Int. J. Nano Dimens., 12 (1): 1-10, Winter 2021

ORIGINAL ARTICLE

# Marine endophytic fungi mediated Silver nanoparticles and their application in plant growth promotion in *Vigna radiata L.*

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Received 16 June 2020; revised 15 September 2020; accepted 23 September 2020; available online 01 October 2020

**Abstract**

Seaweeds tend to have the property of acting as a biofertilizer for plants. Endophytes are organisms that are capable of mimicking and producing secondary metabolites similar to the host. In this report, silver nanoparticles (AgNPs) were synthesized from a marine endophytic fungus, Fusarium equiseti which was isolated from marine seaweed and identified using ITS sequencing. The synthesized Fusarium equiseti nanoparticle (FeNp) was characterized using UV Visible Spectrophotometer and field emission scanning electron microscope (FESEM). Efficacies of these nanoparticles to act as plant growth promoters were tested in laboratory conditions. Two different methods of administrations are nanopriming (NAP) and hydropriming (HYP), which were carried out with varying concentration of the FeNp (1ppm, 2.5ppm, 5ppm and 10 ppm). After comparing both the results, HYP method showed better results by favouring positive effects on wet weight, shoot length, root length, chlorophyll and carotenoid contents even at very low concentration (5ppm).The current results suggested that there is scope for these nanoparticles to be made into a biofertilizer after performing further toxicity studies under field conditions.

**Keywords:** *Biofertilizer; Marine Endophytic Fungi; Mycosynthesis; Plant Growth; Silver Nanoparticles.*

**How to cite this article**

*Adhila Haji Basheerudeen M., Mushtaq Sh.A., Soundhararajan R., Kumar Nachimuthu S., Srinivasan H. Marine endophytic fungi mediated Silver nanoparticles and their application in plant growth promotion in Vigna radiata L.. Int. J. Nano Dimens., 2020; 12(1): 1-10.*

## INTRODUCTION

Nanomaterials are defined as particles with a size less than 100nm in at least one dimension. Nanotechnology is a rapidly developing field which is gaining a lot of fame for the wide application range due to their nano size. Nanoscience has emerged as a field which is of interest for almost all branches of science such as physics, chemistry, biology and polymer technology. Nanoparticles have many applications in a vast variety of industries [1]. Silver nanoparticles are more common as they are chemically stable, possess good conductance and catalytic properties [2]. Silver nanoparticles can be used for drug delivery, implants and as dressing materials [3, 4].

Silver nanoparticles (AgNPs) that are synthesized through biological methods are more biocompatible for clinical applications [5, 6]. Nanoparticles from organisms such as bacteria, fungi and algae can be synthesized using biological synthesis methods [7, 8]. Endophytic organisms are those which are present in a symbiotic relationship with their host plants. They are isolated and cultured due to their various medicinal and industrial properties [9].

Endophyte mediated synthesis of nanoparticles

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is a new area of research which combines biology and nanotechnology. There are about 300,000 different plant species on earth, and they possess different kinds of endophytic organisms. The unique properties of endophytes can be explored for manufacturing AgNPs which can be used for different applications [10, 11]. Many studies can be done to explore the world of endophytes and see how they differ in methods of synthesis and applications.

Recent studies show that these nanoparticles impart some beneficial properties crop growth, development and yield. In the agricultural industry, it has been seen that nanoparticles aid in rapid germination and also improve the growth rate of the seeds [12]. The molecular mechanisms related to nanoparticle and plant interactions are still yet undetermined and the research done in this field is limited.

Nanotechnology is known for its vast application in the agricultural and food industries. One of the main pillars of these industries is seed germination. Seed germination is a crucial factor that affects plant yield and rate of growth. Germination, being one of the initial processes of plant growth is a very sensitive process. It is easily affected by minor changes in the environment. Different studies have shown a positive effect of AgNP on seed germination [13].

According to the recent research done in this field, the most widely used nanomaterials for agricultural purposes are single-walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs). Their effects on tobacco, tomato, barley, and corn have been studied byVerma*et al.*, [14]. Effects of Silver, ZnO [15] and Titanium dioxide nanoparticles($TiO_2$) on rice/ paddy seeds have been studied by Boonyanitipong *et al.,* 2011 [16]. These studies were done to comprehend the effects of nanoscience on agriculture. They mainly focus on toxicity concerns, combating plant disease pathogens and genetic engineering. Nanoparticles are seen to activate or inhibit certain physiological processes in seedlings based on the concentration in which it is administered.

