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Influence of Calcium Phosphate Nano Gel Fertilizer Composite on Enzymes, Biomolecules and Yield of *Abelmoschus Esculentus*

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Abstract

Increase in population accompanied by decrease in the availability of cultivable lands and limited supply of water resources necessitates the urge to improve agricultural productivity in the available land. In the present study, the influence of Calcium Phosphate Nano Gel (CAPNG), urea, diammonium phosphate and dipotassium hydrogen phosphate (FC) and Calcium Phosphate Nano Gel Fertilizer Composite (CAPNGFC) on germination, specific activity of amylase, protease, nitrate reductase, carbohydrates, proteins, free aminoacids and yield performance of *Abelmoschus esculentus* was evaluated. CAPNG was prepared by coprecipatation method. CAPNG was characterized by SEM, FTIR and XRD. Enzyme activities were assessed by the micromoles of product formed/min/mg protein and expressed in Units/mg protein. Aggregated network of CAPNG was observed with the size of 100 nm. The most intense peaks in XRD were in the range of 30 to 35°, which coincides with the peak of the amorphous nature. FTIR spectra showed peaks at 603.68 cm^{-1} corresponding to phosphate. Germination was found to be stimulated by CAPNG from 87% in control to 95%. Amylase activity was stimulated by CAPNGFC and it was increased from 2.31x 10^{-2} Units/mg in control to 5.74x 10^{-2} Units/mg on germination. Protease activity was also increased from 2.56x 10^{-1} Units/mg in control to 5.87 x 10^{-1} Units/mg in CAPNGFC. No substantial increase in peroxidise activity was recorded ascertaining the absence of any stress imposed on the plant. Nitrate reductase activity was 2.50 x 10^{-2} Units/mg protein in control and 4.69 x 10^{-2} Units/mg protein in CAPNGFC. Weight /fruit was 65 g in CAPNGFC where 59 g in control. Thus the study proved that CAPNGFC had a significant positive influence on yield of *Abelmoschus esculentus.*

Keywords: amylase, coprecipitation, protease, SEM, Nitrate reductase

Introduction

India is the second most populous country in the world. The population of India on March 2011 was 121,01,93,422. India with 2.4% of the world's surface area accounts for 17.5% of its population (Chandramouli, 2011). Agriculture is the backbone of rural economy and employment. Traditionally it is the largest contributor to India's GDP accounting for about approximately 50%. Today agriculture has the smallest share in GDP of only about 14%. It can create demand in other sectors and it contributes indirectly to India's GDP growth. Agriculture sector needs to grow at least by 4 percent for the economy to grow at 9 percent. Fluctuations in agricultural production can have large and significant impact on overall GDP growth. Low yield per unit area has become a regular feature of Indian agriculture in recent years (Chakrabarty, 2011).

Agricultural productivity relies on many factors including the type of soil, seeds, optimum dose fertilizers, their mode of application, availability of water for irrigation and climatic factors. To increase productivity farmers apply excess volume of fertilizers. Current practice of fertilizer application includes broadcasting, banding, side-dressing and dusting. Localized application of large volumes of fertilizer in the form of ammonium salts, urea, and phosphate compounds are harmful (Wilson *et al*., 2008). Only a certain concentration of fertilizer is available for plants and the rest is washed away as run-off due to dissolution in soil moisture and leaching. This adds to ground water pollution (Tilman *et al.,* 2002). In addition placing large amounts of fertilizer near the seeds cause salt stress and damage and reduction in moisture content available for seedling emergence. Optimum concentration of fertilizer should be supplemented to soil in a proper method. Therefore there is a pressing need to current fertilizer deployment.

