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Synthesis, characterization of urea coated hydroxyapatite nanoparticles and their effects on seed germination in two varieties of *Brassica juncea* l.

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Abstract
Selection of an optimum fertilizer is mandatory step for proper growth and development of a crop. Many chemical and organic fertilizers have negative impacts on environmental, and human health. Slow releasing fertilizers are recently used fertilizers, urea coated hydroxyapatite nanoparticles are one of them. These release Nitrogen slowly into the soil, and have no side effects on humans and environment. In the present investigation, these nanoparticles were synthesized, and characterized by using X-ray diffraction, FESEM, and FTIR techniques. Then, their effects at different concentrations (250 ppm, 500 ppm, 750 ppm, 1000 ppm) were observed at seed germination attributes of two varieties of *Brassica juncea* L. i.e. GIRIRAJ, and NRC-BH 101. Those were also compared with control plants and plants grown in treatments with the same concentrations of urea. Results revealed that synthesized nanoparticles were 23-32 nm in size, and for all the parameters, seed germination was the maximum at treatment with 500 ppm of urea coated hydroxyapatite nanoparticles when compared to others while higher concentration of these nanoparticles (1000 ppm) lower seed germination attributes. All the results were significantly compared.

Keywords: soil fertilizers, urea coated hydroxyapatite nanoparticles, seed germination *etc*

Introduction

Brassica juncea L., belongs to family Cruciferae, is an oil seed crop, which is generally known as rapeseed mustard. It is one of the economically important crops which is grown in more than 50 countries in the world (Woods *et al.,* 1991) [16].

For the proper growth and development of a crop, soil fertility plays important role. Nitrogen (N) is of prime importance for growing plants as constituent of proteins, nucleic acids, and chlorophylls (Barita, Y. *et al*., 2018) [1]. Plants uptake N from soil organic matter, N fixation, irrigation water, atmospheric dumping, and fertilizer application. Applying N fertilizers in overflow is a common implementation to secure plants. Excessive Nitrogen cannot be utilized by plants (Hopkins, B.G., 2020) [7].

Application of fertilizers is delicate issue for sustainable agriculture to lower the negative effects of farming on the surrounding environment (Zebarth, B.J. *et al*., 2009) [17]. Green leafy vegetables carry high nitrate levels (Prasad, S. *et al*., 2008) which may cause severe malfunctioning in humans (Mensinga *et al*., 2003) [11]. The use of large number of chemical fertilizers have resulted in the development of environmental pollution.

Recent studies have shown that slow-release fertilizers are effective of delivering their nutrients moderately over a certain period of time. These slow release fertilizers increase nutrient uptake proficiency of plants, lower continual application, decrease in money and labour, and upgrade storage and managing properties (Liu *et al*., 2011) [11].

Manufacturing of novel and innovative slow releasing fertilizers using nanotechnology is required to resolve the issues of nutrient losses because of their nanoscale size and high surface to volume ratio (DeRosa *et al*., 2010; Kottegoda *et al.*, 2011; Gunaratne *et al*., 2016; Pulimi and Subramanian, 2016; Dimkpa and Bindraban, 2018; De Silva *et al*., 2020) [3, 8, 9, 12, 4, 2].

Urea coated hydroxyapatite nanoparticles are one of those slow releasing fertilizers. It has been studied that these nanoparticles release N slowly than conventional urea. These nanoparticles are widely renowned for their intrinsic biocompatibility and biodegradability, being the main component of human bones and teeth. So, use of these nanoparticles as fertilizers should not raise any concern on human and environmental health (Gomez-Morales, J., *et al*., 2013; Tampieri, A., *et al.*, 2016; Sprio, S., *et al.*, 2017.) [15, 14].

In the present investigation, urea coated hydroxyapatite nanoparticles were synthesized, and characterized. Effects of those synthesized nanoparticles were observed on different parameter of seed germination for 2 varieties of *Brassica juncea* L. i.e. GIRIRAJ, and NRC-BH 101.