Nanomaterials as fertilizers have the potential to minimize nutrient losses and reduce the occurrence of pollution. Nanoparticles formulated into pesticides and nano-herbicides target specific cellular organelles in the plants to boost specific plant metabolisms and alter their physicochemical properties [17]. Due to their tiny size, they can penetrate the plant and interact with the plant tissues leading to physiological and morphological alterations depending on the properties of the nanoparticle under study [18]. NPs have seen to cause both positive and negative effects on plant growth and development depending on the type of nanoparticle used and plant species targeted [19, 20].

Very less percentage of the total microbial world is known to us and we may get different nanoparticles with useful qualities and applications that may be used in different fields. There is a lot of scope for scientific studies in this field and more research needs to be done. Intense research needs to be done for the usage of AgNPs for increasing food production, ensuring that they do not turn up as toxic or cause any unnecessary side effects. Collaboration among different scientific fields will help in unravelling the working mechanism of endophyte mediated AgNPs.

The main intention of this study was to acquire information regarding the comparative effects seen on *Vigna radiata* seeds when treated with varying concentration of nanoparticles synthesized using marine endophytic fungi. Effects on seed germination, root elongation, shoot elongation, physiochemical changes and phytotoxicity were studied to enhance our understanding the effect on nanoparticles when administered in different ways naming Nanopriming (NAP) and Hydropriming (HYP).

## MATERIALS AND METHODS

### *Isolation of pure culture of endophytic fungi from seaweed*

Isolation of endophytic fungi from seaweed was performed by following the methods given by Tahira *et al.*, 2019 [21, 22]. Healthy thallus of seaweed was washed thoroughly and segmented into fragments of 0.5 cm length. Surface sterilization was done by immersing the fragments in 70% ethanol, followed by 4% Sodium hypochlorite solution, once again in 70% ethanol and finally in distilled water each session lasting for 3minutes. The fragments were placed onto PDA agar plates in aseptic conditions. The plates were incubated at 36 °C for 7 days and fungal colonies were seen to grow around the fragments. The required fungal mycelial colony from the mother culture plate was picked using a sterile inoculation loop and transferred onto fresh PDA plates. The

plates were incubated at 36°C for over a week or until good growth of fungal mycelium is observed. For liquid culture, PDB was inoculated with the fungal strain under aseptic conditions. The flasks were incubated at 27 °C for 10 days.

*DNA isolation and sequencing*

Cetyl trimethyl ammonium bromide (CTAB) method was used to isolate the genomic DNA of the fungi. The extracted DNA was amplified with ITS forward primer (5'TCCGTAGGTGAACCTGCGG3') and reverse primer (5'TCCTCCGCTTATTGATATGC 3') and sequenced to identify the species of the endophytic fungi [23].

*Synthesis and characterization of Mycosilver Nanoparticles*

Fungal mediated AgNPs were synthesized by heat mediated synthesis as given by Ranjani *et al.*, 2020 [23]. The cell-free filtrate of the endophytic fungi was used as the mediator for the synthesis of nanoparticle. It was mixed with an equal volume of 1mM Silver nitrate solution ($AgNO_3$). The mixture was heated on a hotplate at 80 °C for 45-60 min or until colour change from colourless to reddish-brown. Synthesis of AgNPs was confirmed through visual confirmation due to colour change. The nanoparticle synthesis was monitored by measuring UV–vis Spectroscopy (JASCO V-730). The nanoparticle solution was centrifuged at 5000 rpm and the supernatant was discarded. The pellets were carefully collected and dried in the hot air oven. Scanning electron microscopy (Supra 55, FESEM Carl Zeiss Germany) was performed to study the morphological nature of the synthesized nanoparticles [24-26].

