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IMPACT OF NANO EMBEDDED SYMBIOTIC FUNGUS ON BLACK RICE (ORYZA SATIVA L. INDICA) – WITH HIGH MEDICINAL VALUE-MINI REVIEW

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ABSTRACT
The current study focuses on synergistic association of Zinc Oxide nanoparticles with root endophyte Piriformospora indica (Serendipita indica- Basidiomycota, Hymenomycetes), called 'Nano embedded fungus' with emphasis on its correlation with development of Black rice (Oryza sativa L. indica). Zinc Oxide nanoparticles were synthesized by thermal decomposition method at different temperatures which was further characterized by UV-Visible spectroscopy, X-ray diffraction spectroscopy, SEM and FTIR spectra. Zinc Oxide increases the colony size, spore size and hyphae of the fungus. The nanotechnology is used to prepare nano-emebedded fungus by using ZnO and P.indica with an aim to promote germination, growth, medicinal properties and yield of food crops using black rice. This nanotool of ZnO and P.indica showed the remarkable increase in augmentation of seed germination rate of Black rice. So it is recommended that ZnO- nanoparticles may possibly deal with impending medical concerns by used successfully in protecting field of agriculture.

KEYWORDS: *Piriformospora indica*, *Serendipita indica*, Zinc Oxide, Nanoparticle, Black Rice, Interaction.

INTRODUCTION
A novel symbiotic fungus - *Piriformospora indica* (*Serendipita indica*), belonging to class Basidiomycota and order Sebacinales. The fungus was isolated from soil of roots of shrubs of Thar dessert (northwestern part) i.e Jaisalmer, Rajasthan.[1] The fungus has multiple functions as increases in secondary metabolites, growth induction and improve gross yield. This symbiotic fungus can also be used as bio-herbicide, bio-fertilizer, drug enhancer and provide resistance from different types of physical stresses.[2] It also supports the transport of nitrate, zinc, phosphate, iron from soil to the whole plant through roots.[3] *P. indica*-mediated improvements in the biomass, seed germination, plant growth and development and crop productivity under favorable environmental conditions were highlighted, and *P. indica* was argued as a powerful tool for crop improvement.

Morphological Features
P.indica produces pear shaped white balls like spores which having shelf life of about 10-12 months at room temperature. These spores appear single or in bunches measuring 15-44 mm in lenghth and 11-18 mm in width (Fig. 1). The average hyphal and spore wall thickness is ranges from 0.7 and 0.4 mm respectively. The fungal hyphae are interwoven and appears like inter wined cords which sticks together. At initial stage of growth the spores have very thin and hyaline walls but on maturity the walls are almost 1.5 mm thick and appears like double layered and smooth surface.

Taxonomichistory
This story starts with the French mycologists Charles and Louis- René Tulasne, who transferred *Corticiumincrustans* into anew genus *Sebacina*based onlongitudinallyseptatebasidia,[5] Until recently, the genus *Sebacina* included fungi with resupinate or absent fruitbodies (the basid- iospore-bearing structures). Roughly a century later the family Sebacinaceae was erected,[6] basedon micro morphology, such as longitudinally septate basidia, absence of clamp connections and often thick-walled hyphae, particularly within the substrate Fig. 2.