Materials and Methods

Synthesis of hydroxyapatite nanoparticles (HANPs)

Hydroxyapatite nanoparticle were synthesized using the method of Sandhofer *et al*., 2015. All the reagents (Calcium acetate, Orthophosphoric acid, and Ammonium hydroxide) were used of analytical grade. All solutions were prepared in double distilled water. Nanoparticles were prepared by adding solution of H_3 PO_4 (0.21 M) drop by drop into solution of Ca $(CH_3COO)_2$ (0.35 M) at room temperature. For the experiment, pH was maintained at 10 by the addition of NH_4OH. The reaction compound was kept under stirring at room temperature overnight, then the stirring was suspended and the compound was left standing still for 2 h. The reaction mixture was finally centrifuged at 6000 rpm for 7 min and the pellet was continuously washed and suspended in double distilled water up to 10,000 mgL−1

Synthesis of urea coated hydroxyapatite nanoparticles (U-HANPs)

Urea coated Hydroxyapatite nanoparticles were synthesized by using method of Kottegoda *et al*., 2013 [9]. For this, saturated urea solution, HANPs, and double distilled water were used. HA nanoparticles (25 g) prepared as described above were diffused in distilled water (100 ml) under ultrasonic mixing (30 kHz for 45 min). The obtained HA nanoparticle were dispersed at magnetic stirrer in a saturated urea solution (100 ml) at 25°C for 12 h. The reaction mixture was let to settle and the extra liquid was poured out. The by-product was washed with distilled water to draw out extra urea, and dried at 50°C for 7 h. The white powder so obtained was grinded, collected and characterized.

Characterization of U-HA nanoparticles

The synthesized sample was characterized by using following techniques-

a. X-ray diffraction (XRD)

X-Ray Diffraction analysis of synthesized sample was obtained by X-pert pro diffractometer. X-ray diffraction (XRD analysis) is a unique method in determination of crystallinity of a compound. The measurements were taken for powder X-ray diffraction (XRD) a Philips 1710 X-ray diffractometer with CuKα radiation (λ=1.5418 Å) operating at 30 KV and 20 mA was used. Pattern was recorded for the angle (2θ) ranging from 5 to 80° at a scanning rate of 3°/second. The results were compared with the values of standard substances.

The average size of crystal domains along the apatite axis directions (D $_{(002)}$) and (D $_{(310)}$) was calculated by applying Scherrer's equation:

$$D_{[hkl]} = \frac{0.9\lambda}{cos\theta\sqrt{(\Delta_r^2) - (\Delta_0^2)}}$$

b. Field emission scanning electron microscope (FESEM)

Surface morphology was studied using FESEM (Field Emission Scanning Electron Microscope) model (NOVA NANOSEM 450) operated at 18KV.

c. Fourier transform Infra-Red (FTIR) spectroscopy analysis

FTIR analysis were carried out on a Nicolet iS5 spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a resolution of 2 cm^{-1} by accumulation of 64 scans covering the 4000 to 400 cm^{-1} range, using a diamond ATR accessory model iD7.

Plant experiments

a. Plant source and surface sterilization

Seeds of two verities (Giriraj and NRC-BH 101) of *Brassica juncea* L. were obtained from Rajasthan Agricultural Research Institute (RARI), Durgapua, Jaipur, Rajasthan, India. Variety Giriraj was given name S1 while variety NRC-BH 101 was given name S2. Seeds of both the varieties were washed with running tap water. Those were surface sterilized with 5% NaOCl for 5 minutes and then washed repeatedly for two to three times with distilled water to prevent fungal/bacterial contamination. Filter papers were also sterilized in autoclave to reduce any chances of microbial growth. Then seeds were surface sterilized in 0.01% mercuric chloride ($HgCl_2$) solution for 2 min followed by washing with autoclave water and dried on sterilized filter paper.

b. Seed germination protocol

Seeds were germinated in sterile glass Petri dishes of 15 cm diameter lined with filter paper circles moistened with control and four different concentrations (250 ppm, 500 ppm, 750 ppm, and 1000 ppm) of each of urea and U-HANPs. Nearly 10 seeds were sown in each Petri dish and incubated in growth chamber set at 25 ± 2°C for 7 days and each treatment was replicated thrice. Occurrence of Germination was considered when roots were 2mm long. Germination percentage was recorded in every 24h, till the end of experiment. Distilled water was used as control. According to treatments, for both the species, Petri plates were named as mentioned in Table 1.

Table 1: Name of different treatments given for the experiment.