*Determination of plant growth promotion activity*

The synthesized nanoparticle was tested for its efficacy to assist in plant growth promotion. The best method of nanoparticles administration to the plant and the optimal concentration that yielded the best results based on various factors were assessed. Nanoparticle suspension in water was prepared for the concentrations 1ppm, 2.5 ppm, 5 ppm and 10 ppm. Two different methods of administration of nanoparticle was followed. First one is Nanopriming (NAP) is a method which follows soaking the seeds in NP solution before germination and spraying with nanoparticle solution during germination period [27]. The second one is Hydropriming (HYP) which adopts a method of soaking the seeds in water before germination and spraying with nanoparticles solution during germination period [28].

5 g of *Vigna radiata* seeds were soaked in 20 ml of autoclaved distilled water for 5 hours. The seeds were there placed on sterile filter paper in sterilized plastic Petri dishes. 2.5 ml of nanoparticle suspension of the varying concentrations were sprayed onto the seeds everyday and the seeds were incubated in room temperature in normal daylight conditions for 7 days.

4 batches of 1g of *Vigna radiata* seeds each were soaked in 5ml of each of the concentrations of the nanoparticles for 5 hours. The seeds were there placed on sterile filter paper in sterilized plastic Petri dishes. 2.5ml of nanoparticle suspension of the varying concentrations were sprayed onto the seeds everyday and the seeds were incubated in room temperature in normal daylight conditions for 7 days.

*Morphological studies*

To determine the effects of nanoparticle treatment on plants, the plant samples were analysed and the results were compared with the control (without nanoparticle treatment). Data on growth parameters such as whole plant wet weight; root length, shoot length and total length were collected. To determine the wet weight of the whole plant, the plants were first washed with distilled water and dried with a paper towel to remove surface water and weighed on a weighing scale. Root length and shoot lengths of the plant were measured against a standard scale. Both the root and shoot lengths were added to get the total length.

*Biochemical studies*

Biochemical responses of the plant to the treatment given were recorded and compared with the control. The biochemical parameters such as chlorophyll estimation and carotenoid estimation were taken into consideration [29-31]. Chlorophyll content was estimated according to Arnon's method [27] and Carotenoid content was estimated according to the method given by MacLachlan and ZaliK., 1963 [28].

**RESULTS AND DISCUSSION**

The fungi isolated from seaweed was found to be *Fusarium equiseti* according to its morphological characteristics (Fig 1a) and amplification of the

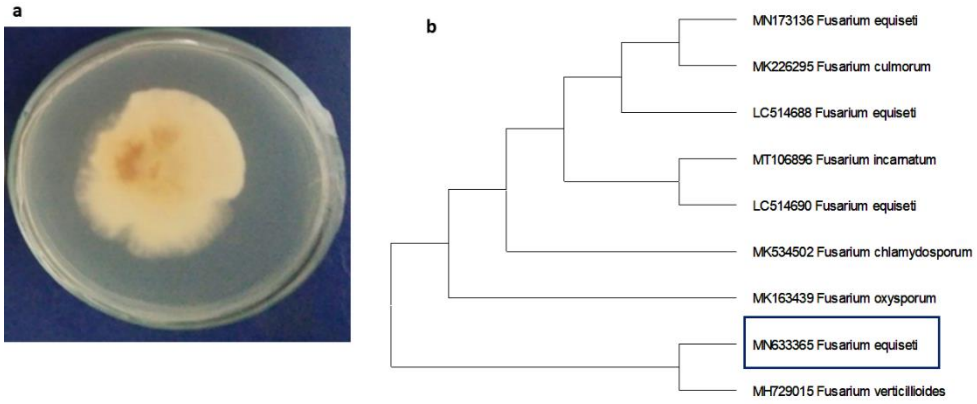


Fig. 1. a. Pure culture of *F. equisetis* isolated from seaweed. b. Phylogenetic relationship of *F. equisetis* with other organism and deposited in NCBI Gen Bank with accession number MN633365.

DNA sequences of the ITS region (Fig 1b).Upon performing BlastN with the identified nucleotide sequence, it was noticed that it showed 100% similarity to over 10 of the *F. equiseti* already present in Genbank. Multiple sequence alignment and construction of a phylogenetic tree with a closely related sequence using MEGA-X confirmed the presence of genome sequences similar to the genus of *Fusarium*. The isolated *Fusarium equiseti* was deposited in NCBI Gen Bank with accession number **MN633365**.

