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# *In vitro* and *in vivo* antifungal properties of silver nanoparticles against *Rhizoctonia solani*, a common agent of rice sheath blight disease

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## Abstract

Sheath blight disease in rice has caused major crop losses worldwide. Managing the causal agent of disease *Rhizoctonia solani* Kühn is difficult because of its broad host range and formation of sclerotia which can survive in harsh environmental conditions; therefore developing innovative disease management methods without application of hazardous chemicals has been considered as the main concern to maintain sustainable agriculture. This presented research has revealed the negative impact of Silver nanoparticles (SNPs) on *R. solani* and disease progress both *in vitro* and *in vivo.* The adverse effects of the SNPs on *R. solani* are significantly dependent on the quantity of SNPs, sprayed at different concentrations *in vitro*. The highest inhibition level against sclerotia formation and mycelia growth are 92 and 85%, respectively, at a SNPs concentration of 50 ppm. *In vivo* glasshouse experiments also showed that SNPs at the same concentration favorably affects

both the fresh and dry weight of rice plants with a remarkable suppressive effect on the lesion development in leaves.

**Keywords**: Nanotechnology, silver nanoparticles, rice sheath blight disease, sclerotia formation, lesion development

## 1. Introduction

Application of nano-materials has widely influenced drug delivery, cancer therapy [1], energy [2], biomedical [3], agriculture [4] and many other high-tech industries over recent years [5]. Nanotechnology has led to the new ways to control diseases using atomic-scale materials [6, 7]. The extremely small-scale particles have emerged as modern agents owing to their large surface to volume ratio which provides a large contact surface with pathogen sources [8]. Nanotechnology applications in agriculture can be successful if natural processes are simulated in greater scientific for successful implementation and examples of successful tools at small scale [9]; plant protection products [10]; Fertilizers [11]; Water purification and pollutant remediation [12]; Nanosensors and diagnostic devices [13]; plant genetic modification [14].

Among nanoparticles, silver NPs (SNPs) can attack microorganisms, including the cell membrane structure in large-scale biological processes [15, 16]. The antibacterial activity of silver ions has been well established and attributed to the ability of ionized SNPs to penetrate into the bacterial cell wall and to modulate cellular signaling [17]. SNPs with fungistatic, bacteriostatic and plasmonic properties are among the eco-friendly inhibitors against plant-pathogens compared to synthetic fungicides [18]; however the antifungal ability of SNPs has received less attention compared to medical and pharmaceutical sciences with only few studies undertaken against phytopathogenic fungi such as *Alternaria alternata, Botrytis cinerea* [19], and *Colletotrichum gloeosporioides* [20].

Rice (*Oryza sativa* L.) is a major food for a large proportion of the world's population, and is an important primary crop in muddy farmlands [21]. Sheath blight disease caused by *Rhizoctonia solani* Kühn AG1 (Teleomorph: *Thanatephorus cucumeris*; anastomosis group 1 IA, AG1 IA), is a common destructive disease of rice in all rice-growing regions in the world. Sclerotia germination is a key factor in the dispersion of rice sheath blight disease, hence any potential inhibitor of sclerotia germination, i.e., SNPs, would be essential in order to decrease the inoculum. This impels rice farmers to use a large amount of anti-nature and harmful chemicals annually to control sheath blight disease, which not only adds further costs in the short term but increases devastative damages in the long term to the human health and environment.

In this study, in order to control rice sheath blight disease with emphasis on the cleaner production at a lower cost, different concentrations of SNPs were examined as a new antifungal substance to suppress the pathogenic activity of *R. solani* under *in vitro* (to evaluate the inhibitory effects of SNPs on sclerotia formation and mycelia growth) and *in vivo* (to investigate the effects of antifungal activity of SNPs on the rice plant in a glasshouse trial) conditions.