Treatment	Distilled water	Urea 250 ppm	Urea 500ppm	Urea 750ppm	Urea 1000ppm	UHANP 250ppm	UHANP 500ppm	UHANP 750ppm	UHANP 1000ppm
Name to Petri-dishes	P_1 (Control)	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9

Observation of seed germination attributes

a. Germination percentage

A mean of 10 seeds were taken and seed germination percentage was calculation by following equation-

Germination percentage = (Total number of seeds germinated × 100)/Total number of seeds

b. Root length and shoot length

The length of primary roots and shoots of seedlings were recorded at 7th day of germination. Mean values were calculated for both root and shoot length and values were expressed in cm.

c. Seedling height

Heights of seedling were recorded at 7th day of germination. Mean values were calculated and expressed in cm.

d. Fresh biomass

The Fresh Weight was recorded from the 7th day old seedling. The whole seedling was surface dried with the blotting paper and their fresh weights were recorded.

e. Dry biomass

The same seedlings were taken and dried in oven for 24 h at 80°C and weighed again. This represented the dry matter.

Data analysis

All the experiments were carried out with 3 triplicates. The results for each parameter were calculated as mean value with standard deviations of each triplicates. The obtained data were analyzed by descriptive analysis. The statistical significance of the treatments was evaluated by one-way analysis of variance (ANOVA). Means were compared, according to Fisher's statistical test by least significant difference.

Results

Characterization of UHANPs

a. XRD

XRD pattern of the urea-modified HA nanoparticles Indicated the presence of peaks due to UHANPs. The main compound diffraction peaks corresponding with d spacing as mentioned in Table 2.

Sharp single peaks show that major compounds of Calcium phosphate and complex with nitrogen and urea particles majorly at 100% intensity. Remaining part of phosphors and nitrogen complex shown in different peaks of XRD pattern.

Table 2: The main compound diffraction peaks corresponding with d spacing by XRD method.

Compound name	Obs. Max	d (Obs. Max)	Net Height	FWHM	Intensity
	2-Theta°	Angstrom	cts	2-Theta°	%
Urea	22.4899	3.95344	2224.09	0.1476	100.00
Calcium phosphate complex	24.8876	3.57773	263.77	0.1476	11.86
Calcium phosphate complex	26.1309	3.41026	116.77	0.2952	5.25
Urea	29.5101	3.02699	335.07	0.0886	15.07
Calcium phosphate	31.8967	2.80575	448.80	0.1181	20.18
Urea	35.7779	2.50978	176.94	0.1771	7.96

b. FESEM

By using results of FESEM studies, surface morphology and size distribution of UHANPs were determined. Images of UHANPs with different magnification are shown in Figure 1.

The results revealed that the average particle size of UHANPs ranges from 23-32 nm. It was also observed that FESEM results are in good agreement with size distribution of UHANPs measured by XRD.