Mycelium on maturity produces character- istic pear-shaped chlamydospores, which appear single or in clusters measuring 16– 45 mm in length and 10–17 mm in width and they are distinctive due to their pear-shaped structure. The average spore and hyphal wall thickness is 0.7 and 0.3 mm, respectively. Very young spores have thin, hyaline walls. At maturity, these spores have walls up to 1.5 mm thick, which appear two-layered, smooth and pale yellow. The cytoplasm of the chlamydospores is densely packed with granu- lar material and usually contains 8–25 nuclei. Neither clamp connections nor sexual struc- tures are observed Mycelium on maturity produces character- istic pear-shaped chlamydospores, which appear single or in clusters measuring 16– 45 mm in length and 10–17 mm in width and they are distinctive due to their pear-shaped structure. The average spore and hyphal wall thickness is 0.7 and 0.3 mm, respectively. Very young spores have thin, hyaline walls. At maturity, these spores have walls up to 1.5 mm thick, which appear two-layered, smooth and pale yellow. The cytoplasm of the chlamydospores is densely packed with granu- lar material and usually contains 8–25 nuclei. Neither clamp connections nor sexual struc- tures are observed Mycelium on maturity produces character- istic pear-shaped chlamydospores, which appear single or in clusters measuring 16– 45 mm in length and 10–17 mm in width and they are distinctive due to their pear-shaped structure. The average spore and hyphal wall thickness is 0.7 and 0.3 mm, respectively. Very young spores have thin, hyaline walls. At maturity, these spores have walls up to 1.5 mm thick, which appear two-layered, smooth and pale yellow. The cytoplasm of the chlamydospores is densely packed with granu- lar material and usually contains 8–25 nuclei. Neither clamp connections nor sexual struc- tures are observed.

A material of great interest – ZnO

As Zinc is an vital material for natural world with small size (1-100nm) and high volume ratio to use as a fertilizer in plants to enhance its metabolic activities.[7] It has been stated that application of NPs like ZnO, TiO_2, Ag as a micronutrient fertilizers is an essential way to liberate all the necessary nutrients step by step.[8] to modify plant physiological processes to endorse the growth and development of plant. There are numeral of researchers which have reported the importance of function of zinc to boost plant growth and gross yield. Zn is compulsory for production of chlorophyll, germination, fertilization and pollen function. It also plays an imperative part in biomass production.Due to their distinctive properties and versatile applications in spin electronics, transparent electronics, piezoelectric devices, UV (ultraviolet) beam emitters, and chemical sensors.[9] ZnO nanoparticle exhibit strong adsorption capability and elevatedcatalytic efficiency.

Zinc is known to play a critical role in normal functioning of body and is integrated with several enzyme systems. Adequate dietary intake of zinc has been shown to exert ameliorating effect on the skin, and this attenuates the likelihood of restricted linear growth in young children.[10] Neuro-behavioral disturbances among infants and repeated infections are common among zinc- deficient subjects of all ages. Pregnant women with zinc deficiency are at the risk of complicated pregnancy outcomes. This is further complicated by the fact that Zinc deficiency is one of the major factors limiting the yield of agricultural crops. This necessitated the need to fortify black rice with zinc using zinc oxide nanoparticles.[11] It is believed that black rice augmented with zinc would not only ameliorate the health of the marginalized sections of the Indian populace but pave the way to improve the agri- cultural yield thereby sustainably improving the carrying capacity of the arable land.[12]

As compared to Titanium dioxide, Carbon nano tubes the ZnO nanoparticles are among the most popular nanomaterials manufactured at industrial scale and are extensively studied material due to their high electron mobility, strong room-temperature luminescence, low toxicity, broad band gap, high-quality clarity and photochemical stability.[13]

In the field of agriculture nanotechnology is most promising new technologies in the 21st Century to conquer the challenges which are allied with conventional farming such as unbalanced ecosystem and low productivity. Earlier the use of nanotechnology in the field of agriculture for the most partis theoretical. However, to accomplish the demand of agricultural needs for ever-increasing population in current world, the green revolution technology using biosource in nanotechnology could bring significant contribution than the existing technologies.[14]

Agriculture is backbone of third world economics but unfortunately now, the largerange of challenges is faced by agricultural scientist which includes urban sprawl and change in climatic conditions which promote the other environmental problems like acid rain, decompose in soil organic matter and misuse of natural resources. These challenges are going to be more serious because of raise in world's population from 6to 9 billion by 2040.[15]

So there is a need to implement more resourceful techniques to build agriculture more sustainable. Application of nanofertilizers to plants at the time of sowing is required to alleviatevarious problems like soil pollution which is due to overloadof chemical fertilizers. The best part of these nanofertilizersis that they are required in a very little quantity to be used as a fertilizers e.g. for the growth of matured tree 40-50 kg of nanofertilizer is required as compared to other common fertilizers.[16] The Zinc Oxide in combination with *P.indica* is considered to be the perfect fertilizer for plants.[17]