Nanotechnology offers an attractive alternative to challenges in all branches of Science and Technology. Nanoparticles have size ranging from 10 to 1000 nm in any one dimension (Nakache *et al.*, 1999). As all properties of materials are size dependent, nanoparticles have unique properties and they offer great promise for the improvement of agriculture (Navrotsky, 2000; Kuzma, 2007; Baruah and Dutta, 2009). Their high surface volume ratio can assist the controlled release of agrochemicals for nutrition, delivery of nanocides–pesticides, stabilization of biopesticides and micronutrients for efficient use. Use of nanosized aqueous dispersion enhance the bioavailability of pesticides (Storm *et al*., 2001). The nanomaterial used for agricultural purpose must be nontoxic, biocompatible and biodegradable and it should have safe and easy mode of application (Green, and Beestman, 2007).

Materials and Methods

Preparation of Calcium Phosphate NanoGel (CAPNG)

***Solution A*:** 18.36 g of disodium hydrogen phosphate, 12.5 g of Sodium hydroxide ,7.5 g of sodium bicarbonate were dissolved in 325 ml of distilled water.

***Solution B*:** 10.75 g of calcium nitrate was dissolved in 125 ml of distilled water.

Solution A was added to solution B and the total volume was made upto 1.0 L with distilled water and sonicated for 15 minutes.

Preparation of Fertilizer Composite (FC)

10 mg of urea, 20 mg of diammonium phosphate and 20 mg of dipotassium hydrogen phosphate/1l of distilled water.

Preparation of Calcium Phosphate Nano Gel Fertilizer Composites (CAPNGFC)

950 ml of Fertilizer Composite was added to 50 ml of CAPNG and made upto 1.0 L with distilled water.

Characterization of CAPNG

Size and morphology of CAPNG was recorded in Scanning Electron Microscope.FTIR Spectrum was recorded in Shimadzu FTIR Spectrophotometer.XRD was measured in Bruker D8 powder diffractometer.

Seed Germination and Biochemical Analysis

Certified seeds were purchased from Central Agrobiotech, Tirumangalam, TamilNadu, India. Germination percentage (ISTA, 1995), Amylase activity (Miller, 1972), Protease activity **(**Folin and Ciocalteau, 1929), Peroxidise activity (Chance and Maehly, 1955), Nitrate reductase activity (Camm and Stein, 1974), Total carbohydrates (Hedge and Hofreiter, 1962), and Proteins (Lowry's *et al*., 1951) of leaves on 10th day,20th and 30th day of plant growth were assessed.

Statistics

The data represented is a mean of four replicates. It was statistically analyzed by Two way ANOVA. Tukey-Kramer multiple comparison test was used to determine the stastical significance between the means (SAS, 1998).

Results and Discussion

Calcium phosphate has been used for more than 30 years to deliver genetic material to mammalian cells. Apart from using calcium phosphate nanoparticles (CAP) in gene delivery systems, it has also been utilized as an adjuvant for protein-based vaccines (He *et al*., 2000). Among the various methods of manufacture of calcium phosphate nanoparticles, the co-precipitation method is easier and can be utilized in the industrial scale (Shantanu Tamuly and

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Mumtesh Kumar Saxena, 2012). The following equilibrium takes place during the precipitation process.

$H_2PO_4^-H^+ = HPO_4^{2-} + 2H^+$,

$Ca^{2+} + H_2PO_4^- = CaH_2PO_4^+$,

$Ca^{2+} + H_2PO_4^{2-} = CaH_2PO_4$.

The consumption of phosphate ion in the formation and growth of brushite would decrease the pH of the solution because of the release of the hydrogen ions from $H_2PO_4^{2-}$ dissociation. The higher the initial reagent concentration, the higher was the concentration of the remaining acid and the lower was the nucleation pH (Hlabse and Walton, 1965; Boistelle and Lopez-Velero, 1990; Christoffersen *et al*., 1990).

Calcium phosphate Nano Gel was synthesized by coprecipitation showed agglomeration with a particle size around 100nm as represented in Figure 1. Large agglomerates of several hundred nanometer of calcium phosphate nano material was also observed by Lis Sopyan *et al*., (2008). XRD spectrum of CAPNG is given in Figure 2.The most intense peaks in XRD are in the range of 30 to 35°, which coincides with the peak of the amorphous nature.