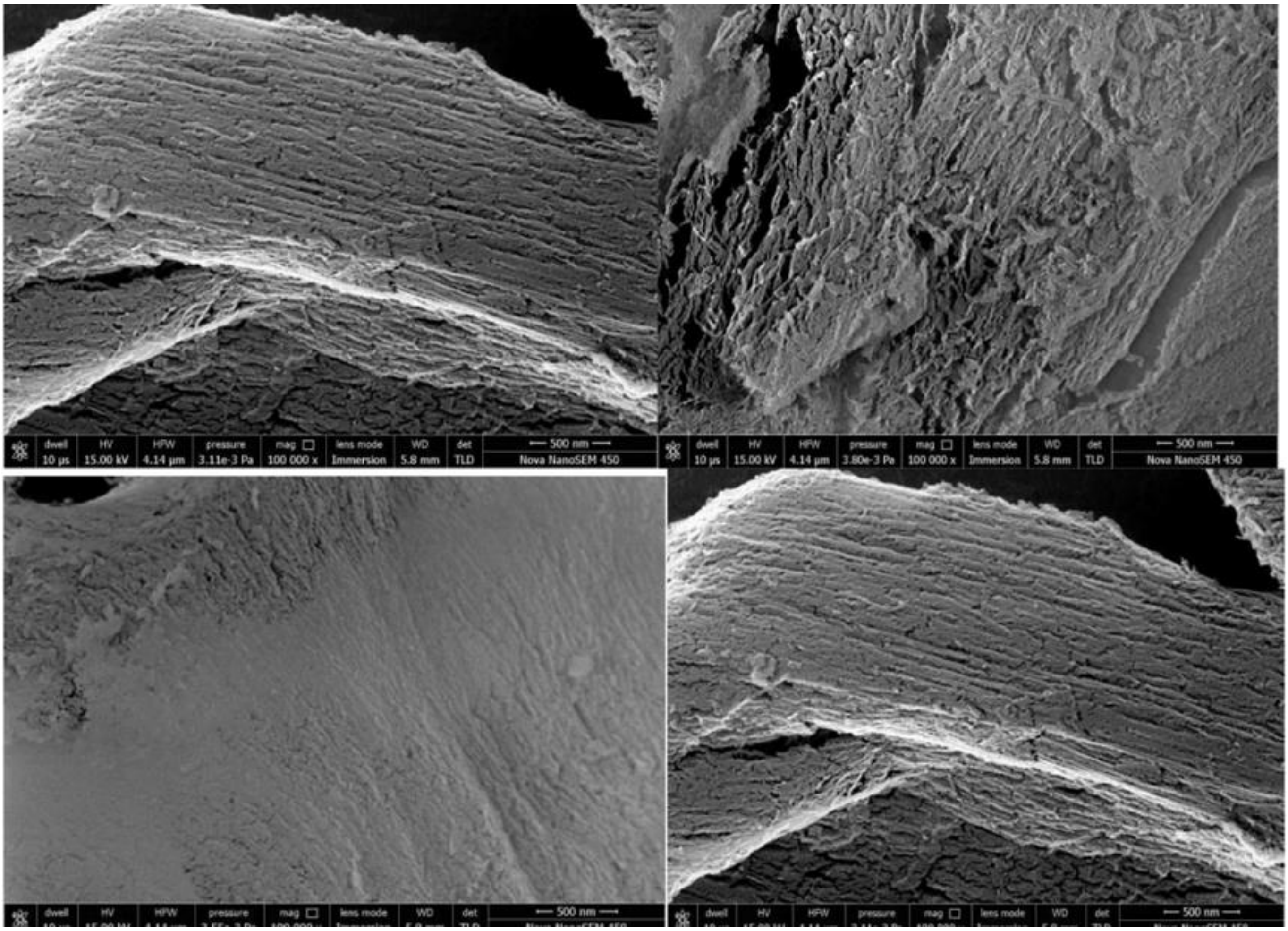

Fig 1: FESEM images of synthesized Urea coated hydroxyapatite nanoparticles.

c. FTIR

Chemical nature and molecular bonding were observed by FTIR studies. Results are shown in Figure 2.

Results of FTIR studies showed the presence of total 14 peaks with 6 major peaks including 3430.75 cm-1(N-II stretching), 3332.54 cm-1 (O-II stretching), 1738.76 cm-1 (carbonyl stretching), 1678.54 cm-1(N-H bending), 1455.96 cm-1 (N-C-N stretching), and 1025.37 cm-1 (P-O stretching of PO_4^{-3}). These results confirmed the structure of UHANPs.