Synthesis and Characterization of ZnO Nanorods

To synthesize Zinc Oxide nanorods, 2g of $Zn(CH_3COO)_2.2H_2O$ (zinc acetate dihydrate) (LobaChe- mie, purity:99.5%) was placed in a quartz crucible enclosed with the lid. The covered crucible was heated to its appropriate melting temperature which is 4ºC per minute in a muffle furnace in the presence of air. Hereafter, the treated samples were kept for about 12 hour at different temperatures (275, 350, 425, and 500°Celsius) for 4 h to obtain four ZnO nanopowders with different aspect ratios.[18,19] The obtained powders were then washed twice with distilled water, followed by drying in an oven at 80^0C for 8 hours.The surface morphology of the prepared sample of Zinc oxide powder was studied using SEM.[20] The images of synthesized Zinc Oxide nanoparticle obtained by using SEM (Scanning Electron Microscopy). The SEM image at X 25,000 magnification shows that the nanoparticles were spherical, oblong and rod in shape (Figure 3). The diameter varied from 9.6-25.5 nm in size. Table 1 indicates ZetaPotential at different temperatures. This varied from -0.455, -2.50, -2.50 and - 6.51.

Cultivation of *P.indica* by using Jaggery as Sole Carbon Energy Source

Most important advantage of *P.indica* over AM fungi is that it grows axenically on wide range of synthetic solidified and broth media e.g., Aspergillus, Modified Aspergillus Medium, Potato Dextrose Agar, woody Plant Medium, Yeast Extract Mannitol Peptone Agar, Czapek's Dox Agar, Malt Extract, M4N, Modified Melin Norkrans, MMN 1/10, MMNC, MS, MYP Mycelium of *P. indica* forms rhythmic rings on solid agar medium. It is propagated by chlamydospores or by mycelium. Among the tested media, most optimum was aspergillus. The cost of this media is high due to which mass production becomes expensive. In earlier study locally available cheap media was used as an alternative culture media for mass production of entomopathogenic fungi. *P. indica* was cultivated in a 10L bioreactor (with Bio Eco Spin, GAK equipment and technology), which is of 7L working capacity. Special focus was given to minimize the use of expensive nutrients and grow the fungus on cheap energy source i.e., Jaggery (Gud) which can be easily obtain from Sugarcane (*Saccarum officanarum*) (Figure 4). The 4% Jaggery was used and it itself is a complete energy source for growth of *P.indica*.[21] The fermenter contained 7 l of Jaggery medium 4% (w/v), which is corresponding to about 70% of working volume. Other environmental parameters of bioreactor were as follows: inoculums size: 5%, initial pH: 6.5±2, agitation speed: 180rpm, temperature: 28±2, aeration rate: 0.40 vvm. Out of Jaggery 4% (w/v) and Hill and Kaefer which are used for the mass cultivation of *P. indica* in a bioreactor, the maximum dry biomass and spore count of 6.27g and 1×10^9–1×10^{10} repectively was obtained in Jaggery medium (Table 2). Moreover, in case of Jaggery medium the colonies get enlarged as compare to Hill and Kaefer due to rapid glucose consumption (Figs. 5 and 6).

A Crop of great interest – Black Rice (Oryza sativa L. indica)

Rice (Oryza sativa L.) is one of the most important cereal crops in the world. The major area of production is Asia, with more than 6.6 billion tonnes produced in 2016, followed by America (36 million tonnes) and Africa (32 million tonnes) (FAOSTAT, http://www.fao.org/faostat/en/#home). One of the advantages of rice is that it can be grown in a wide range of environments, even in areas not suitable for other crops .

