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Antimycotic efficacy of Zinc nanoparticle on dark-spore forming Phytopathogenic fungi

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Abstract
We investigated the antimycotic activity of zinc nanoparticle (ZnNP) against the dark-spore formers, *Bipolaris sorokiniana* and *Alternaria brassicicola*. This study is a primary investigation to observe whether ZnNP is effective to interfere with fungal metabolism in such phytopathogenic fungi. The efficacy of ZnNP was assessed using four different concentrations (10, 20, 50 and 100 ppm) on the spore germination as well as mycelial growth of these fungi. ZnNP at 20 ppm was effective to inhibit spore germination in *B. sorokiniana*. However, 10 ppm was found effective for *A. brassicicola*. Further, mycelial growth of both the fungi upon application of ZnNP at four different concentrations of 10, 20, 50 and 100 ppm was tested. Again, 100 ppm concentration of ZnNP was reported to inhibit the growth of both the pathogens significantly.

Keywords: *Alternaria brassicicola*, *Bipolaris sorokiniana*, Nanoparticle, Spore germination, ZnNP

Introduction
Bipolaris sorokiniana and *Alternaria brassicicola* are two well known foliar pathogens characterized by multiple cycles of conidia production. Rain, dew and wind aid their spores dispersal causing secondary infections (Acharya *et al*., 2011) [1]. *B. sorokiniana* isolates are observed to differ among themselves in virulence and aggressiveness (Duveiller and Altamirano, 2000) [8]. Therefore, the presence of collateral hosts intensifies the disease epidemic of *B. sorokiniana* (Bashyal *et al*., 2010) [4]. Similarly, the other foliar pathogen *A. brassicicola* is a necrotrophic fungus capable of infecting a huge number of economically important crops of brassicaceae family. Both the foliar pathogens are hence responsible for the enormous losses made to rabi crops grown in gangetic plains and northern regions of India. The global climate change prohibits desperate use of toxic agrochemicals for their management now-a-days. Also, efficacy of biological method of management is not always effective against these pathogens (Vallad and Goodman, 2004) [23]. An alternative way of their management is thus the need of the hour. Application of nanoparticle in the field of plant health management may find its way in such situations (Mishra *et al*., 2014; Kim *et al*., 2012) [16, 13]. Nanoparticles differ from their bulk counterpart in terms of their chemical reactivity, mechanical as well as physical properties. Any material at nanoscale possesses far distinct properties due to which application of nanoparticles has already attracted attention in different fields like medicine, pharmaceuticals, cosmetics, and electronics including little experimentation in agriculture. However, the research in the field of fungal disease management is still on its way to explore the future opportunities provided by nanoparticle in this field. Zinc has long been an essential micronutrient needed for the overall growth and development of plants. Compounds containing zinc are commonly used as fungicides (Waxman, 1998) [25]. Also, zinc simultaneously is biocompatible to human cells and has long been shown to exhibit antimicrobial activity (Padmavathy and Vijayaraghavan, 2008; Yamamoto, 2001; Sawai and Yoshikawa, 2004) [18, 26, 22]. Zinc at nanoscale is currently being used as fertilizer for slow and controlled release of vital plant nutrients. Few studies have even shown its inhibitory aspect on fungal as well as bacterial pathogens. The present study was thus targeted to test the applicability of ZnNP in the field of plant health management.

Materials and methods: Isolates of *B. sorokiniana* and *A. brassicicola* were collected from infected Barley and Mustard crops respectively from Agricultural Farm of Bihar Agricultural University, Sabour. The specimen was washed under running tap water, cut into small pieces, again washed thrice with 1% sodium hypochlorite solution for 20-30 seconds and finally washed with distilled water. Thereafter, the specimens were transferred onto PDA plates, allowed to grow for 2-3 days and incubated at 25±2 °C. Fungal mycelium from the edge was transferred to PDA slants, grown for 5-6 days in the incubator and stored at 4 °C in household