FTIR Spectrum of CAPNG is shown in Figure 3. The peaks observed at 603.68 cm^{-1} is ascribed to the triply degenerate v4 bending vibration of the PO_4^{3-}. Peak at 144.58 cm^{-1} confirms the alkalinity of the gel prepared by coprecipitation. This might be due to the solubility of atmospheric CO_2 yielding CO_3^{2-}. The peak at 3313 cm^{-1} attests the presence of octacalcium phosphate. The bands in the region 900-1200 cm^{-1} is due to í1 and í3 phosphate (Beganskiene, 2003).

The influence of fertilizers on germination of seeds is represented in Table.1.Germination was found to be stimulated by calcium phosphate Nano Gel from 87% in control to 95%. In CAPNG applied plants germination was 89%. CAPNG has a stimulatory role than FC. The stimulatory effect of CAPNGFC might be due the effect of both calcium and phosphorus and nitrogen, potassium. Calcium restricts peroxidation of lipids and prevents membrane deterioration. In addition it plays a key role in maintaining the structural integrity of membranes. Phosphorus is an essential mineral for plant growth and development next to nitrogen. Readily available soluble phosphorus stimulates plant growth (Legge *et.al*.,1982).

Influence of fertilizers on amylase activity is given in Table 2. Amylase activity was stimulated by CAPNGFC and it was increased from 2.31x 10^{-2} Units/mg in control to 5.74x 10^{-2} Units/mg on germination analysis. FC alone expressed specific activity of 5.65 x 10^{-2} Units/mg and CAPNG alone showed specific activity of 5.76 x 10^{-2} Units/mg. Increase in amylase activity might be due to the stimiulatory influence of calcium and phosphate on the translation of amylase genes

Influence of fertilizers on protease activity is given in Table 3. Protease activity was also increased from 2.56x 10^{-1}Units/mg in control to 5.87 x 10^{-1} Units/mg in CAPNGFC where as the activity was 3.79 x10^{-1} Units/mg in FC and 2.86 x 10^{-2} Units/mg in CAPNG respectively (Table 2). The supporting effect observed in CAPNG and CAPNGFC when compared with control is due to a simultaneous uptake and translocation of calcium and phosphate (Svend Tage Jakobsen, 1993). The capability of plants to absorb calcium ions is then increased by application of soluble phosphate fertilizers.

