Study on Antifungal Activity and Ability Against Rice Leaf Blast Disease of Nano Cu-Cu₂O/Alginate

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10.18805/IJARe.A-582

ABSTRACT

Background: Rice blast disease due to Pyricularia oryzae fungus is one of the most destructive ones for rice, causing serious losses in yield and quality in Vietnam and other countries. Studying to develop new fungicides to control the fungal disease effectively is essential.

Methods: The sodium alginate-stabilized Cu-Cu₂O nanocolloidal solution with a size of about 4 nm and the Cu concentration of 5,000 mg/L was prepared by chemical method. The antifungal activity in vitro against P. oryzae and the ability to control the rice blast disease in the greenhouse of nano Cu-Cu₂O/alginate were assessed.

Result: The nano Cu-Cu₂O/alginate exhibited highly fungal activity with the IC₅₀ of 17.8 mg Cu/L. The disease severity of nano Cu-Cu₂O/alginate treatments was in the range of 9.38 - 18.54% in comparison with 47.57% of the untreated control. The grain yield of rice plants treated with nano Cu-Cu₂O/alginate increased compared with that of the untreated control, and the Cu content in polished rice was almost the same as that of usually cultured polished rice. Thus, the nano Cu-Cu₂O/alginate can be used in agriculture as a plant fungicide, especially for rice cultivation.

Key words: Alginate, Antifungal activity, Nano Cu-Cu₂O, Pyricularia oryzae, Rice blast disease.

INTRODUCTION

Rice (Oryza sativa L.) is a major staple food crop in the world. In Vietnam, rice is the first staple food and importance for local consumption and export. Among the rice-growing countries in the world, according to the Ministry of Agriculture and Rural Development, Vietnam has an area of 7.57 million hectares with a production of 43.98 million tons in 2018 (Vi and Liu, 2019). However, yield production of rice can be reduced by 30 - 50% due to the rice blast disease during epidemic seasons (Koutroubas et al. 2009). Furthermore, the losses may be up to 90% depending upon the infected components of the plant (Kulmitra et al. 2017).

Rice blast is one of the severe diseases due to the Pyricularia oryzae fungus, which caused a heavily devastative production of rice worldwide (Anwar and Bhat, 2005; Chen et al. 2019). In recent years, resistance to commercially available fungicides by phytopathogenic fungi has been increasing and becoming a serious problem and also conflicting with the public concern for fungicide residues on human health and environment (Elamawi and El-Shafey, 2013). Nanotechnology would be one of the strategies that can raise the antimicrobial activity of materials by converting them to nanoparticles. The improved antimicrobial activity of nanoparticles is due to their unique properties, e.g., large surface area to volume ratio (Kanmani, 2018).

In late years, the researches on Cu-based nanoparticles had a lot of interest by scientists due to their electrical, heat conductivity, and high antifungal activity for crop pathogens (Kanhed et al. 2014). Cu-based nanomaterials are a reasonable cost compared to that of Ag or Au nanomaterials (Rusjan, 2012). In addition, Cu₂O nanoparticles were able

...to produce on a large scale feasibly. Copper oxide (CuO, Cu₂O) nanoparticles have been widely used as an anti-microorganism agent (Rusjan, 2012; Giannousi et al. 2014).

In agriculture, copper is one of the micronutrients for plant growth (Rusjan, 2012). Furthermore, nano Cu₂O exhibited a highly anti-microbial activity (Giannousi et al. 2014). Recently, some researches on antifungal activity of...
Cu$_2$O nanoparticles on the plant published, typically Huang et al. (2015) used Cu$_2$O nanoparticles to inhibit Alternaria solani/fungus causing blight of tomato and root rot of peppers.

The objectives of this study were to determine the inhibitory property of their efficacy on rice leaf blast disease in vitro and the greenhouse of sodium alginate-stabilized Cu$_2$O/alginate colloidal solution (nano Cu$_2$O/alginate).

**MATERIALS AND METHODS**

**Materials**

CuSO$_4$.5H$_2$O was a product of Guangdong Sci-Tech Co., China. Alginate was extracted from brown seaweed described in our previous paper (Du et al. 2019). The fungal strain of Pyricularia oryzae, isolated from blast disease in rice leaf, was kindly provided by Cuu Long Delta Rice Research Institute (CLRRI), Cantho City, Vietnam. The Potato Dextrose Agar (PDA) medium for fungal incubation was purchased from Himedia, India.