During FeNp synthesis the colour change in the mixture of *F. equiseti* cell-free filtrate and $AgNO_3$ from colourless to dark brown was observed and it confirmed the synthesis of *F. equiseti* mediated myco-silver nanoparticles (FeNp) (Inlet fig 2a). To confirm this, the cell-free filtrate and silver nitrate solution were individually heated using the same parameters and they retained their original colour. The colour change had occurred as the secondary metabolites of fungal extract have reduced the metallic silver to silver ions. They stabilize themselves by acting as a capping agent for the synthesis of *Fusarium equiseti* mediated synthesis of FeNp. When UV spectra analysis was performed, Surface plasmon resonance was recorded at $A_{300}$ which confirms that FeNp have been synthesized. AgNPs are usually seen to for absorbance peaks from 300nm – 500nm range and the SPR peak was observed at 300nm (Fig 2b). Further, FESEM analysis was carried out to study the morphological features of the FeNp. Surface morphology, size, and shape of FeNp were observed as spherical shaped with the size ranged from 2nm–50nm. Spherical shape is the most seen shape of FeNp with smooth surface (Fig 2c). EDAX analysis confirms the presence of Ag in FeNp by showing the peak at 3KeV (Fig 2d) [22-24].

During soaking, the seeds that followed NAP appeared bigger than the seeds that followed HYP. As per a study done by Noshad., 2019 by, it could be hypothesized that the level of solution absorption is higher in NAP because FeNp is capable of piercing and penetrating through the seed coat. This might have created bigger pores for the water to enter at a higher rate compared to the untreated control seeds. Water uptake is a very important process since the seeds are dry and there is a minimum water requirement to kick start the cell metabolic processes. This could be a possible explanation for the bulky appearance of the seeds soaked in FeNp solution.

NAP seeds were the first to sprout out on comparison with HYP seeds. A possible explanation could be that, due to higher rates of seed bulging due to increased water entry, the seed coats of the NAP seeds broke during the soaking process itself. Therefore, it has paved away and made it easier for the roots of the NAP seeds to sprout out earlier than the HYP seeds. Earlier appearance of the sprouting could also be due to increased nutrient uptake due to the pore size in NAP seeds. In a study conducted [27], AgNPs primed seeds had a higher content of dehydrogenase due to the early and