Black rice is a prevailing fashion among wellbeing oddities and this is simply because of its umpteen medical advantages. Black rice speaks to one of the most significant grain in Asia, with high content of vitamins like riboflavin, niacin and vitamin B, proteins, minerals which are high source of antioxidants.[22] It has a long life cycle.[23] it is a grain that has a place with the *Oryza sativa L.* species, with in excess of 200 varieties around the world. Black Rice was originally cultivated in Odisha (India) but is presently extensively cultivated in North-Eastern States of India such as Assam and Manipur,[24,25] where despite its low yield, it is consumed for its medicinal value.[26] The black rice shows black colour because of high content of anthocyanin (Fig. 7). Some varieties of this black rice species are gluten rich. Rice is indeed the staple diet for a vast majority of Indians inhabiting the 15 agro-climatic regions and is a source of nutritional supplement to the poorest of the poor. Curiously, Black rice in antiquated occasions was a prized ownership and was prevalently known as the Forbidden rice or Emperor's rice, the name of this rice is as intriguing as its story. As the name infers, an appropriate consent from regal specialists was an order to devour this rice. Eating this rice without looking for authorization had perilous outcomes. In China, black rice was solely utilized for the utilization of imperial families, which is the reason it was otherwise called Emperor's rice. This kind of rice is viewed as a decent panacea for some, stomach related ailments on account of its therapeutic impact. However, its ability to foster the nutritional needs of the marginalized populace has not been successfully harnessed to its fruition.

One serving of black rice contains around 156 calories, in addition to high levels of flavonoid phytonutrient anthocyanin, it is a good source of fiber as well as micro-nutrients such as iron and copper, along with plant based proteins,[27] The fact that black rice is fortified with phytonutrients such as anthocyanin. The anthocyanins are compounds that occur naturally in black rice, being a gluten free, cholesterol-free, low in sugar, salt and fat type of cereal, in addition to being responsible for lowering the cholesterol in the human body.[28] For quite a while, black rice anthocyanins (BRACs) were viewed as a critical piece of various wellbeing advancing practical nourishments due to their cancer prevention agent movement,[29] hypoglycemic, anticancer and anti-inflammatory effects[30] These compounds are found in the pericarp (Shen et al., 2009), the aleurone layer and seed coat and of black rice,[31] and they can also be isolated as fractions to use them as food ingredients and functional colorants. These compounds are considered as one of the most efficient antioxidants in the whole plant kingdom.[32]

Rich in fibers suggest that local inhabitants of the North-eastern parts of the country consume it to reduce not only the burden of free radicals in their body but also ease out the bowel movements thereby preventing episodes of bloating, diarrhea as well as constipation. Another important trait of black rice pertains to its unique ability to detoxify the body leading to weight loss. Black rice is gluten free and can also be used as a staple diet for patients suffering from Celiac Disease, can supplement their diet with black rice to fulfill their daily requirement of protein and fiber. Some reports showed that by including black rice in diet will help to improve the lipid profile, reduce the oxidative stress and also modulated the atherosclerotic lesion.[33] As black rice has many health benefits so it also used in the food industries as an organic dye.

A CASE STUDY

Interaction of *P.indica* with ZnO Nanoparticle

The growth of *P.indica* in the presence of different ZnO Nanoparticles was signified in terms of increase in dry weight and spore count. *P.indica* was inoculated in 100 ml of Jaggery medium containing ZnO prepared at different temperatures i.e., 275, 350, 425 and 500°C, respectively. The inoculated *P.indica* culture was kept in dark at 28°C at 120 rpm for 7days. *P.indica* without ZnO nanoparticle was also grown separately which was considered as control against treated sample. On 7th day, fungal biomass was filtered by using Whatman filter paper No. 1. After removing all moisture the fungal biomass was dried in hot air oven for about 24 h at about 85°C and weighed the dry biomass by using weighing balance. The dry biomass was calculated by using w= C-Co/Co x 100 in which W= increase in dry weight of fungal biomass in the presence of ZnO nanoparticle, Co is dry weight of fungal biomass without ZnO nanoparticle and C is the dry weight of fungal biomass in thre presence of ZnO. Treatment with ZnO nanoparticles stimulated fungal growth evidenced with large numbers of small, uniform globular fungal pellets in comparison to control. Maximum fungal biomass was obtained with ZnO synthesised at 500° C (Fig. 8).