refrigerator. Isolates were maintained and subsequently used in the experiment whenever required. Zinc (ZnNP) nanoparticle was prepared following the procedure suggested by Aneesh *et al*. (2007) [2]. Stock solutions of $Zn(CH_3COO)_2.2H_2O$ (0.1 M) was prepared in 50 ml methanol with continuous stirring. To this, stock solution of 25 ml of NaOH (0.2 M to 0.5 M) solution prepared in methanol was added with continuous stirring to obtain pH value of reactants between 8 and 11. Solution was then transferred into Teflon lined sealed stainless steel autoclave, and were maintained at various temperature in the range of 100 ºC to 200 ºC for 6 and 12 h under autogenous pressure. Solution was then allowed to cool naturally at room temperature. TEM (Transmission electron microscopy) revealed zinc nanoparticle to be in the range of 29-37 nm. For testing efficacy of zinc on mycelial growth cultures and spore production, four different concentrations of zinc (ZnNP) nanoparticle were prepared by diluting the initial concentration of 100 ppm solution which was later used in *in-vitro* studies. Four different concentrations along with control were maintained in cavity slides for counting of spores under microscope. Also similar concentrations were mixed in PDA media for conducting poisoned food experiment. Effect of nanoparticle on spore germination% as well as inhibition% was tested by preparing conidial suspension of 15-20 days old cultures of both pathogens viz. *B. sorokiniana* and *A. brassicicola* in 0.025% Tween20 solution (Ghatak *et al*., 2013) [9]. Conidial suspension was further mixed with different concentrations of nanoparticles (viz. 10 ppm, 20 ppm, 50 ppm and 100 ppm) and placed over the cavity as stated earlier. Conidial suspension in Tween20 without nanoparticle was maintained as Control set. Thereafter, the spore suspension containing cavity slides were incubated at 25±2 °C for 24 h (*B. sorokiniana)* and 48 h (*A. brassicicola)* in moist chambers developed with Petri plates (90 mm diameter) containing blotter papers soaked in sterile distilled water. The slides were examined under microscope (40×, Olympus) at 24 h interval for *B. sorokiniana* and 48 h for *A. brassicicola*. Similarly, the efficacy of zinc (ZnNP) nanoparticle (at concentrations of 10 ppm, 20 ppm, 50 ppm and 100 ppm) on mycelial growth of 15-20 days old cultures of *B. sorokiniana* and *A. brassicicola* were observed by conducting poisoned food assay technique. Control set with no use of inhibitory chemical was compared with the rest. Actively growing mycelium of diameter 5 mm was picked with the help of cork borer and placed at the centre of a PDA plate having particular concentration of nanoparticle and the control plate. Radial growth was measured by averaging the horizontal and vertical measurements for four times at an interval of 48 h (Kumar *et al*., 2018) [14].

Results and Discussion: Efficacy of zinc (Zn) nanoparticle at different concentrations was evaluated against *B. sorokiniana* and *A. brassicicola*. Among various potential nanoparticles used in the field of plant health management, ZnNP may be preferred owing to its excellent chemical and thermal stability, low cost and environmental-friendliness (Sabri *et al*., 2013) [21]. Further, Zinc being one of the essential micronutrients needed for the overall growth and development of plants may be utilised for its applicability in controlling pathogenic growth. ZnNP has been found to exhibit antimicrobial activity against bacteria *Bacillus subtilis* (Meruvu *et al*., 2011) [15], *Staphyllococcus aureus* (Sabir *et al*., 2014) [20], *Aspergillus fumigates* and *Candida albicans* (Jasim, 2015) [11]. The antifungal as well as antibacterial property of ZnNP is attributed to its higher surface to volume ratio at nanoscale (Cassaignon and Colbeau, 2013; Kahru and Dobourguier, 2010; Ray *et al*., 2009) [6, 12, 19] and is known to induce deformation in the structure of fungal hypha of *Fusarium oxysporum* and *Penicilium expansum* (Yehia and Ahmed, 2013) [27] suggesting ZnNP to be responsible for structural changes of microbial cell membrane causing cytoplasm leakage and death of pathogen (Brayner *et al*., 2006; Sawai and Yoshikawa, 2004) [5, 22]. The present study was thus targeted to analyse inhibitory property of ZnNP against two foliar pathogens *B. sorokiniana* and *A. brassicicola*.