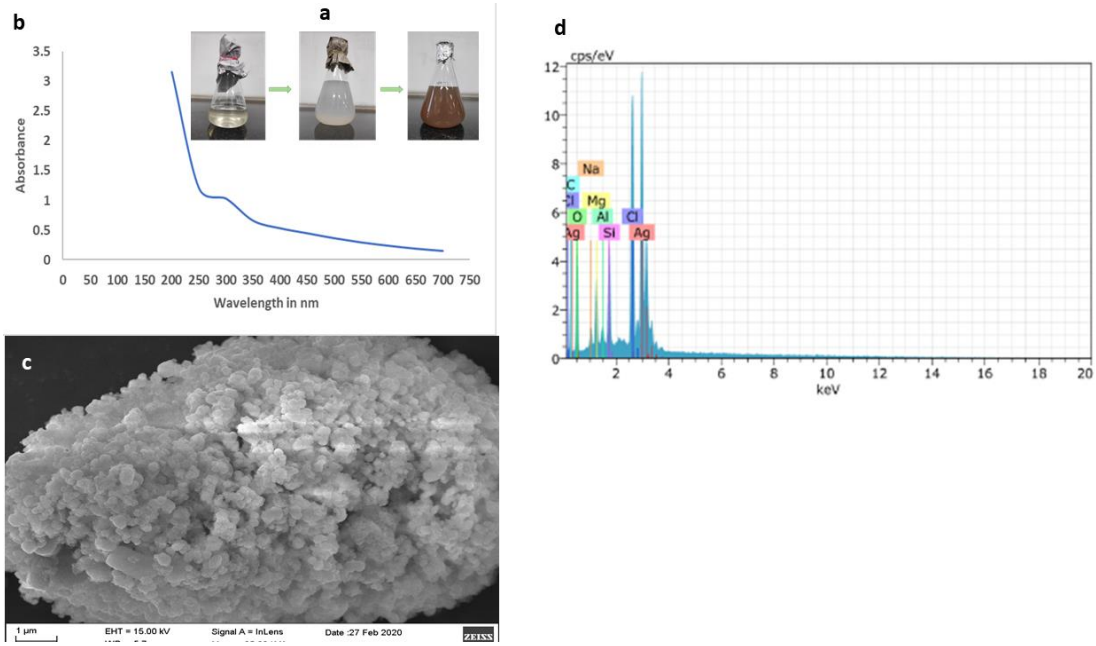


Fig. 2. Inlet figure a. Colour change during FeNp synthesis. b. UV Spectral analysis of synthesized nanoparticles. Spectrum shows the maximum absorbance at A300 for the synthesized nanoparticles from *F. equiseti*. c. Characterization of FeNp by FESEM. d. EDAX analysis of FeNp.

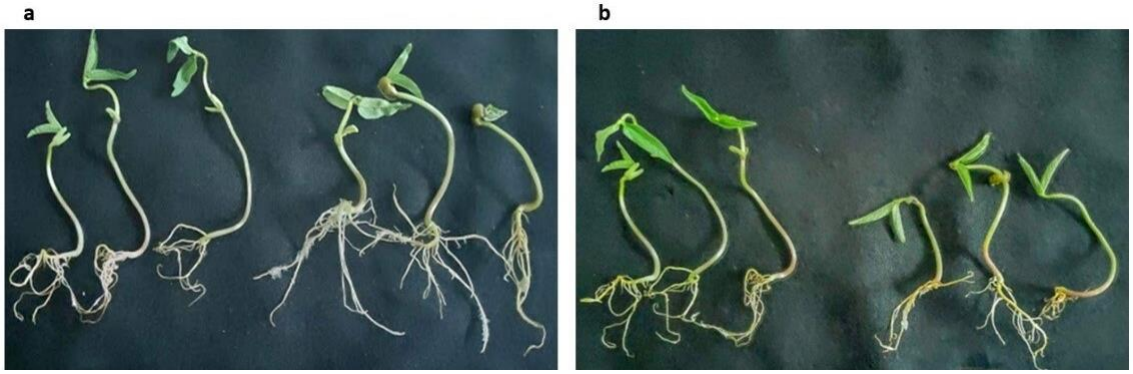


Fig. 3. Morphological changes in *V. radiata* seedlings under HYP treatment and NAP treatment with control in triplicates. a. Shows HYP plants with healthy, long roots and higher shoot length when compared to NAP plants and control. b. Shows NAP plants with shorter shoot and thin, dry roots when compared to HYP plants and control.

increased access to water. Dehydrogenase is an enzyme that plays a key role in cellular respiration. Hence, aid in the cellular and metabolic processes.

Root size of HYP seeds appeared comparatively longer (Fig 3a) than NAP seeds (Fig 3b) despite a delay in sprouting. Previous studies suggest that the accumulation of AgNPs tends to delay the root growth process due to an internal change in pH due to the release of OH. Gene expression studies of nano-primed seeds by [27] show that with increased water uptake, the gene coding for aquaporin was expressed more which led to $H_2O_2$ accumulation. Specific aquaporin isoforms enable the diffusion of $H_2O_2$ into the membranes of the cells and increase the membrane permeability for $H_2O_2$. This alters the redox potential and increased reactive oxygen species (ROS) production [27].