Synergistic affect of Black Rice and symbiotic fungi Interaction of Seeds with Nanomaterials

The seeds of Black Rice var. Chakhao Poireiton obtained from Manipur, North India were germinated on the wet filter paper in aPetriplate impregnated with 100 micro liter of 100 ppm nano material prepared at different temperatures (275, 350, 425 and 500^{0}C). The glass petri plates were incubated in dark for 4-5 days at 28^{0}C. Table.3 indicates the average number of root length and branches measured after 6 days of incubation. The maximum number of root branches and root length were recorded for those treated with nano zinc material synthesized at 500^{0}C. Early seed germination was also recorded while fortified with nano material. The preliminary work justifies the impact of nano zinc on seed germination and root development.[34]

Co-cultivation of Black Rice with Nano embedded *P.indica* in Green House

In pot experiments, the Black Rice responded variably towards the different treatments i.e Pi-ZnO, Pi, ZnO and Control (without any treatment).It was observed that Nano embedded fungus had more pronounced effect on the overall plant growth as evidenced by increased seed germination rate, fresh root and shoot weight, dry root and shoot weight and root and shoot length of the seedlings in comparison to treatment with *P. indica* alone or the control.

Black Rice seeds brought from Manipur, North India of same sizes were planted in pots of same size. The pots were kept at temperature 28°C, humidity 60%, light (16h light and 8h dark).After 30 days, the roots and shoots were separated and washed under running tap water to remove soil and then dried to remove moisture. The significant difference in root and shoot length of Black Rice plants with different treatments were analyzed. Radical and shoot lengths shows that plants when treated with combinations of Pi and ZnO nanoparticle attained maximum root and shoot height as compared to plants with Pi and ZnO alone.

Table 3 represents the most considerable data i.e plants with the treatment of ZnO and P.i were the tallest at 10.94 cm as compared to plants with *P.indica* and ZnO alone (i.e 7.31 and 6.91, respectively). This increase in plant height is because of the presence of ZnO which closely interact with plant and act as an important base to provide essential nutrients which leads to the vital plant growth. Similarly, root height of treated plants also gives significant response to ZnO as ZnO and Pi inoculated plants possessed the longest roots (3.94 cm), while plants with *P.indica* and ZnO alone possessed the shortest roots (2.01 cm and 1.43 cm, respectively).

The results were confirmed by taking fresh & dry weight of seedlings which also shown the same trend as other parameters i.e. fresh weight of root and shoot and dry weight of root and shoots of treated plants co-cultured with *P.indica* and ZnO embedded fungus is maximum describes in Table 3. The *Pi* and ZnO interaction effects the fresh and dry root and shoot weight of plants as treated plant has maximum fresh and dry weight 10.91 and 3.26 respectively. This above observations were according to shoot and root length of seedlings i.e. plants with maximum root and shoot length have maximum biomass.

Based on results obtained, by deploying nano-embedded fungus plant showed high biomass, reflects early flowering and enhanced seed germination. These results depict that this nano and fungus interaction play important role to increase plant biomass, enhance early flowering improve seed germination rate. This is due to the fundamental role of nanoparticle to maintain structure of cell wall, provide protection, enzyme synthesise.

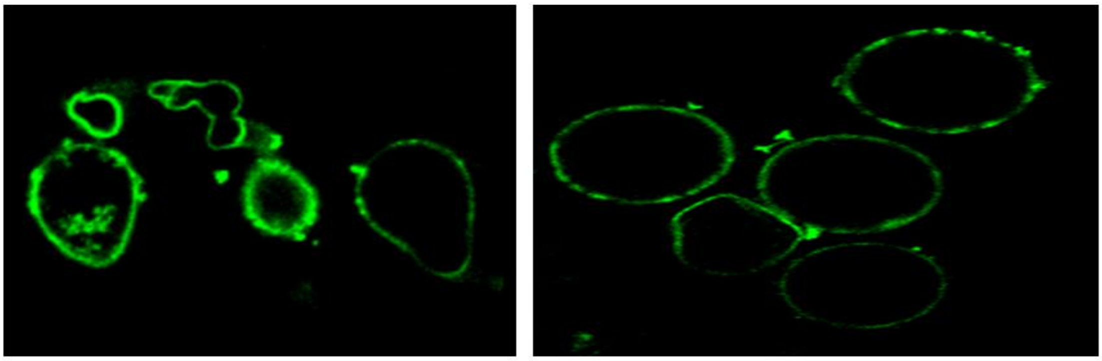


Fig. 1: A view of *P.indica* spores under Confocal Microscope.