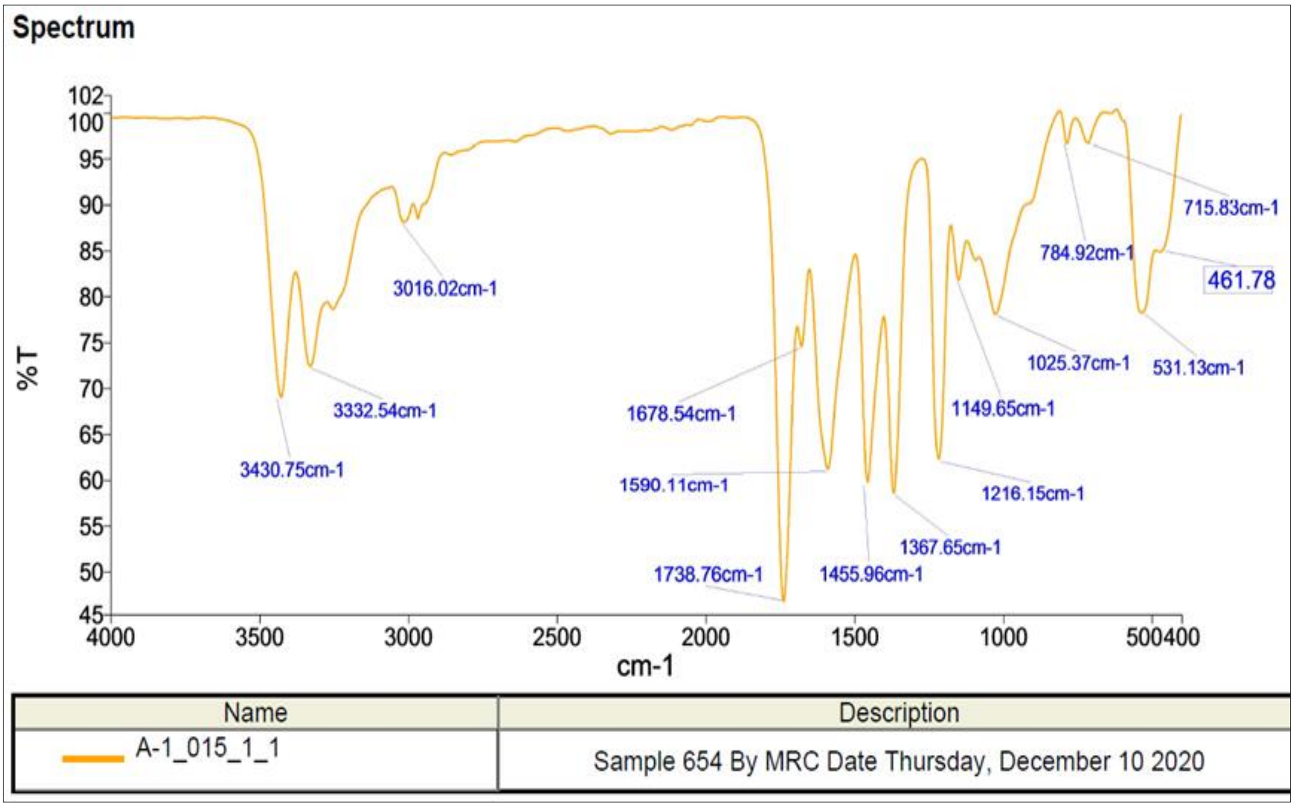

Fig 2: FTIR Spectrum of the synthesized urea coated hydroxyapatite nanoparticles.

Seed germination parameters

a. Germination percentage

Results for seed germination percentage of each treatment are shown in Table 3 and graphically represented in Figure 3.

The maximum seed germination in both the selected species was observed in P_7 (U-HANPs 500 ppm) (90±0.75 % for S1, and 90±0.75 % for S2) while the minimum seed germination was found in P_5 (Urea 1000 ppm) (54.13±0.26 % for S1, and 56.34±0.36 % for S2).

Seed germination percentage in P_7 was significantly higher than that of other treatments including P_1 control while values are not significantly different for other treatments for both species.

Table 3: Effects of Urea coated hydroxyapatite nanoparticles on seed germination on two varieties of *Brassica juncea* L.

Treatment	Germination percentage		Root Length (cm)		Shoot length (cm)		Fresh matter		Dry matter		Seedling size	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
P_1	66.14±0.18	62.22±1.16	1.53±0.62	1.36 ±0.06	1.77±0.14	1.32±0.07	0.244±0.11	0.281±0.10	0.054±0.02	0.055±0.02	3.46±0.08	2.30±0.12
P_2	68.86±0.27	63.43±0.73	5.93±0.18	4.09±0.18	1.95±0.22	2.11±0.11	0.303±0.7	0.292±0.05	0.054±0.02	0.062±0.01	4.04±0.12	7.05±0.180.23
P_3	77.71±1.14	75.14±0.27	7.64±0.27	7.61±0.16	2.16±0.17	2.92±0.15	0.327±0.04	0.313±0.08	0.055±0.01	0.063±0.05	9.70±0.16	9.85±0.21
P_4	66.14±0.47	61.86±0.13	1.67±0.38	1.46±0.22	1.77±0.85	1.32±0.72	0.192±0.10	0.202±0.16	0.051±0.03	0.055±0.01	3.14±0.17	2.02±0.17
P_5	54.13±0.26	56.34±0.36	0.67±1.13	0.85±0.27	1.68±0.42	1.19±0.16	0.187±0.16	0.196±0.02	0.049±0.01	0.049±0.01	2.33±0.05	1.98±0.02
P_6	68.86±0.11	66.14±0..26	6.6±0.28	5.87±0.37	2.26±0.17	2.83±0.64	0.307±0.04	0.312±0.11	0.061±0.02	0.063±0.02	8.39±0.12	3.06±0.17
P_7	90±0.75	90±0.27	8.77±0.18	8.56±0.25	2.96±0.63	2.85±0.17	0.333±0.07	0.319±0.16	0.062±0.02	0.064±00.03	11.2±0.26	12.66±0.26
P_8	65.75±1.12	63.16±0.18	1.14±0.06	1.74±0.07	1.64±0.14	1.66±0.06	0.211±0.13	0.235±0.06	0.058±0.01	0.063±0.03	2.87±0.18	3.06±0.62
P_9	61.21±0.04	59.65±0.76	1.04±0.28	1.03±0.11	1.35±0.26	1.31±0.17	0.226±0.10	0.225±0.15	0.053±0.01	0.058±0.01	1.87±0.16	2.37±0.27