## 2. Materials and Methods

### *2.1. Reagent, rice seeds and fungal pathogen source*

SNPs suspension was obtained from Nanocide Co., Tehran, with a concentration of 4,000 ppm and an average particle size of 5~10 nm, in dark brown colloid physical form. Rice seeds of *Oryza sativa* L. var Hashemi and pure culture of *R. solani* AG-1 IA were obtained from Iran Rice Research Institute (IRRI), Rasht [22]. The *Oryza sativa* L. var Hashemi potentially has a high yield, and is susceptible to sheath blight disease. The fungus was maintained on potato dextrose agar (PDA, Merck Co.) at room temperature.

*2.2. In vitro examination of inhibitory effects of silver nanoparticles on mycelia and sclerotia of R. solani*

To evaluate the *in vitro* antifungal effects of SNPs against *R. solani* AG1, four different concentrations of SNPs suspension (5, 10, 25 and 50 ppm) were added to Petri dishes before pouring plates with PDA. Uniform agar plugs with a diameter of 6 mm containing fungal mycelia were inoculated simultaneously at the center of each Petri dish containing SNPs, followed by incubation at 28 ± 1°C for three days. The mycelia growth inhibition rate was calculated using Eq. 1 [4]:

$$Inhibition\ rate\ (RH)\ \% = \frac{(R-r)}{R} \times 100 \quad (1)$$

The parameter *RH* is the inhibition rate, *R* for the mycelium inhibition growth is the expansion in diameter of the mycelial fungus in the control dish (cm), and for the sclerotia formation growth under the inhibition process, *R* is the weight of the sclerotia in the control dish (mg). The parameter *r* for mycelium inhibition growth is the expansion in diameter of the fungus mycelial when treated by SNPs (cm), and for the sclerotia formation growth under the inhibition process, *r* is the weight of sclerotia when treated by SNPs (mg).

The antifungal effect of SNPs against *R. solani* sclerotia formation was measured after adding the four concentrations of SNPs to the PDA media content. Inoculated *R. solani* plates were maintained at room temperature for two weeks to manifest sclerotia formation, and the sclerotia formation inhibition rate was calculated using Eq. 1. All tests were carried out in triplicate.

The effect of SNPs on the germination of sclerotia was assayed using the following procedure [23]. The sclerotia of *R. solani* were formed on PDA at 15 °C through incubating the inoculated plates for a week. Uniform sclerotia were collected from PDA plates, and the surface was sterilized in

1.5% sodium hypochlorite solution for 3 minutes. Then, three surface sterilized sclerotia were treated by SNPs of various concentrations and placed in a petri dish, and then they were incubated for a week at 25°C in the dark. The germination rates of sclerotia were measured and compared with the control (without SNPs). The percentage of inhibition against sclerotia was calculated using Eq. 1.

*2.3. In vivo examination of silver nanoparticles on sheath blight disease under glasshouse conditions*

Rice seeds were sown 3-4 cm below the soil surface of the pots (1 L) and they were separated into six groups with four pots in each group as follows: (a) pathogen alone, (b) pathogen + SNPs (5 ppm), (c) pathogen + SNPs (10 ppm), (d) pathogen + SNPs (25 ppm), (e) pathogen + SNPs (50 ppm), and (f) control (without SNPs). Rice plants were grown in pots under glasshouse conditions at 30°C and 85-95% relative humidity. As the plants reached their late tiller stage (three-week-old plants) were treated by the inoculation process with *R. solani*. To achieve this, mycelia suspension of *R. solani* ($5\times10^8$ CFU/ml) was evenly sprayed using a hand sprayer on the rice plants [24]. To maintain a fair coverage of SNPs on the foliage throughout the evaluation period, two sprays applied including 24 hrs post inoculation and seven days, subsequently. After inoculating with pathogen and SNPs spraying process, the seedlings were covered with plastic bags for three days to maintain the high humidity. After 15 days, the disease severity was recorded via measuring the fresh weight, dry weight and relative lesion height (*RLH*), according to the 1996 IRRI standard. The relative lesion height of each tiller was calculated using Eq. 2 [25]:

$$RLH\% = \frac{Lesion\ height\ (cm)}{Plant\ height\ (cm)} \times 100 \quad (2)$$

118 *2.4. Statistical analysis*

119 Recorded data were subjected to analysis of variance with SAS software (SAS Institute, version 9).
120 Duncan's Multiple Range Test was utilized to compare means.