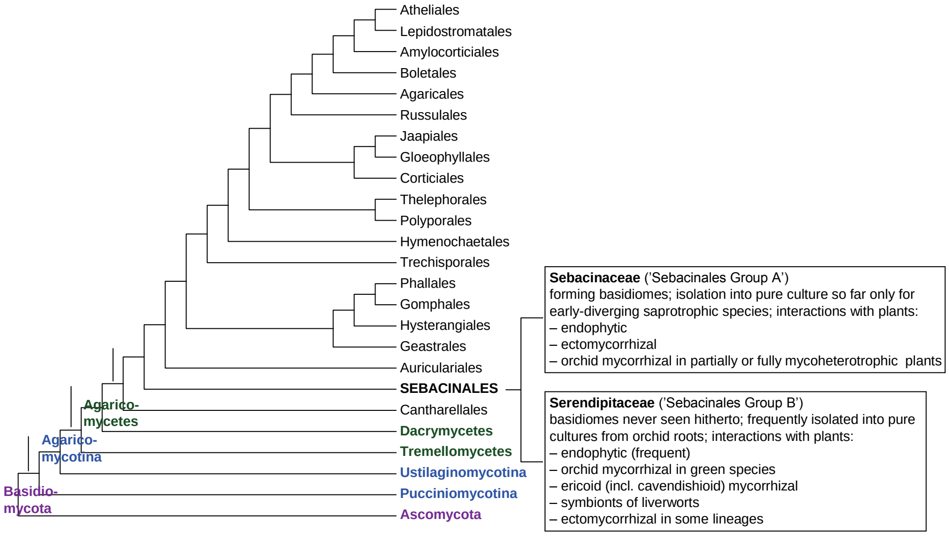


Fig. 2: Tree of phylogenomic analysis taken from Weis et al 2016.

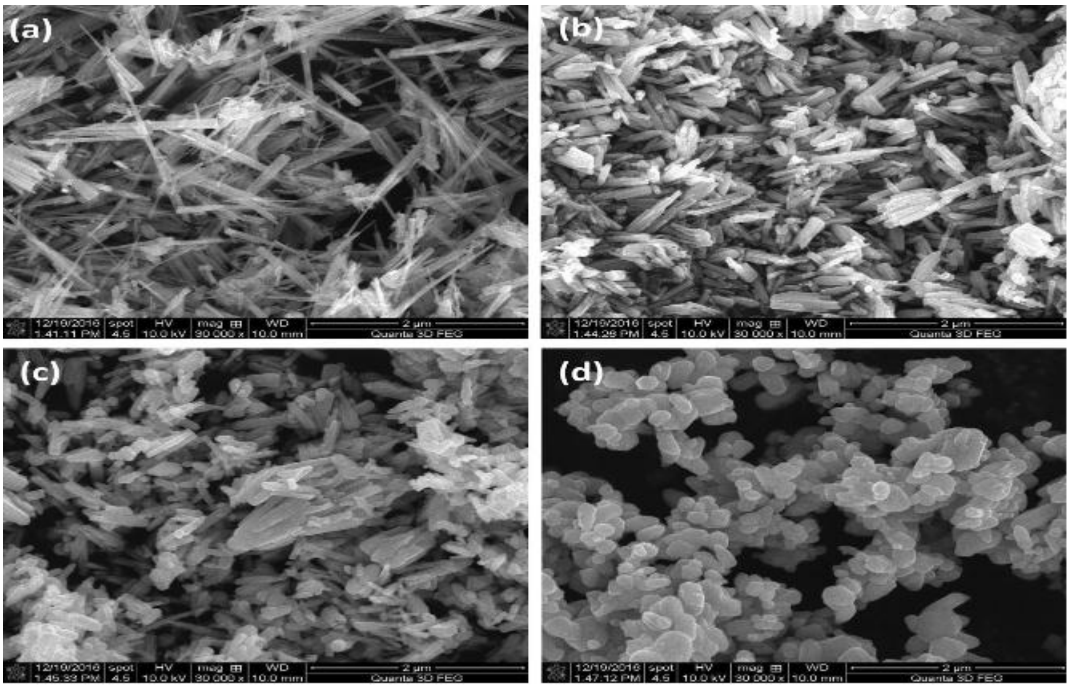
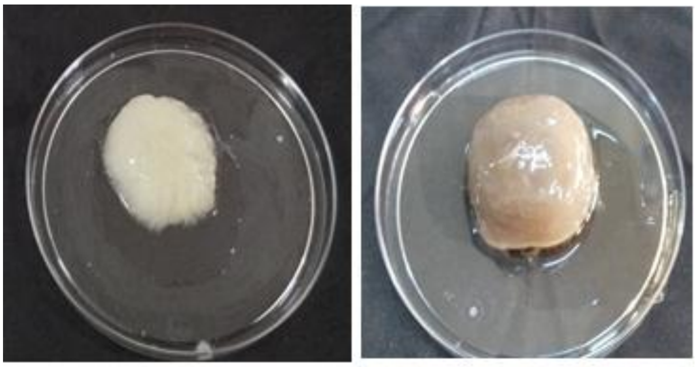