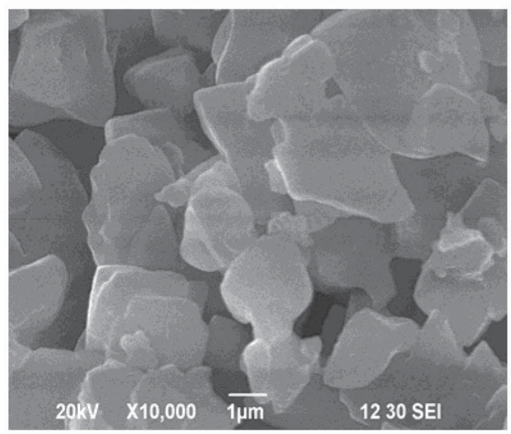

Figure 1: Sem Image Of Calcium Phosphate Nano Gel

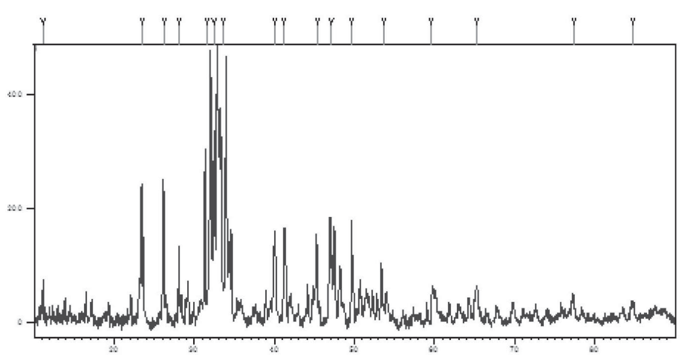

Figure 2: Xrd Spectrum Of Calcium Phosphate Nano Gel

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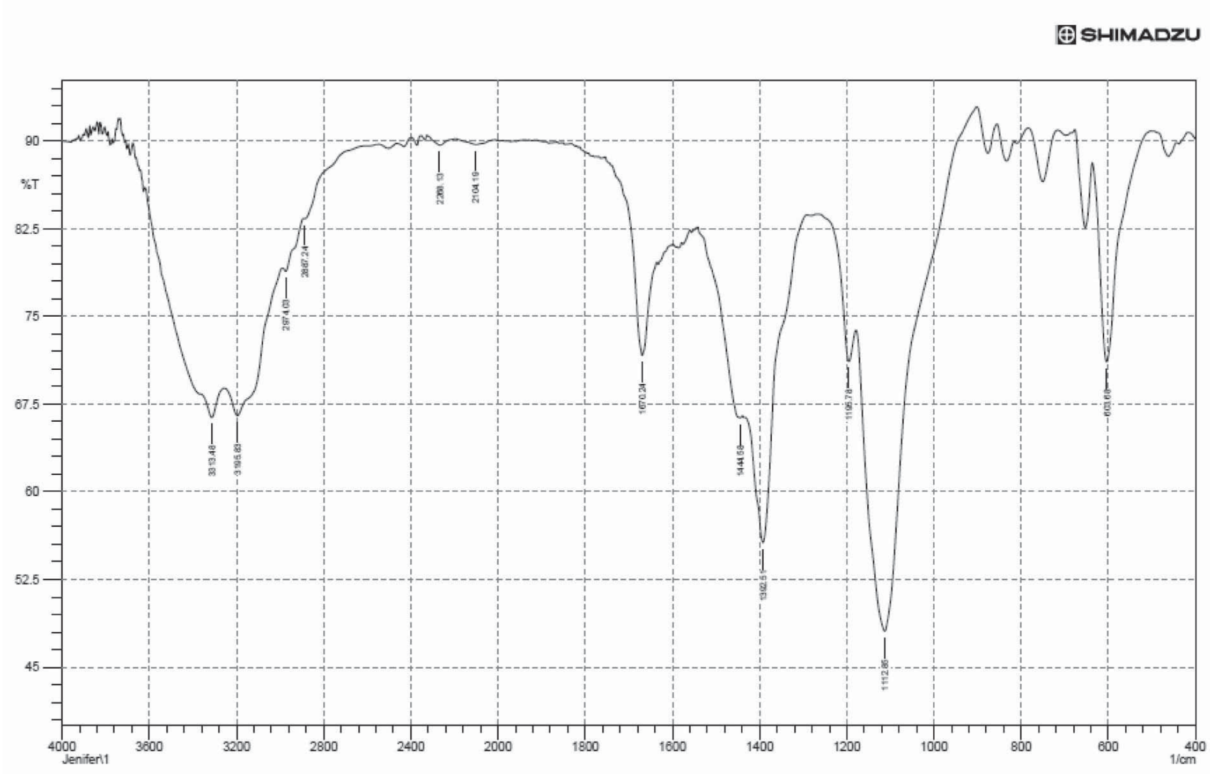

Figure 3: Ftir Spectrum Of Calcium Phosphate Nano Gel

Influence of fertilizers on peroxidise activity is given in Table 4. No substantial increase in peroxidise activity was recorded ascertaining the absence of any stress imposed on the plant. As oxidative enzymes, peroxidases utilize H_2O_2 as a final electron acceptor in order to generate radical species which then act as catalysts for biological reactions (Passardi *et al*., 2007). Through this process, peroxidases aid in the degradation of lignin, the removal of hydrogen peroxide within the cell, and the oxidation of toxic substances (O'Brien, 2000; Erman and Vitello, 2002). There are conflicting reports on the influence of fertilizers on peroxidise activity. Several other studies have shown that reduction in peroxidise activity could be attributed to nitrogen inputs which limit carbon mineralization, microbial growth, and enzyme production.(Saiya-Corka *et al*., 2002; Henry, 2005; Allison, 2005).