121

122 **3. Results**

123 *3.1. In vitro examination of inhibitory effects of silver nanoparticles on mycelia and sclerotia of R.*
124 *solani*

125 The effects of tested SNPS concentrations on mycelium growth, sclerotia formation and
126 germination are presented in Fig. 1. Plates treaded with 50 ppm SNPs revealed the minimum
127 number of sclerotia. For the, the *RH*s of 12, 23, 56 and 92 % were found for the 5, 10, 25 and 50 of
128 SNPs concentrations, respectively. With regards to the mycelia growth, *RH*s of 8, 35, 67 and 85
129 were recorded for the SNPs of concentrations of 5, 10, 25 and 50 ppm, respectively. The *RH*s of 15,
130 26, 57 and 98 % were found for the SNPs concentrations of 5, 10, 25 and 50 ppm, respectively
131 related to sclerotia germination. These results indicate that SNPs has strongly suppressed *R. solani*
132 under *in vitro* condition (Fig. 2).

133

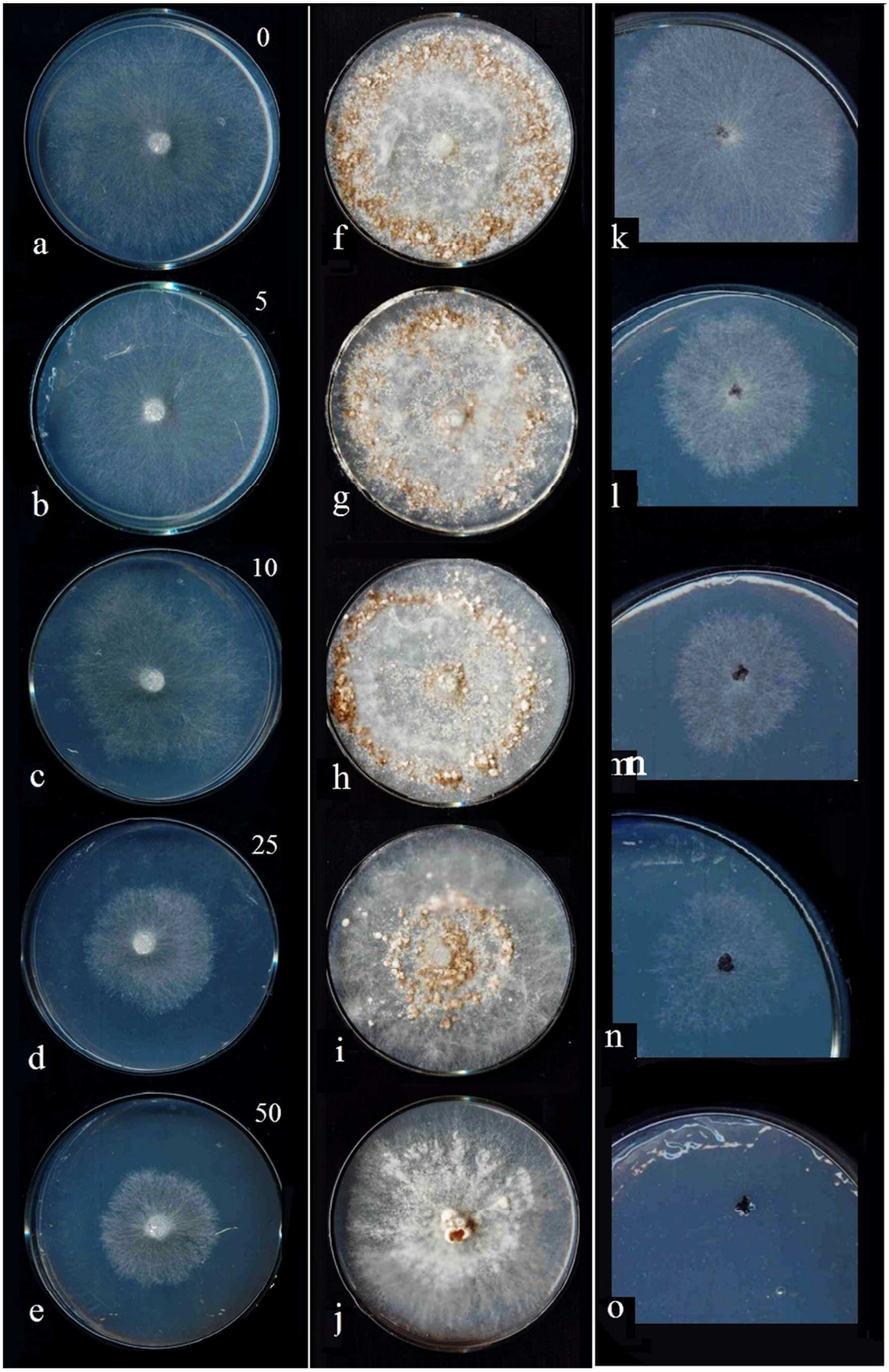

Fig. 1. *In vitro* inhibitory effects of different concentrations of SNPs (indicated in the top right of left column) on *Rhizoctonia solani* AG1. Mycelia growth stage (left column, a-e); sclerotia formation (middle column, f-j) and sclerotia germination (right column, k-o).

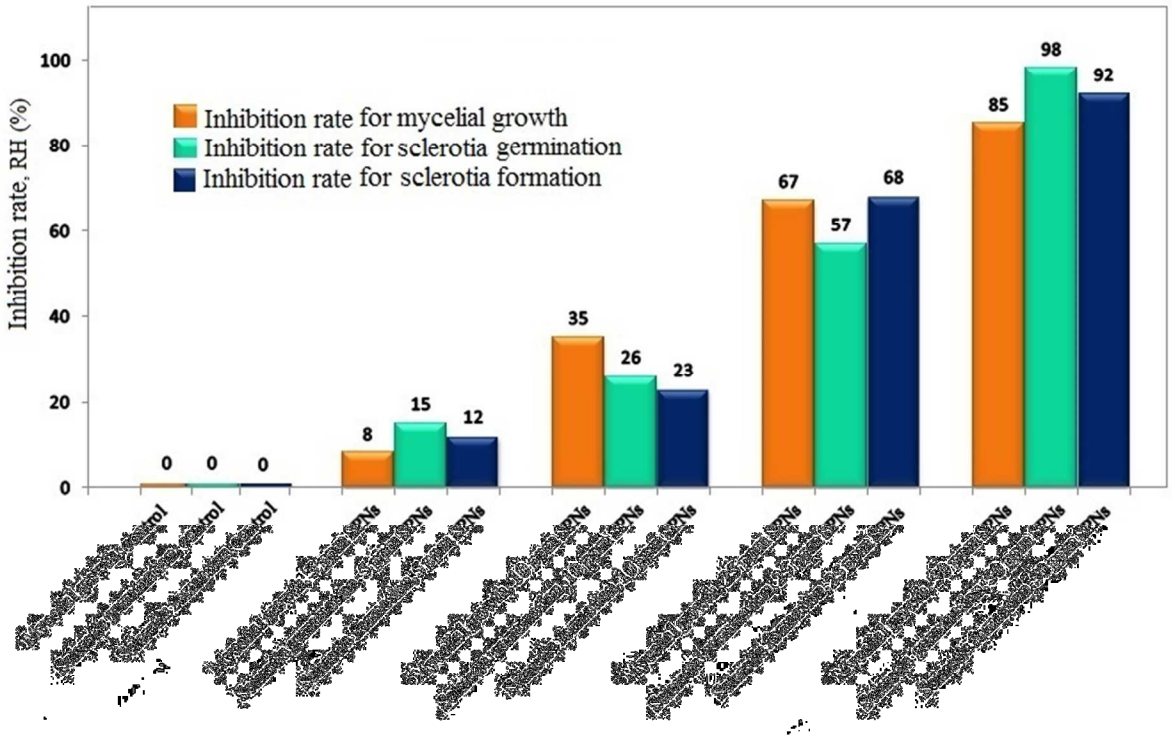


Fig. 2. *In vitro RH* of SNPs effects on mycelia growth, sclerotia germination and sclerotia formation of *Rhizoctonia solani* AG1-IA.