Fig. 3: Morphology under SEM A. ZnO at 275°C B. ZnO at 325°C C. ZnO at 450℃ D. ZnO 500°C, courtesy Dr Manika Khanuja, Jamia Islamia University, New Delhi.

Fig. 4: Innovation: Jaggery (gur).

Hill and Kaefer Jaggery

Fig. 5: Multiplication of *P.indica* in Fermenter by using different nutrient medium.



Hill and Kaefer Jaggery

Figure 6: *P.indica* Biomass at Day 7.

Figure 7: Black Rice seed A. With Husk B. Without Husk C. Boiled D. Anthocyanin.

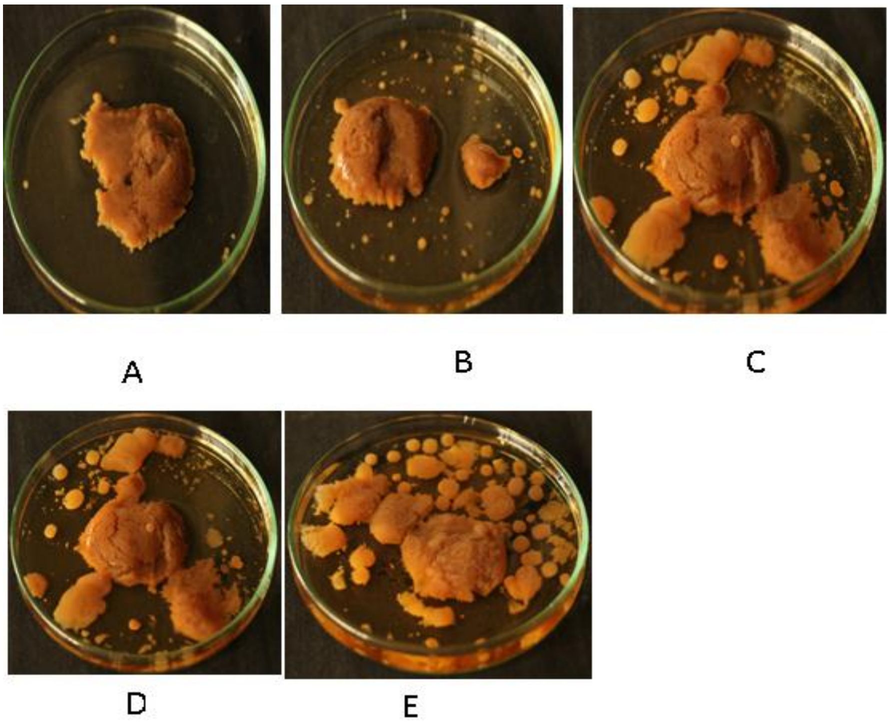


Figure 8: ***Piriformospora indica*** **grown with nanomaterialsA. Control B. ZnO at 275°C C. ZnO at 325°C D. ZnO at 450℃ E. ZnO at 500° C.**