Changes in Spore Germination %: In general, lower germination% was observed in *B. sorokiniana* when compared to *A. brassicicola*. Germination% at concentrations of 10 ppm, 20 ppm, 50 ppm and 100 ppm was noted at 24 h and 48 h of incubation for *B. sorokiniana* and *A. brassicicola* respectively. For both the foliar pathogens, lower germination% was recorded at 100 ppm concentration of nanoparticle over control. This result draws conclusion from the earlier experiment conducted by Wani and Shah (2012) [24] which states that application of ZnNP reported for high inhibition in the germination of fungal spores of *Alternaria alternata*, *Fusarium oxysporum*, *Rhizopus stolonifer* and *Mucor plumbeu*. Result revealed that 20 ppm concentration of ZnNP may be observed suitable for germination test considering greater inhibition in spores of *B. sorokiniana*. Effect of zinc (ZnNP) nanoparticle on germination% of *A. brassicicola* was found to be non-significant among 10, 20 and 100 ppm but significantly higher germination was detected in 100 ppm. Hence, 10 ppm (minimum dose) of Zinc (ZnNP) is suggested for further test when targeting greater inhibition in germinating spores of *A. brassicicola*. Further, *A. brassicicola* was observed to be resistive to ZnNP as against *B.* sorokiniana. The significant difference in antifungal activity of ZnNP among the two fungi may possibly be due to difference in the level of tolerance of *B. sorokiniana* and *A. brassicicola*.

Effect of ZnNP on mycelial growth: In order to find out the effect of ZnNP on the mycelial growth of both the foliar pathogens *B. sorokiniana* and *A. brassicicola*, poisoned food assay was conducted. Radial growth was measured at 48, 96, 144 and 192 h after inoculation (hai). For determining significance, last observation i.e. at 192 dai was considered for the purpose of analysing the deviation in radial growth of the fungal culture, reason being slow growing character of fungus. Larger mycelium was visualised on control set as compared to the other four concentrations of ZnNP for both the pathogens. All the four concentrations of ZnNP revealed lower radial growth of the mycelia when compared to control. This result is in agreement with the earlier research work which shows that ZnNP significantly inhibited growth of *Botrytis cinerea* (63-80%) and *Penicillium expansum* (61-91%) in a plating assay due to the systemic disruption of cellular function within both pathogens (He *et al*., 2011) [10]. Greater than 80% inhibition in the growth of mycelia is reported for *Colletotrichum gloeosporioides* on ZnNP application (De la Rosa-García *et al*., 2018) [7]. ZnNP at 10 ppm in the present study was found to inhibit the spore germination of *B. sorokiniana* and *A. brassicicola*. Lower level of germination% of spores suggests the fact for employing zinc nanoparticles for restricting the spore germination of pathogens, paving scope for control at primary

level of infection. Further, the observance of mycelial growth inhibition of both the pathogens at 100 ppm concentration of ZnNP is in aggrement with the earlier report of zinc exhibiting antimicrobial activity against *Aspergillus flavus* and *Aspergillus fumigates* (Navale *et al*., 2015) [17]. The interaction between the nanoparticles and biological protein present in the microbes is attributed to the fact that microorganisms consider nanoparticles as foreign materials that induce them to emit pro-inflammatory signals, this activity has been utilised to prove microbial inhibition in *Erythricium salmonicolor* in terms of reduced mycelial growth on application of ZnNP (20-45 nm) (Arciniegas-Grijalba *et al*., 2017) [3]. The lower mycelial growth on nanoparticle applied plates suggests the implication of nanoparticles in effectively reducing the growth of fungus. Commercial fungicides are nearly threatened in their use due to rise of microbial resistance in recent times, hence an alternative management tool in the form nanoparticles is the current demand of phytopathological research. However, future studies regarding the optimum time and concentration of nanoparticle application needs to be stressed for more and better use of nanoparticle in restricting the growth of fungus. Till today, the study of nanoparticles in managing plant diseases is limited to *in vitro* studies hence more extensive field trials may be considered in future for developing nanoparticles, zinc being one among them to restrict the growth of pathogens at the very initiation of disease.