*3.2. In vivo examination of silver nanoparticles on sheath blight disease under glasshouse conditions*

Fig. 3 shows the rice seedlings at the late tiller stage of 90 days under the greenhouse conditions (30°C, 85-95% humidity). The *in vivo* results of SNPs against *R. solani*, the causal agent of sheath blight disease, are presented in Fig. 4. In this figure, A indicates the leaf symptoms resulting from

infection by *R. solani* alone, and b-e indicate the pathogen plus the applied 5, 10, 25 and 50 ppm of SNPs and their different degrees of inhibition in the leave-lesion development. The treatment of plants with the pathogen without SNPs resulted in typical sheath blight symptoms, but the treated plants with pathogen + SNPs show different levels of inhibitory effects. According to the results of ANOVA for different traits, all traits were different at significance level at $P \leq 0.05$. There are significant reductions in the symptoms of pathogen in pots treated with SNPs. The 5 ppm SNPs concentration has a small effect on the dry weight of the rice plants (Table 1); by increasing the concentration of SNPs to 50 ppm, the fresh weight and dry weight increased significantly. At 50 ppm SNPs concentration, the *RLH* of each tiller decreased which evidence that applying SNPs has a strong antifungal influence on and minimizes lesion in the rice tillers. The comparative results of the inhibition activity of SNPs against sheath blight revealed significant reduction of lesions on the rice sheath (Fig. 5).

Table 1. The *in vivo* inhibitory effect of SNPs on rice sheath blight under glasshouse conditions

| Treatment | Fresh Weight (g) | Dry Weight (g) | RLH % |
|---|---|---|---|
| **Control** | $22.6^{*a}$ | $5.8^{a}$ | - |
| **Pathogen** | $12.5^{b}$ | $2.3^{b}$ | 95% |
| **Pathogen + SNPs (5 ppm)** | $13.7^{b}$ | $2.5^{b}$ | 90% |
| **Pathogen + SNPs (10 ppm)** | $14.2^{bc}$ | $2.7^{bc}$ | 80% |
| **Pathogen + SNPs (25 ppm)** | $18.3^{d}$ | $3.8^{d}$ | 55% |
| **Pathogen + SNPs (50 ppm)** | $20.3^{ad}$ | $4.4^{ad}$ | 15% |

*The presented data are the means of four replications, and they are subjected to the analysis with the variance *n=5*

Fig 3. Rice seedlings after 90 days under greenhouse conditions, with a temperature of 30°C and constant humidity of 85-95%


Fig 4. Effect of SNPs treatment on the lesion development by *R. solani* on rice leaves: a) pathogen alone; b) pathogen + SNPs (5 ppm); c) pathogen + SNPs (10 ppm); d) pathogen + SNPs (25 ppm); e) pathogen + SNPs (50 ppm); f) untreated control.


Fig 5. Effect of SNPs on the lesion development by *R. solani* on rice sheath:

a) pathogen alone; b) pathogen + SNPs (5 ppm); c) pathogen + SNPs (10 ppm); d) pathogen + SNPs (25 ppm); e) pathogen + SNPs (50 ppm); f) untreated control.

188
189 *3.3. Discussion*

190 SNPs can denature cells by attacking their membranes and structures. A previous research found
191 that SNPs disrupts transport systems, including ion efflux [26]. The dysfunction of ion efflux causes
192 a rapid accumulation of silver ions, interrupting cellular processes such as respiration and
193 metabolism by reacting with the molecules. Ji Seon et al. (2009) showed that upon the treatment by
194 SNPs spray, the hyphal walls were seriously damaged and resulted in the plasmolysis of hyphae.
195 Considering the cellular effects of silver ions, SNPs mediated the collapse in *Sclerotinia*
196 *sclerotiorum* hyphae which damaged the hyphal walls [27].