The effect of fertilizers on nitrate reductase activity is given in Table 5. During germination, nitrate reductase activity was 2.50 x 10^{-2} units/mg protein in control and 4.69 units/ mg protein in CAPNGFC applied plant.On 10^{th} day the activity was 6.71 x 10^{-2} in control and 9.97 x 10^{-2} in CAPNGFC. The enzyme is responsible for nitrate assimilation. The expression of nitrate reductase genes and the rate of nitrate reduction in the plant tissues can be considerably modified by some factors such as light, presence of NO_3 ions, day length, sugar level or certain nitrogen metabolites (Lillo *et al*., 2004). Moreover, the form of nutritive nitrogen also influence the activity of enzyme.

The effect of fertilizers on carbohydrate is given in Table 6. Towards the beginning of yield stage, foliar carbohydrate concentration was reduced from 6.51x 10^{-1} mg/g of fresh tissue in control to 7.59 x 10^{-1} mg/g of fresh tissue in CAPNGFC applied plants. This might be due to the greater accumulation of photosynthates in CAPNGFC applied plants.

The effect of fertilizers on proteins is given in Table 7. Protein concentration was 3.12 x 10^{-2} in control and was markedly increased to 7.49 x 10^{-2} in CAPNGFC on 20^{th} day of analysis. Increased availability and utilization of nitrogen and other nutrients could have favoured the assimilation of nitrogen, synthesis of aminoacids and finally translation . As it is clear that irrespective of the increase in protease activity protein concentration was high.Increase

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Table 1: Influence Of Fc, Capng And Capngfc On Germination Of Crop Plant (%)

Plant	Control	Fc	Cap	Capngfc
Abelmoschus esculentus	$87^{a} \pm 5.6$	$87^{a} \pm 5.9$	89 ± 4.2	95 ± 2.5

**Means sharing the common superscript are not significantly different from each other (Tukey's HSD, P<0.05)*

Table 2: Influence Of Calcium Phosphate Nano Gel Fertilizer Composite On Amylase Activity (μmoles Of Reducing Sugars Released/Min/ Mg Protein)

Days	Control	Npk Mix	Capng	Capngfc
Germination	2.31×10^{-2}	4.89×10^{-2}	3.52×10^{-2}	5.74×10^{-2}
10	6.5×10^{-2}	5.65×10^{-2a}	5.76×10^{-2a}	7.59×10^{-2}
20	4.83×10^{-2}	6.5×10^{-2}	5.98×10^{-2}	6.89×10^{-2}
30	7.0×10^{-2}	6.58×10^{-2}	6.23×10^{-2}	5.13×10^{-2}

**Means sharing the common superscript are not significantly different from each other (Tukey's HSD, P<0.05)*

Table 3: Influence Of Calcium Phosphate Nano Gel Fertilizer Composite On Protease Activity (μmoles of amino acid released/min/mg protein)

Days	Control	Fc	Capng	Capngfc
Germination	2.56×10^{-1}	3.79×10^{-1}	2.86 X 10-1	5.87x 10-1
10	6.58×10^{-2}	5.41×10^{-2}	4.98×10^{-2}	9.86×10^{-2}
20	3.12×10^{-2}	6.91×10^{-2}	6.12×10^{-2}	7.49×10^{-2}
30	6.87×10^{-2a}	7.13×10^{-2}	6.74×10^{-2a}	8.59×10^{-2}

**Means sharing the common superscript are not significantly different from each other (Tukey's HSD, P<0.05)*

Table 4: Influence Of Calcium Phosphate Nano Gel Fertilizer Composite On Peroxidase Activity (μmoles of purpurogallin formed /min/mg protein)