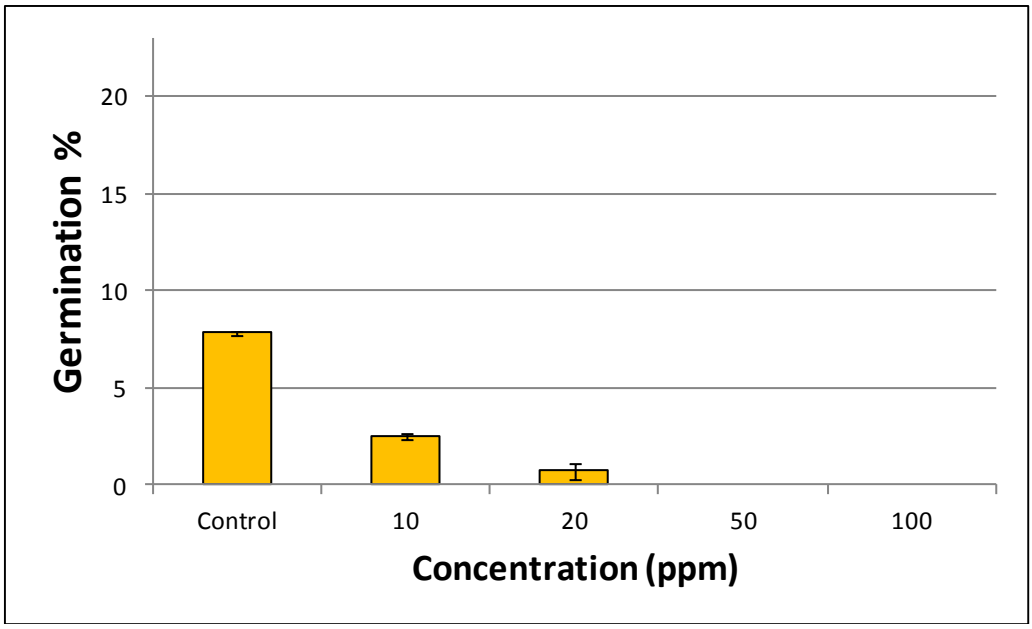

Fig 1: Germination% of *Bipolaris sorokiniana* on ZnNP application

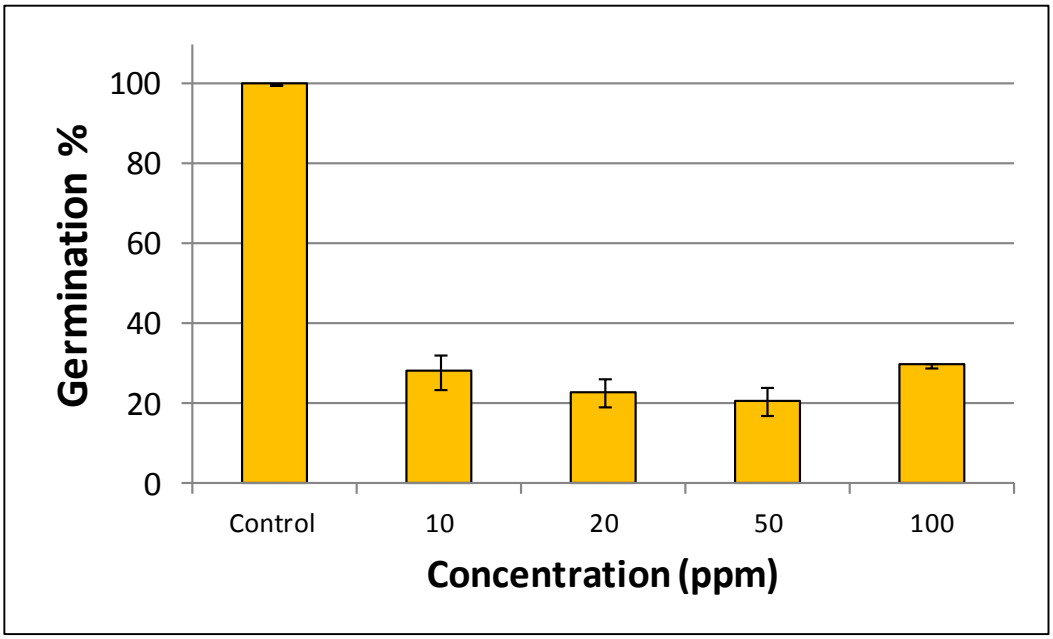

Fig 2: Germination% of *Alternaria brassicicola* on ZnNP application

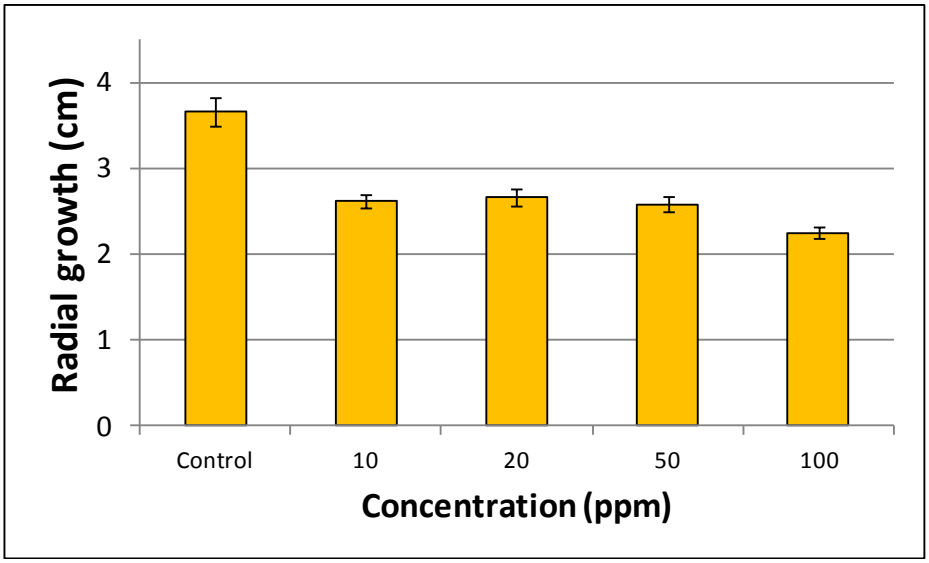