197 Silver ions are known to deactivate cellular enzymes and DNA by coordinating with electron-
198 donating groups such as thiols, carboxylates, indoles, amides, hydroxyls, etc. [28, 29] According to
199 past studies on SNPs, the smaller the SNPs, the more Ag+ ions they release which affects the

performance of microorganisms [30, 31]. As discovered in our investigation, SNPs in an aqueous solution with small sizes can penetrate into the cells of microorganisms and destroy their membrane integrity [32-34].

NPs have a vast surface to volume ratio which significantly enhances their property of cell membrane permeability compared with non-NPs forms of the same material [35-37]. NPs are able to penetrate the membranes of microorganisms, leading to cell deformation [38]. NPs, with their large surface to volume ratio, exhibit active antimicrobial properties due to their higher ability to interact with cellular membranes through disruption of the cell wall structure, affecting the respiratory chain and cell division in DNA and proteins as a microorganism [39]. It is likely that the size of SNPs similarly plays a key role in their permeability and antifungal activity. In short, SNPs have an active antifungal activity with great biocide properties, thus, they have the potential to be considered as an economical and eco-friendly pesticide. The application of chemical fungicides adds additional indirect long-term and hidden costs as it causes dangerous side effects in both human health and the environment [40]. The editors of Nature estimated that any technology takes some 20 years to emerge from the laboratory and be commercialized [41]. Application of nanotechnology in agriculture might take a few decades to move from laboratory to land however reasonable expectations would be crucial for this nascent field to blossom [42].

The efficacy of SNPs is increased by conjugating the antifungal drug miconazole with SNPs which exhibits significant fungicidal activity [43]. SNPs with chitin inhibits the spore germination of the examined pathogens [44, 45]. Moreover, bioactive capped SNPs was found to be able to control the endophytic fungus of *Colletotrichum gloeosporioides, in vitro* [46]. The antifungal activity of SNPs is comparable to those of ionic silver NPs; however, ionic silver remains cytotoxic at the concentrations that inhibit the growth of the examined yeasts [47]. According previous research

both positive and negative effects on plant growth and development was suggested [48]. In some plants SNPs can be increase growth as shoot and root length and biochemical attributes such as: chlorophyll, carbohydrate and protein contents, antioxidant enzymes [49].

## 4. Conclusion

*In vitro* and *in vivo* study of the antifungal activity of SNPs at concentrations of 5, 10, 25, and 50 ppm was conducted against fungal pathogen *R. solani* to reduce and prevent the sheath blight in rice seedlings. The *in vitro* results showed the *RHs* for mycelial growth, sclerotia formation and sclerotia germination were respectively (8, 35, 67, 85), (12, 23, 56, 92) and (15, 26, 57, 98) for their corresponding SNPs concentrations (5, 10, 25 and 50 ppm). The results clearly show that the *RHs* strongly depend on SNPs concentration, and substantially increase upon an increase in SNPs concentration. In the vitro examination part, we can conclude an increasing trend in the inhibition rate for mycelial growth, sclerotia germination and sclerotia formation with the increasing amount of SPNs. By spraying SNPs on the rice plants, the sheath disease's symptoms on the leaves decreased, and at 50 ppm SNPs concentration, the symptoms completely vanished. In the vitro examination part, we can conclude an increasing trend in the inhibition rate for mycelial growth, sclerotia germination and sclerotia formation with the increasing amount of SPNs. However the author haven't give the results under condition of more than 50 ppm, which can be used to confirm the relationship between inhibition rate and SPNs concentration

The *in vivo* results show that the SNPs solution created an antimicrobial layer around the rice plants which protected the plants from pathogens. It was also demonstrated that SNPs highly affect sclerotia formation and germination. SNPs can penetrate into the fungal cell membrane and cell wall, killing microorganism cells. This investigation suggests SNPs can replace chemical pesticides in controlling and inhibiting sheath blight, a common disease in rice.

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