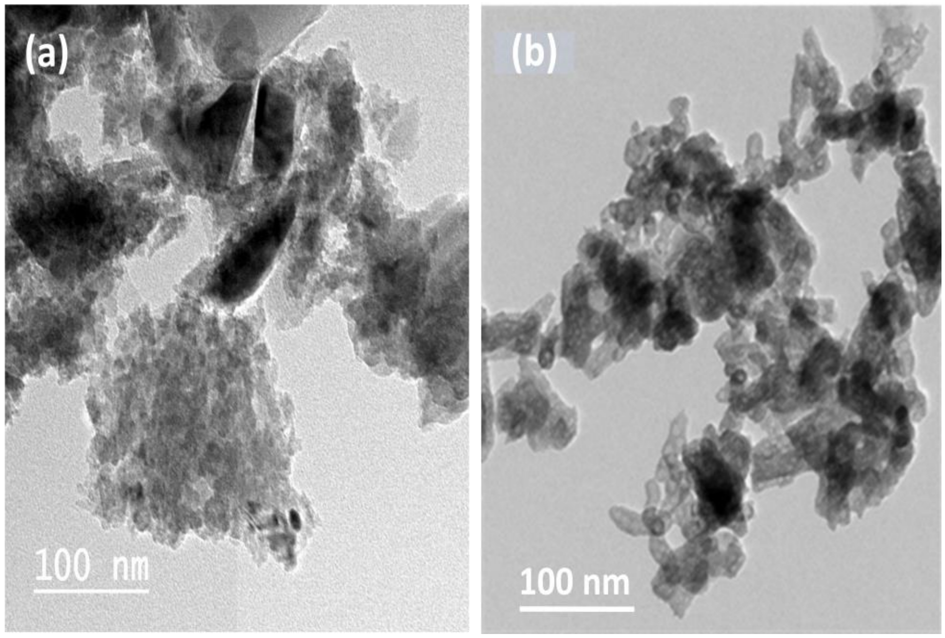


Figure 3.TEM images of (a) Fish -HAp and (b) Egg –Hap

Table.1.Average morphological measurements of soybean plant after 7 weeks from sowing under different treatments: water without fertilizer (W), synthetic fertilizer with triple super phosphate (S), synthetic fertilizer with nHAp from fish (F), and synthetic fertilizer with nHAp from egg shell (E).

	Sample treatment			
	W	S	F	E
Number of leaves	19 ± 1.5	21± 1.5	23±1.5	23±1.5
Number of Pods	7± 0.5	7 ± 0.5	10± 0.8	7± 0.5
Length of shoot (cm)	21± 2	40 ± 2	75± 2	72± 2
Length of roots (cm)	4 ± 0.3	5.5± 0.3	4.5± 0.3	5.5± 0.3
Length of leaves (cm)	2.2 ± 0.3	2.2 ± 0.3	3± 0.3	3.5± 0.3
Width of leaves (cm)	1.5 ± 0.2	1.6± 0.2	2.4± 0.2	2.2 ± 0.2

Table.2. Concentration of macronutrients percentages of soybean plant after 7 weeks from sowing under different treatments: water without fertilizer (W), synthetic fertilizer with triple super phosphate (S), synthetic fertilizer with nHAp from fish bone (F), and synthetic fertilizer with nHAp from egg shell (E).

	N%	P%	K%
Water without fertilizer (W)	1.35 ± 0.05	0.3060± 0.05	1.450± 0.05
Triple superphosphate (S)	1.275± 0.05	0.3055± 0.05	1.540± 0.05
Fish nHAp (F)	1.540± 0.05	0.404± 0.05	1.800± 0.05
Egg nHAp (E)	1.400± 0.05	0.3125± 0.05	1.455± 0.05
LSD 0.05	0.62	0.13	0.90