**Preparation of nano Cu$_2$O/alginate solution**

The nano Cu$_2$O/alginate solution with 5,000 mg/L and the particle size of 4.1 ± 1.4 nm was prepared according to the procedure of Du et al. (2019). Briefly, 5.5 ml of 25% NH$_4$OH solution was added into a mixture of 5 g CuSO$_4$.5H$_2$O and 5 ml water to create Cu(NH$_4$)$_2$O$_4$ complex to avoid gel formation. Then, the solution of Cu(NH$_4$)$_2$O$_4$ complex was added into the sodium alginate solution (12.5 g of sodium alginate dissolved in 230 ml distilled water) while stirring to prepare a homogeneous mixture. Finally, 10 ml of 8% hydrazine solution was slowly added into the alginate-CuO$_4$ solution while stirring to prepare a nano Cu$_2$O/alginate solution.

**The in vitro antifungal effect of nano Cu$_2$O/alginate against P. oryzae**

The antifungal activity of nano Cu$_2$O/alginate was tested against P. oryzae by culture medium toxicity method (Du et al. 2019; Xin et al. 2020), in 2019 - 2020 at the Institute of Tropical Biology, Ho Chi Minh City, Vietnam. Firstly, the 9 cm Petri plates were filled with 15 mL of sterile PDA medium amended with 15.0; 22.5; and 30.0 mg/L Cu. For the trials, each concentration was represented by four plates in replication. Secondly, mycelial plugs (5 mm) cut from the margin of a 5-day-old colony were transferred into the center of the PDA plates surface. The Petri dishes containing the PDA medium only were used as the control. After incubation for 7 days at 30 ± 2°C in the dark, colony diameter was measured and the IC$_{50}$ values (50% inhibition concentration) were calculated according to Xin et al. (2020) as follows:

\[
\text{Inhibition efficiency (\%)} = \frac{100 \times (\text{do} - \text{d})}{\text{do}}
\]

where do and d are the fungal growth diameter (mm) of the control and studied samples, respectively.

**The greenhouse test of nano Cu$_2$O/alginate against rice leaf blast disease**

The experiment of nano Cu$_2$O/alginate against rice blast disease under the greenhouse condition was carried out at the Cuu Long Delta Rice Research Institute, Cantho City, Vietnam, in 2019 - 2020 following the method of Elamawi and El-Shafey (2013). Six rice seedlings of OM 5451 cultivar were transplanted in plastic pots (25 cm diameter × 25 cm depth) and kept in the greenhouse. Two-third of the pot was filled with 5 kg fertilized soil with 3 g of NPK (16-16-8) at planting. After the transplant, NPK fertilizers were applied three times on the 10th, 20th day after the transplant (2 g of NPK (16-16-8)/pot) and the 5th day before the appearance of panicles (2 g of NPK (21-5-21)/pot). Soil moisture in the pots was regulated by daily irrigation. Four pots for each treatment were randomly arranged. For fungal inoculation, conidial P. oryzae suspensions (10$^6$ conidia/mL + 0.2% Tween 80) prepared before were sprayed on 21 days old rice seedlings. Nano Cu$_2$O/alginate (0, 20, 30, 40 mg/ ml based on Cu concentration) were applied at third days post-inoculation. Then, fifth, tenth, fifteenth and twentieth-day post-inoculation, the disease severity was scored using the (0-9) scale of IRRI (1996). The disease severity (DS) was assessed from each leaf with the following formula (Chen et al. 2019):

\[
\text{DS (\%)} = \left(\frac{\Sigma n_i \times v_i}{N \times V}\right) \times 100
\]

where: ni: number of leaves with i score; N: number of total leaves observed; V: the highest scale of disease severity; v: scales of disease severity (0 - 9)  

**Scale description**

0 = no lesions.
1 = small brown, specks of pinhead size.
2 = larger brown specks.
3 = small, roundish to slightly elongated, necrotic gray spots about 1 - 2 mm in diameter.
4 = typical blast lesions, elliptical, 1 - 2 cm long, usually confined to the area of the two main veins infecting < 2% of the total leaf area.
5 = typical blast lesions infecting < 10% of the leaf area.
6 = typical blast lesions infecting 10% - 25% of the leaf area.
7 = typical blast lesions infecting 26% - 50% of the leaf area.
8 = typical blast lesions infecting 51% - 75% of the leaf area.
9 = all leaves dead.