Since NAP seeds had a higher exposure to

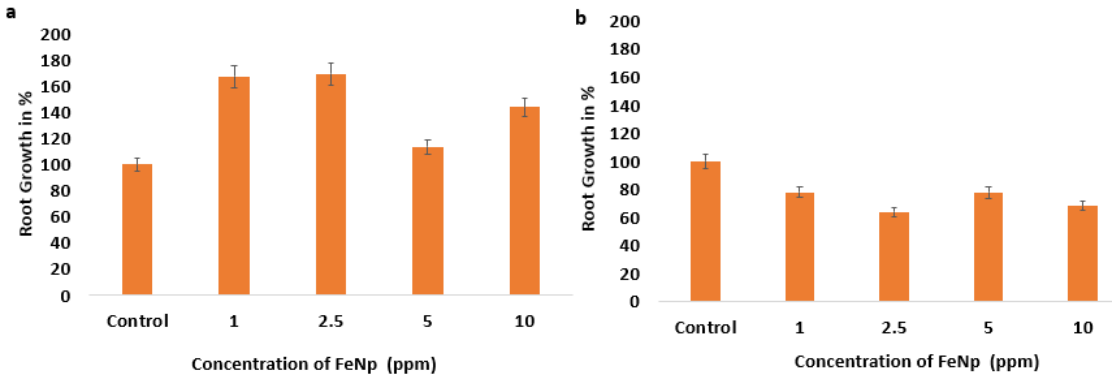


Fig. 4. a. Effect of FeNp on root length in *V. radiata* seedlings through HYP. b. Effect of soaking the seed in FeNp on fresh root length of *V. radiata* seedlings through NAP.

FeNp compared to HYP seeds, their germination process could have been affected by the increased $H_2O_2$ content. This statement is also supported by the brown patches that appeared in the NAP seeds around day 4 of germination. This patching is a common indicator of higher amounts of ROS production which leads to a delay in biological processes [31].

Studies also suggest that the levels of ROS can either effect germination both positively and negatively depending on their levels. But excess increase of ROS along with the lack of antioxidant potential of the cells leads to faster seed ageing and reduction in the ability to germinate properly. This could also be a reason for the delay in the growth of NAP seeds. In recent studies, it has been proposed that, when ROS content is within an oxidative window, it leads to ROS signalling which regulates seed germination. Difference in levels of ROS in NAP seeds and HYP seeds could explain why the HYP seeds showed faster growth than NAP seeds [32].

Roots are the first parts to gain exposure to the nanoparticles. This could explain why the influence of NPs is more on the roots when compared to shoots. Ag is known to enhance the levels of plant hormones due to their ability to upregulate the gene responsible for auxin regulation. This is the hormone responsible for root growth acceleration. This could explain the increase in thickness and fibrous nature of the HYP seeds. Roots of the control plant were of medium thickness and less fibrous. Roots of NAP seeds were comparatively thinner and dried very easily. Smaller quantities of nanoparticles are easy to transport within a plant and unlike higher concentrations, they do not accumulate in a single place. This could be the reason why HYP seeds treated with 2.5 ppm of NP solution showed better root growth when compared to the higher concentrations. From the graphical representation, it was observed that upon nanopriming the length of root growth was decreased when compared with the control (Fig 4a). Upon hydropriming, the average root length was increased by 67%, 69%, 14% and 44% for 1, 2.5, 5, 10 ppm of FeNp, when compared with the control (Fig 4b).

AgNPs are capable of leading to upregulation of genes encoding plant growth regulators like cytokinin and gibberellins. These hormones aid in cell elongation by cell division. They mostly target the shoot and an upwards growth [33]. The graphical representation of nanopriming method showed a decrease in the shoot length when compared with the control plant (Fig 5a). Based on hydropriming method, seeds treated with 5ppm and 10ppm showed 7% and 40% of the increase in shoot length when compared with the control (Fig 5b).

Plants from NAP seeds seemed to weigh lesser (Fig 6a) than HYP seeds (Fig 6b). Based on nanopriming and hydropriming treatment with FeNp, the fresh weight of hydropriming based treatment increases by 75% when compared to control. Nano-priming seems to enhance starch degradation when compared to control seeds and Hydro primed seeds. But a faster degradation combined with the decrease in pH due to the increased $H_2O_2$ leads to a higher risk of decay of the endosperm material. But hydropriming seed treatment with 5ppm turned out to yield the highest rates of wet weight of plants