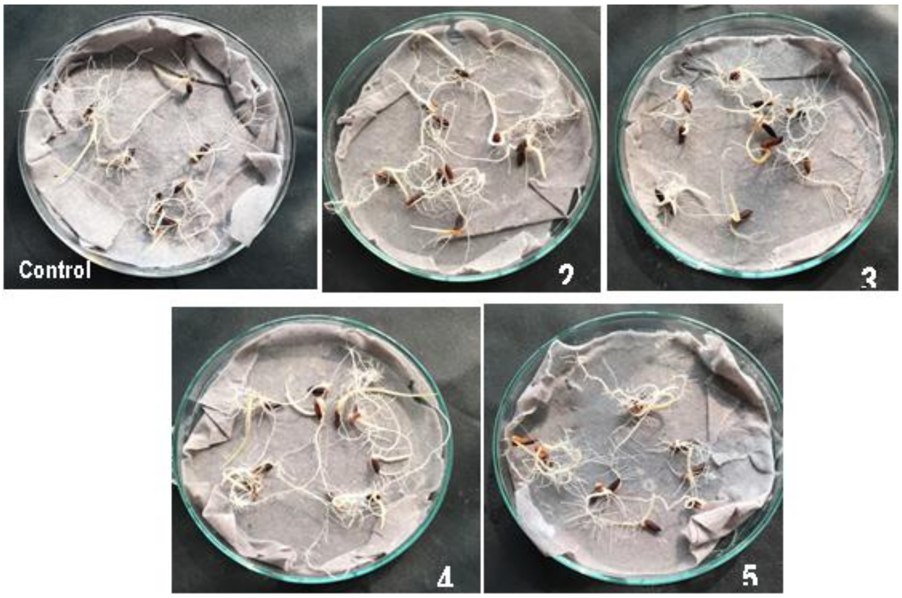


Fig. 9: Seeds were treated with 100ppm nanomaterials prepared at 275, 325, 450 and 500℃, respectively.Profuse rooting was observed after 7 days.

Table 1: Zeta Potential at different temperatures.

Zeta Potential Analyzer	Charge
Zeta potential of ZnO at 275°C	-0.455
Zeta potential of ZnO at 300°C	-2.50
Zeta potential of ZnO at 425°C	-2.50
Zeta potential of ZnO at 500°C	-6.51

Table 2: Effect of Nutrient medium on groeth and sporulation.

Medium	DCW (g/100ml)	Spore Count/100ml
Hill and Kaefer	6.01	$1 \times 10^7 - 1 \times 10^8$
Jaggery	6.27	$1 \times 10^9 - 1 \times 10^{10}$

Table 3: Effect of ZnO NPs on different parameters of Black Rice.

Growth Parameters	Control	*P.indica*	*P.indica*+ZnO
Plant Height (cm)	6.91	7.31	10.94
Root Length (cm) (±SE)	1.43 ± 0.025	2.01±0.02	3.94±0.15
Fresh weight (g) (±SE)	5.6±0.05	6.15±0.3	12.04±0.32
Dry weight (g) (±SE)	0.94±0.01	1.1	1.3

CONCLUSION

We can conclude that this nano and fungus interaction play important role to increase plant biomass, enhance early flowering improve seed germination rate. This is due to the fundamental role of nanoparticle to maintain structure of cell wall, provide protection, enzyme synthesise.

FUTURE PROSPECTIVE

In future we can study about the mechanism that how this nano material enhance the fungal biomass and spore count. Moreover, how it can enhance the overall plant growth. For this the genomics, proteomics and metagenomics studies can be carried out to find out the triggering factor.

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