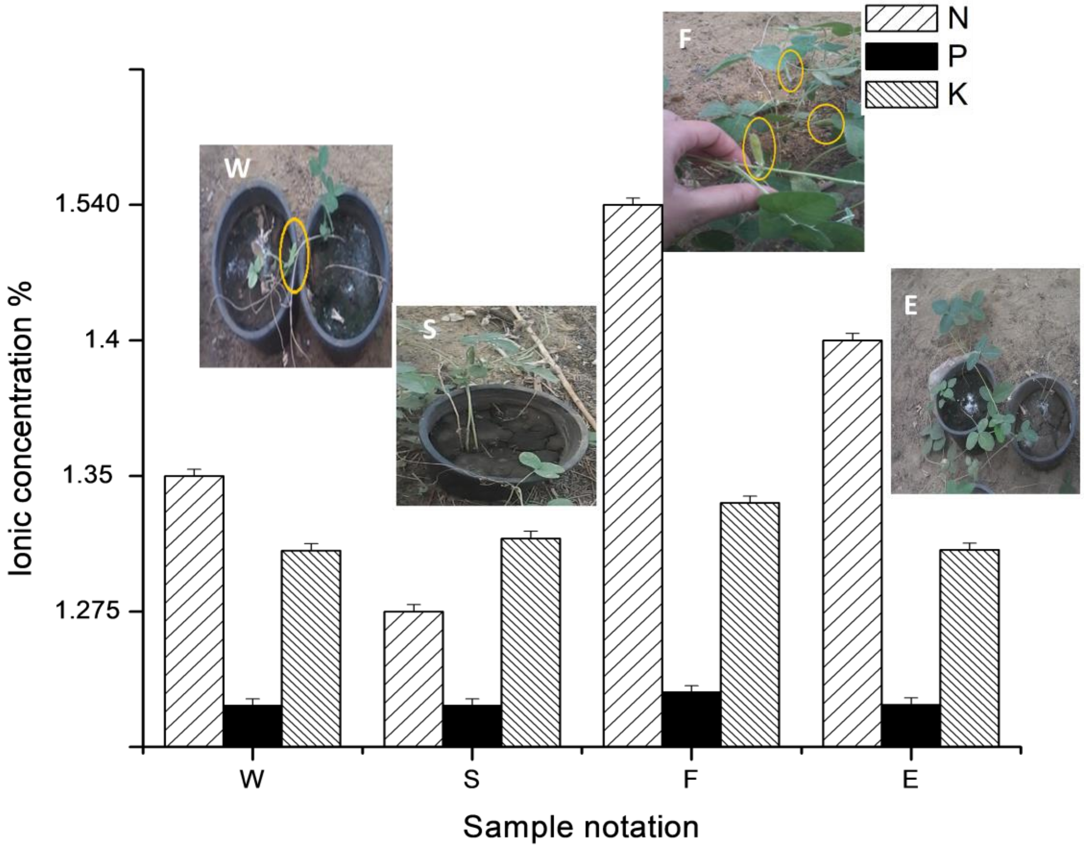


Figure 4.Concentration of macronutrients percentages of soybean plant after 7 weeks from sowing under different treatments: water without fertilizer (W), synthetic fertilizer with triple super phosphate (S), synthetic fertilizer with nHAp from fish bone (F), and synthetic fertilizer with nHAp from egg shell (E).

It is worth mentioning that in many cases, the problem associated with crop mineral nutrition is not the amount of one or more elements present in the soil but rather their availability to plants (Pilbeam, 2015). In a substrate or soil, the presence of an element essential for plants in the form of nano-fertilizers allow better dissolution, faster absorption and assimilation by the plant compared to traditional fertilizers. This has been demonstrated for N, P and K (Ditta and Arshad, 2016). To achieve this, nanoparticles containing the nutrients should idealy respond to any chemical or physical stimulation that indicates that to the plants requires mineral nutrients. Examples of these stimuli would be specific time periods or signals such as rhizosphere acidification or ethylene production by the roots, which occur when there is a deficiency of P or K.

The effect of treatments on plant biomass of shoot and seed production of soybean plants after 78 days was shown in Table 1. However, significant difference fresh shoot were found between different treatment and control. Increasing treatment (nHAp) from fish bone (F) a highly increased in plant biomass of shoots compared with different treatments followed by egg shell (E). In this way, plants that grow rapidly, with a higher uptake of phosphate and potassium, may store little P in their shoot and stimulate cluster-root formation. By contrast, species that have an inherently low growth rate may accumulate high levels of P in their shoot, even at a relatively low phosphate supply. it is therefore of interest to evaluate changes in P concentration and cluster-root formation in different plants with inherently different growth rates as affected by phosphate supply in their root medium.

Phosphorus contributes to many vital

functions in the plant, such as early root and seedling growth, improving winter hardiness, promotion of early heading and uniform maturity, seed formation and quality, and increased water-use efficiency. Plant height, grain yield, biomass yield and P uptake efficiency of soybean increases at high levels of P application (Manje et al., 2011). Phosphorous and potassium deficient plants often have slow growth, poor drought resistance, weak stems and are more susceptible to lodging and plant diseases [Jack and Sara, 2001)

The application of P on soybean increases the amount of N derived from the atmosphere by the soybean-Brady rhizobium symbiotic system (Chien et al., 1993, Sanginga et al., 1986). Nitrogen nutrition in soybean is ensured by di-nitrogen fixation and mineral nitrogen assimilation, which is important for high vegetative growth, high productivity and high seed protein content of soybean(Ronis et al., 1985). Only 25 to 65% of N in soybean dry matter originates from symbiotic nitrogen fixation, the remainder comes from soil-N [Harper, 1974). Previous work noted that soybean plants act as sinks for soil-N and effectively use N regardless of source (Varvel and Peterson, 1992).Therefore N fertilization could benefit soybean. Another work also found out that N fertilization of soybean increases seed protein or oil concentration (Varvel and Peterson, 1992). Starter N application is aimedat providing soybean with readily available soil-N during seedling development, and has been shown to increase soybean grain yield (Helms and Watt, 1991)

CONCLUSION

As a conclusion, production from the natural sources seems much simple and available than synthetic sources for HAp production. On the other hand, it must not be forgotten, that HAp production from calcitic (egg shells) source gives us nHAp production possibility for various applications. A simple, low cost, eco-benign method for the synthesis of Nano-hydroxyapatite of varying particle size, morphology and degree of crystallinity could be developed from fish bone and egg shell wastes. We can use the produced HAp in the agriculture field as a phosphorus fertilizer to increase rate of plant growth, biomass production and seed yield compared to traditionalist P treatment. Also nHAp minimizes the secondary contamination risks (e.g., eutrophication) and the delivery problems associated with solid phosphate. Since the method promotes the extensive use of waste egg shell this will contribute to minimize the pollution especially in the areas of hatchery and catering units. Field studies needed to confirm the fertilizing effect of nHAp on various plants and in various soil environments. More research is needed to systematically elucidate the interaction of nHAp with plants and soil. Field studies also required to approve the fertilizing effect of nHAp on various plants and in several soil environments. The eutrophication potential of nHAp needs to be especially addressed.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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