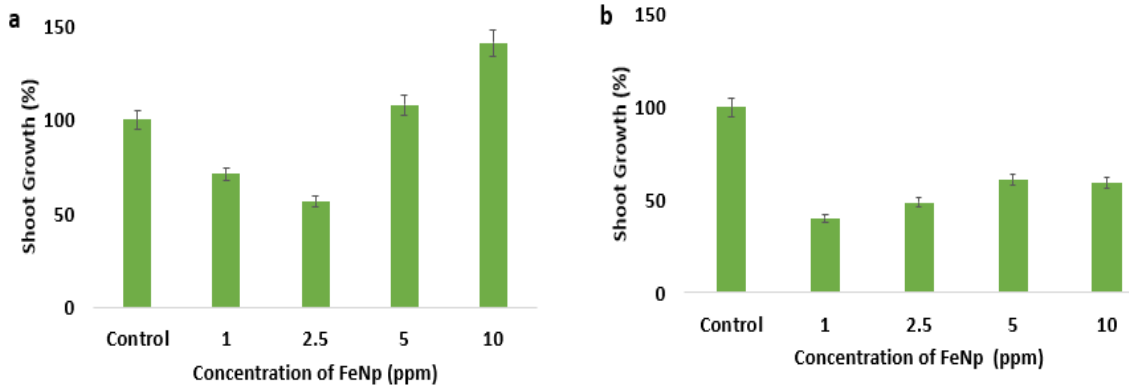


Fig. 5. a. Effect of FeNp on shoot length in *V. radiata* seedlings through HYP. b. Effect of soaking the seed in FeNp on fresh shoot length of *V. radiata* seedlings through NAP.

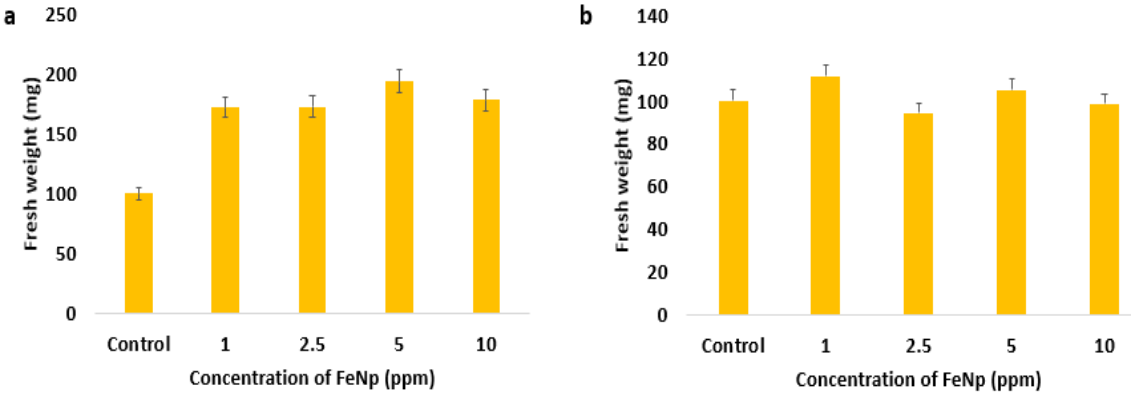


Fig.6. a. Effect of FeNp on fresh weight in *V. radiata* seedlings through HYP. b. Effect of soaking the seed in FeNp on fresh weight of *V. radiata* seedlings through NAP.

when compared to the very high and very low concentrations. α-amylase is a hydrolytic enzyme that breaks down starch to soluble sugars for the respiratory mechanism for seedling growth until the plant can photosynthesize by itself. Existing literature suggests that AgNPs lead to increased activity of α-amylase and hence a faster rate of starch hydrolysis. This increases the bioavailability of soluble sugars for the germinating seeds for generating energy for growth. This could be the reason why the HYP seeds showed an increased speed of germination. Literature survey shows that hydro primed seeds tend to have a better antioxidant system than the nano-primed ones. This increases the general fitness of the seedlings [34, 27]. This could explain the better survival rates of HYP seeds.

According to these discussions, it was concluded that HYP method was better than the NAP method. Therefore, only the plants that came out of the HYP seeds were used for further analysis on measuring the content of chlorophyll A, chlorophyll B, total chlorophyll and total carotenoid. Comparative studies were done by administering the FeNp in different dosages through HYP method and comparing with the untreated control seeds.