Fig 3: Mycelial growth on ZnNP application in *Bipolaris sorokiniana*

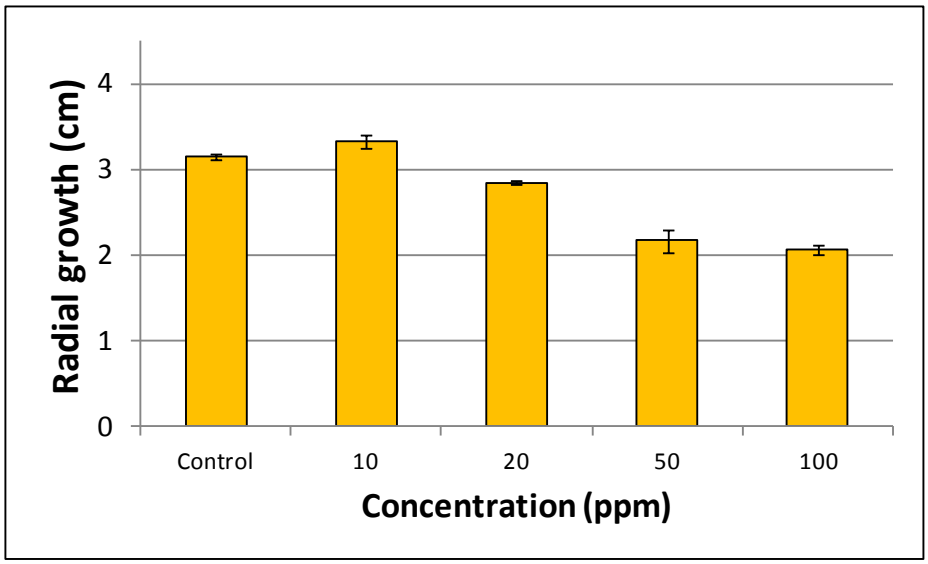

Fig 4: Mycelial growth on ZnNP application in *Alternaria brassicicola*

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