**Determination of Cu content in rice products**

The matured rice grains were harvested and air-dried to constant weight for calculation of grain yield. The samples of chaff, cortex (embryo), brown rice, and polished rice separated by machines were oven-dried at 60°C to constant weight and ground into a fine powder. The Cu content in the samples was analyzed by collision cell inductively coupled plasma mass spectrometer (Perkin-Elmer’s ICP-MS model NexION 2000) operated in helium gas mode (Punshon and Jackson, 2018).

**Statistical analysis**

Nan Cu$_2$O/alginate size was statistically calculated from the TEM image by Photoshop CS6 and Microsoft EXCEL.
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2010 software. All data were expressed as mean ± standard error (SE) and subjected to statistical analysis with the SAS 9.1 software (SAS Institute Inc., USA). One-way ANOVA was performed for each treatment with three replicates. Significant differences between means determined by Duncan’s multiple range test at $P < 0.05$.

RESULTS AND DISCUSSION
Characterization of nano Cu-Cu$_2$O/alginate

The morphology and particle size distribution of nano Cu-Cu$_2$O/alginate are shown in Fig 1. It had a spherical shape, an average size of 4.1 ± 1.4 nm, and a narrow distribution in the range of 1 - 7 nm. The content of Cu in the nano Cu-Cu$_2$O/alginate solution was determined by the ICP-MS method to be 5,087 mg/L. The results revealed that alginate seemed to be a suitable stabilizer for the synthesis of nano Cu-Cu$_2$O with a small size (~4 nm) and relatively high Cu concentration.

Antifungal effect of nano Cu-Cu$_2$O/alginate against P. oryzae

Results in Fig 2 indicated that P. oryzae fungus grew normal on the control plates by the diameter reached 46 mm, after 7 days of incubation. Meanwhile, the growth inhibition with nano Cu-Cu$_2$O/alginate was 39.1%, 69.6% and 100% for Cu 15.0, 22.5 and 30.0 mg/L, respectively.

The relationship between inhibition efficiency and nano Cu-Cu$_2$O/alginate concentration was fitted with the equation: $y = 0.0439x^2 + 2.0424x - 0.1980$ ($R^2 = 0.9992$). So, its IC$_{50}$ value for P. oryzae was calculated to be 17.8 mg/L. The IC$_{50}$ value of Cu$_2$O nanoparticles against S. cerevisiae yeast was reported to be 34.35 mg/L Cu$_2$O@oleylamine nanoparticles (Giannousi et al. 2014). It is higher than that of this work. The difference may be due to the nanomaterial used in our experiment contained nano Cu and Cu$_2$O with a small size. According to Hans et al. (2013), the antibacterial efficacy mainly depended on a copper ion release in which the highest ion release was for pure copper, followed by Cu$_2$O and CuO.

Effect of nano Cu-Cu$_2$O/alginate on blast disease and rice grain yield

The results of nano Cu-Cu$_2$O/alginate against P. oryzae at different concentrations in the greenhouse are presented in Table 1 and illustrated in Fig 3. The disease severity of the control rice was 47.57% at 20th-day post-inoculation, which was considerably higher than that of nano Cu-Cu$_2$O/alginate treatments ranged from 9.38% to 18.54%. The lowest disease severity was for the treatment of nano Cu-Cu$_2$O/alginate at 40 mg/L. As the results in Table 1, all treatments of nano Cu-Cu$_2$O/alginate showed a significant effect against

![Fig 1: TEM image (left) and particle size distribution (right) of nano Cu-Cu$_2$O/alginate.](image1)

![Fig 2: The inhibition efficiency (left) and photograph (right) of nano Cu-Cu$_2$O/alginate against P. oryzae growth after 7 days of incubation.](image2)

![Fig 3: Blast disease symptom on rice leaf: control (NT1) and treatments of nano Cu-Cu$_2$O/alginate (NT2: 20 mg/L, NT3: 30 mg/L and NT4: 40 mg/L Cu).](image3)
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Table 1: Effect of nano Cu-CuO/alginate on severity of blast disease on rice.