Days	Control	Fc	Capng	Capngfc
Germination	4.71×10^{-2}	4.95×10^{-2}	5.1×10^{-2}	5.62×10^{-2}
10	5.54×10^{-2}	5.72×10^{-2}	6.04×10^{-2}	5.89×10^{-2}
20	2.22×10^{-2a}	2.33×10^{-2}	2.17×10^{-2a}	2.54×10^{-2}
30	2.52×10^{-2}	2.78×10^{-2}	2.69×10^{-2}	2.49×10^{-2}

**Means sharing the common superscript are not significantly different from each other (Tukey's HSD, P<0.05)*

Table 5: Influence Of Calcium Phosphate Nano Gel Fertilizer Composite On Nitrate Reductase Activity (μmoles of nitrate released /min/ mg protein)

Days	Control	Fc	Capng	Capngfc
Germination	2.50×10^{-2}	5.76×10^{-2}	4.74×10^{-2}	4.69×10^{-2}
10	6.71×10^{-2}	7.87×10^{-2}	6.72×10^{-2}	9.97×10^{-2}
20	7.45×10^{-2}	8.54×10^{-2}	6.91×10^{-2}	9.98×10^{-2}
30	3.84×10^{-2}	5.85×10^{-2}	5.98×10^{-2}	6.73×10^{-2}

**Means sharing the common superscript are not significantly different from each other (Tukey's HSD, P<0.05)*

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Table 6: Influence Of Calcium Phosphate Nano Gel Fertilizer Composite On Total Carbohydrates (mg/g)

Days	Control	Fc	Capng	Capngfc
Germination	2.31×10^{-2}	3.52×10^{-2}	4.89×10^{-2}	5.74×10^{-2}
10	6.5×10^{-2}	5.76×10^{-2}	5.95×10^{-2}	7.59×10^{-2}
20	4.83×10^{-2}	5.98×10^{-2}	6.5×10^{-2}	6.89×10^{-2}
30	7.0×10^{-2}	6.23×10^{-2}	6.78×10^{-2}	5.13×10^{-2}

**Means sharing the common superscript are not significantly different from each other (Tukey's HSD, P<0.05)*

Table 7: Influence Of Calcium Phosphate Nano Gel Fertilizer Composite On Proteins (mg/g)

Days	Control	Fc	Capng	Capngfc
Germination	2.56×10^{-2}	3.79×10^{-2}	2.86×10^{-2}	5.87×10^{-2}
10	6.58×10^{-2}	5.41×10^{-2}	4.98×10^{-2}	9.86×10^{-2}
20	3.12×10^{-2}	6.02×10^{-2}	6.91×10^{-2}	7.49×10^{-2}
30	6.87×10^{-2a}	6.70×10^{-2a}	7.13×10^{-2}	8.59×10^{-2}

**Means sharing the common superscript are not significantly different from each other (Tukey's HSD, P<0.05)*

in foliar protein concentration by fertilizer application was supported by Brohi *et al*., (2000) and Atia (2005). This clearly indicated greater mobility of biomolecules to the seeds. Flower setting was earlier by three days in CAPNGFC applied plants and the yield performance was homogeneous. Nitrogen is a part of chlorophyll, the green pigment of the plant that is responsible for photosynthesis. Helps plants with rapid growth, increasing seed and fruit production and improving the quality of leaf and forage crops. Phosphorus Effects rapid growth, encourages blooming and root growth. Calcium, an essential part of plant cell wall structure, provides for normal transport and retention of other elements as well as strength in the plant. It is also thought to counteract the effect of alkali salts and organic acids within a plant.

Weight /fruit was significantly higher than control. In CAPNGFC applied plants bud initiation occurred in 32 days and flower formation in 38 days.Average weight of fruit was 65 g. But in control plants, initiation of bud was observed on 36^{th} day and , flower formation in 43^{rd} day. Average weight of fruit was 59 g in control. Thus the study proved that CAPNGFC had a significant positive influence on the biochemical and yield characteristics of *Abelmoschus esculentus***.**

Conclusions

Plant productivity can be improved well with the application of CAPNGFC in the industrialization era where productivity should be increased in the limited availability of land and water resources.

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