Note* S1- Variety GIRIRAJ, S2- variety NRC-BH 101

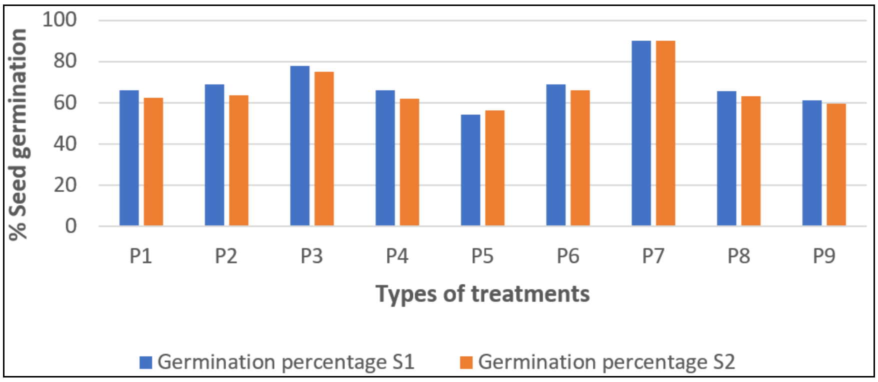

Fig 3: Effects of urea coated hydroxyapatite nanoparticles on germination percentage of seeds of two species of *Brassica juncea* L.

b. Root length and shoot length

Observed data for root length and shoot length are shown in Table 3 and these are graphically represented in Figure 4.

The maximum root length in both species was found in P_7 (8.77±0.18 cm for S1, and 8.56±0.25 cm for S2) while the minimum root lengths were recorded in P_5 for both the selected species (0.67±1.13 cm for S1, and 0.85±0.27 cm for S2).

The maximum root length in P_7 was significantly higher than that of P_1 (control), P_4, P_5, P_8, and P_9, while these are not significantly different from other treated plants (P_2, P_3, P_6).

The maximum shoot length was also recorded in P_7 in both the species (2.96±0.63 cm for S1, and 2.85±0.17 cm for S2) while the minimum shoot length was observed in P_9 in both the selected species (1.35±0.26 cm in S1, and 1.31±0.17 cm in S2).

The maximum shoot length in P_7 in both plants are statistically different for that in P_1 (control), P_2, P_5, P_8, and P_9.