| Treatment | Cu conc. (mg/L) | Disease severity, % |
|-----------|----------------|---------------------|
|           | 5 dpi          | 10 dpi              | 15 dpi | 20 dpi |
| Control   | 0 (water)      | 6.26 ± 0.93          | 23.21 ± 3.07 | 30.04 ± 3.52 | 47.57 ± 4.20 |
| Cu-CuO/alginate | 20        | 4.12 ± 0.99           | 11.19 ± 2.00 | 15.72 ± 1.81 | 18.52 ± 2.40 |
|            | 30               | 4.03 ± 0.88           | 9.85 ± 1.21 | 10.05 ± 1.75 | 10.44 ± 1.76 |
|            | 40               | 3.62 ± 0.62           | 8.07 ± 1.29 | 9.38 ± 1.37  | 9.38 ± 1.22  |
| LSD_0.05   |                   | 2.72                  | 4.69     | 5.26      | 6.01       |
| CV, %      | 4.02 ± 0.18      | 4.46                  | 7.3      | 7.7       | 6.2        |

Note: Different letters in the same row indicate significant differences at P < 0.05; dpi: days post-inoculation.

Table 2: Influence of nano Cu-CuO/alginate on the grain yield of rice.

| Cu treatments, (mg/L) | Control (0) | 20 | 30 | 40 | LSD_0.05 | CV, % |
|-----------------------|-------------|----|----|----|-----------|-------|
| Grain yield (g/pot)   | 27.0 ± 1.8^a| 40.5 ± 3.4^b| 50.0 ± 2.6^c| 54.5 ± 4.2^c| 5.43     | 8.78  |
| Increase for control (%) | -           | 50.0      | 85.2    | 101.9   |           |

Note: Different letters in the same column indicate significant differences at P < 0.05.

Table 3: The distribution of copper in different parts of rice grains.

| Cu treatments, (mg/L) | Cu concentration (mg/kg DW) |
|-----------------------|-----------------------------|
|                       | Chaff | Cortex (embryo) | Polished rice |
| Control (0)           | 4.12 ± 0.31                  | 8.35 ± 0.66 | 4.22 ± 0.23 |
| 20                    | 4.21 ± 0.29                  | 8.52 ± 0.47 | 4.35 ± 0.17 |
| 30                    | 4.02 ± 0.18                  | 8.45 ± 0.52 | 4.31 ± 0.21 |
| 40                    | 4.36 ± 0.41                  | 8.73 ± 0.81 | 4.46 ± 0.38 |
| Commercial rice grain | 4.06 ± 0.26                  | 8.37 ± 0.63 | 4.63 ± 0.29 |

The Cu content in different parts of the rice grain in Table 3 indicated that the Cu content in the cortex (embryo) was higher (~2-fold) than that in chaff and polished rice. And the Cu content in rice grain was almost the same for nano Cu-CuO/alginate treated and untreated rice. The obtained results were almost similar to that of Xu et al. (2006), who studied to mix copper salt (CuCl) in the soil for rice culture. The commercial rice grain (OM 5451 variety) collected in Cantho City, Vietnam had Cu content of 4.06, 8.37 and 4.63 mg/kg in the chaff, cortex, and polished rice, respectively. Xu et al. (2006) also reported that the Cu content in conventional polished rice was 4.54 mg/kg. This study proclaimed that the nano Cu-CuO/alginate (20 - 40 mg/L) used for rice blast disease control had almost a negligible effect on the cumulative Cu in rice grain. Therefore, the nano Cu-CuO/alginate solution may be considered as a potential agrochemical (fungicide) for the protection of the rice plants. In addition, the nano Cu-CuO/alginate also provides copper micronutrients for plants because Cu is an essential element for plant growth and plays a significant role in many physiological processes such as photosynthesis, respiration, and carbohydrate distribution (Rusjan, 2012).

CONCLUSION

In this study, the nano Cu-CuO/alginate solution with a high Cu concentration of 5,000 mg/L and the particle size of 4.1 ± 1.4 nm was prepared successfully. The nano Cu-CuO/alginate product exhibited highly antifungal efficiency against leaf blast disease on rice. The results of this study revealed that nano Cu-CuO/alginate is a potential agro-chemical for use as a fungicide in sustainable agriculture production, especially for rice cultivation.

ACKNOWLEDGEMENT

This research is funded by the program of science and technology for the sustainable development of the Mekong Delta region under grant number TNB.DT/14-19/C38.

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