Based on spectrophotometric data, it can be seen that higher amounts of chlorophyll A (Fig 7a), chlorophyll B (Fig 7b) and total chlorophyll (Fig 7c) are present in leaves of the plants administered with 5ppm of When ROS is present within the oxidative window, it influences the presence of electron acceptors which play a crucial role in photosystem II during photosynthesis. Better efficiency of these electron acceptors indicates a higher amount of chlorophyll in the leaves [35, 36]. This could be the reason why higher chlorophyll content

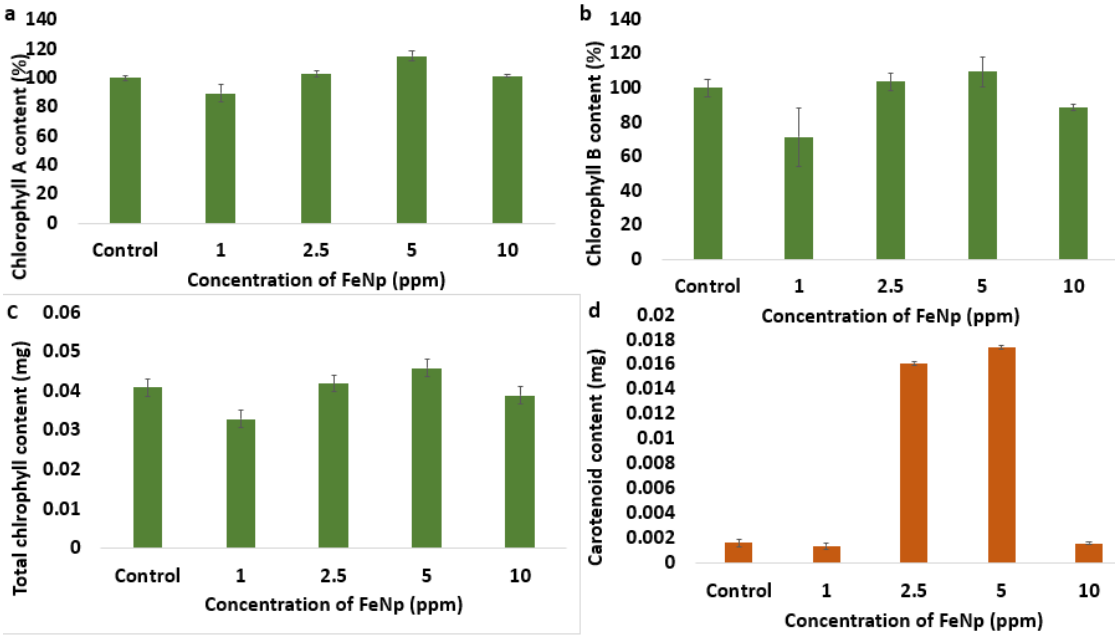


Fig.7. a. Effect of FeNp on Chlorophyll A content in leaves of *V. radiata* seedlings. b. Effect ofFeNp on Chlorophyll B content in leaves of *V. radiata* seedlings. c. Effect of FeNp on total chlorophyll content in leaves of *V. radiata* seedlings. d. Effect of FeNp on total carotenoid content in leaves of *V. radiata* seedlings.

was observed in the seeds treated with 5ppm of NP when compared to the control. At very high concentrations like 10ppm, there might have been very high ROS and in lower concentrations, there might not have been enough ROS to accelerate the increase of electron acceptors. Similar results were observed for carotenoid estimation as well. Plants administered with 5ppm of FeNp solution yielded a higher number of carotenoids when compared to the control plants (Fig 7d).

## CONCLUSION

According to the results, it can be summarized that hydropriming (HYP) method is better than nanopriming (NAP) method for administration of FeNp into seedlings. Comparatively, the HYP method leads to lesser accumulation of AgNPs within the plant. After comparison of results yielded by HYP seeds and control seeds, it can be summarised that mycosynthesized nanoparticles FeNp can be given to plants at lower concentrations of 5ppm and yield good results in plant growth promotion. Higher concentrations of FeNp seem inhibitory to plant growth. Nanotechnology combined with the power of marine endophytic organisms could be a solution to the current challenges we are facing in terms of sustainability in the food industry. Further studies need to validate to get an in-depth analysis of the molecular processes involved and toxicity studies also need to be done before implementing these on a field level.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Ministry of Science and Technology, Department of Science and Technology (KIRAN Division) (GoI), New Delhi. (Ref. No. DST/WOSB/2018/1583-HFN (G)). The authors are also thankful to B.S. Abdur Rahman Institute of Science and Technology, Chennai for providing research facilities in the school of life sciencesand the Department of Biotechnology (DBT), New Delhi for the Bioinformatics Infrastructural Facility (BIF–BTISNeT), Mizoram University.

## CONFLICT OF INTEREST

Authors have no conflict of interest.

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