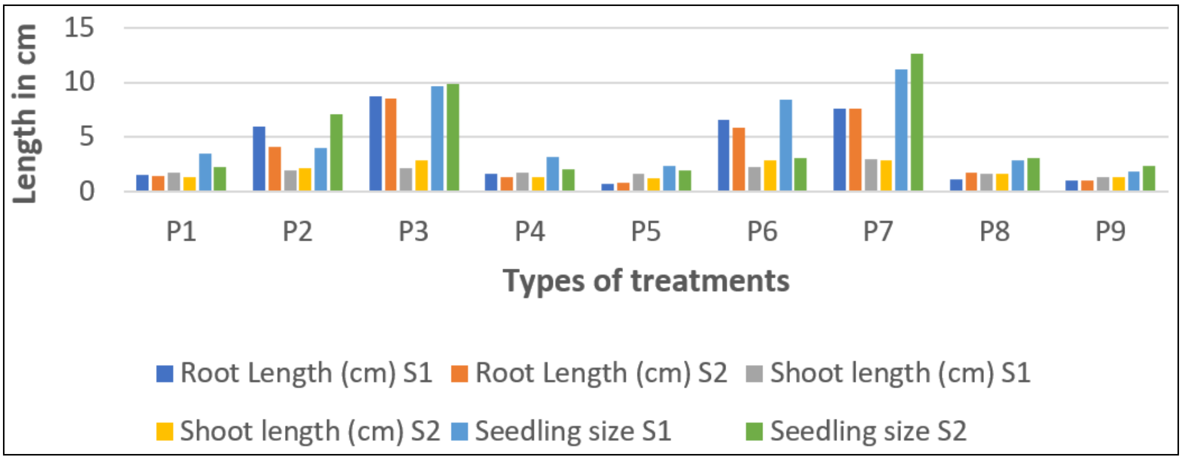

Fig 4: Effects of urea coated hydroxyapatite nanoparticles on root length, shoot length & seedling size of two species of *Brassica juncea* L.

c. Seedling size

Observed seedling size in al treated plants for both species are shown in Table 3 and graphically represented in figure 4. The maximum seedling size in both species were observed in P_7 (11.2±0.26 cm in S1, and 12.66±0.26 cm in S2) while the minimum seedling size were recorded in P_9 (1.87±0.16 cm) in S1, and P_5 (1.98±0.02 cm) in S2.

For both the species, the maximum seedling size was significantly different than P_1 (control), P_4, P_5, P_6, P_8, and P_9 while there is no significant difference when compared to P_2, and P_3.

d. Fresh matter and dry matter content

Results obtained for fresh matter content, and dry matter contents are shown in table 3, and these are graphically represented in Figure 5. The maximum fresh matter content was recorded in P_7 for both the species (0.333±0.07 g for S1, and 0.319±0.16 g for S2) while the minimum fresh matter content was recorded in P_5 for both the species (0.187±0.16 g for S1, and 0.196±0.02 g for S2).

Fresh matter content in P_7 was significantly higher in all plants (including control) other that P_2, P_3, and P_6.

The maximum dry matter content was recorded in P_7 for both the species (0.062±0.02 g for S1, and 0.064±00.03 g for S2) while the minimum dry matter content was recorded in P_5 for both the species (0.049±0.01 g for both S1, and S2).

There was no significant difference in dry matter content in all the treated plants.

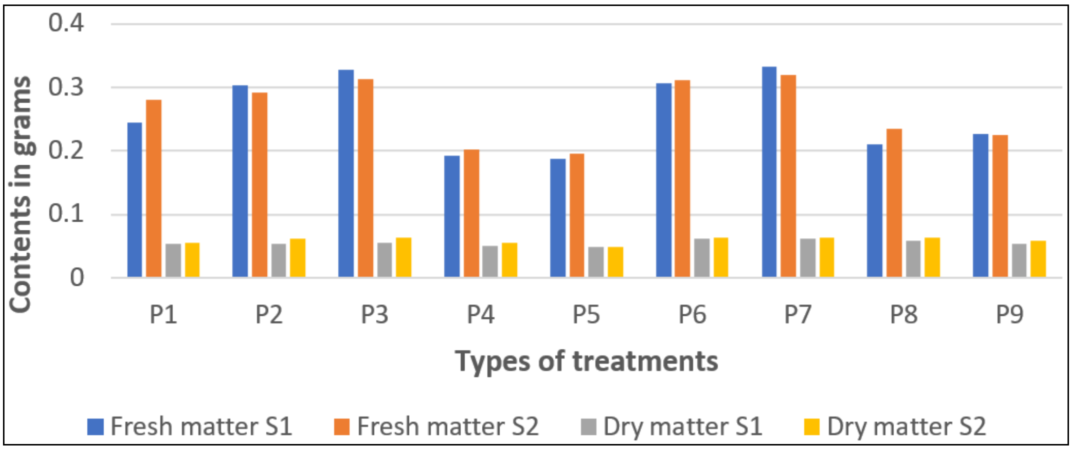

Fig 5: Effects of urea coated hydroxyapatite nanoparticles on fresh matter & dry matter content of two species of *Brassica juncea* L.

Conclusion

Results of the present investigations, revealed that urea coated hydroxyapatite nanoparticles at the concentration of 500 ppm can increase seed germination parameters in all the selected aspects including seed germination percentage, root length, shoot length, seedling size, fresh matter content, and dry matter contents when compared to urea treated plants while the higher concentration of these can interfere with seed germination attributes in the selected plant species. This study may help farmers to use optimum fertilizer at optimum concentration to grow high yield plants.

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