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Production of copper nanoparticle-immobilized chitin nanofibers and their role in plant disease control

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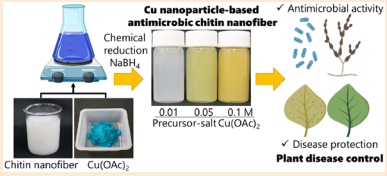
Chitin is used in agriculture to improve crop production; however, its use is limited due to difficulties in its handling. A chitin nanofiber (CNF) overcomes this issue and, due to its elicitor activity, has great potential for crop protection. To expand CNF utilization, a copper nanoparticles–based antimicrobic CNF (CuNPs/CNF) was prepared using a chemical reduction method. The formation of CuNPs was confirmed *via* scanning electron microscopy. Thermogravimetric analysis revealed that the amount of CuNPs on the CNF was dose-dependent on the precursor salt, copper acetate. CuNPs endowed the CNF with strong antimicrobial activity against *Alternaria brassicicola* and *Pectobacterium carotovorum*. Moreover, the CuNPs/CNF reduced pathogen infection in cabbage. The antimicrobial activity and disease prevention of the CuNPs/CNF was increased compared to the corresponding CNF or commercial agrochemical Bordeaux treatment. These results indicate that CuNPs conferred antimicrobial activity on the CNF and increased the efficacy of plant disease protection.



Keywords: chitin nanofiber, copper nanoparticles, chemical reduction, antimicrobial activity, plant protection.

Introduction

Chitin is a highly abundant natural carbohydrate polymer occurring mainly in the exoskeletons of arthropods, including crustaceans and insects, and in the cell walls of fungi.[1)] Chitin has a history of use in the agricultural field due to its potency, which can contribute to improved crop production. A well-known beneficial effect of chitin on plants is the elicitation of plant defense systems against pathogen infection.[2)] In plants, chitin in fungal cell walls (fungi being a potential pathogen) is recognized by pattern recognition receptors as a microbe- or pathogen-associated molecular pattern (MAMP/PAMP). This triggers the generation of reactive oxygen species (ROS), activation of mitogen-activated protein kinases, and expression of defense-related genes,[3,4)] the so-called PAMP-triggered immunity.[5)]

Chitin has great potential for agricultural applications but its uses are limited due to its insolubility in most solvents. We have previously prepared chitin nanofiber (CNF) from crab shells using a simple mechanical disintegration treatment.[6)] CNF has a highly uniform structure, with a thickness of approximately 10 nm, and shows high dispersing ability in water, which allow to shape into desired forms, such as film, hydrogel, and aerogel, depending on the application.[7)] We have reported the elicitor activity of CNF that increases ROS production, and the expression of defense genes in Arabidopsis, cabbage, rice, strawberry, and tomato.[8–11)] Moreover, CNF treatment was found to induce disease resistance, either locally or systemically, against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 in Arabidopsis and the fungal pathogens *Alternaria brassicicola* in Arabidopsis and cabbage, *Bipolaris oryzae* in rice, *Colletotrichum fructicola* in strawberry, and *Fusarium oxysporum* f. sp. *lycopersici* in tomato.[8–11)]

Chitin has several promising applications in various fields, such as biomedical, food, cosmetics, pharmaceuticals, and agriculture, owing to its biocompatible and biodegradable eco-

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friendly biopolymer. However, chitin does not have antimicrobial properties. To increase chitin utilization, surface modification has been used to endow CNF with antimicrobial properties. Surface-deacetylated chitin was prepared from chitin whose surface had been transformed into chitosan by deacetylation, which has antifungal activity against *A. alternata*, the causal agent of black spot disease on susceptible Japanese pear cultivars.[12] N-halamine CNF film was prepared by the reaction of CNF film with sodium hypochlorite solution, which showed microbicidal activity against the bacterial pathogens *Escherichia coli* and *Staphylococcus aureus*, involved in human health care, and the plant pathogenic fungi *A. alternata* and *Penicillium digitatum*.[13] Moreover, CNF was used as a scaffold for silver nanoparticle immobilization, with the silver nanoparticles endowing CNF with antifungal activity against many plant pathogenic fungi.[14]

Copper has been utilized as an antimicrobial agent for thousands of years. Some studies have demonstrated the preparation of a copper-based chitin, chitosan (deacetylated chitin), and cellulose (structurally related to chitin) nanomaterials for exploitation of antimicrobial applications.[15–18] Assorted forms of copper-based nanomaterial, such as mat, hydrogel, aerogel, and solution show antibacterial activity against *E. coli*, *Listeria monocytogenes, S. aureus*, and *P. aeruginosa*, and antifungal activity against *A. alternata*, *Aspergillus niger*, *F. oxysporum*, and *Trichoderma viride*, making it a valuable material for biosensor, clinical wound healing, food packaging, and wastewater treatment.[15–18] In agriculture, copper has long been used as a fungicide, known as Bordeaux, in orchards and vineyards.[19] Copper-based agrochemicals are widely used, not only in conventional agriculture but also in organic farming, because of their favorable properties, which include wide-spectrum antimicrobial activity and long-lasting protection. Furthermore, since copper is an essential micronutrient for normal plant growth, it is a constituent of fertilizer. However, long-term and excessive usage of copper-based agrochemicals lead to high accumulated concentration of copper in soil and water and raise concerns about environmental impact on soil fertility, aquatic life, and plant and animal health including human.[20] Accordingly, the reduction of copper use is necessarily desired.

Recently, there has been much interest in the use of copper nanomaterials for fertilizer and agrochemicals to improve crop yield.[21] Growth enhancements caused by copper nanoparticles (CuNPs) treatment have been shown in tomato and eggplant.[22] The antimicrobial activity of CuNPs has been demonstrated against the plant fungal pathogens *A. alternata*, *Botrytis cinerea*, *C. gloeosporioides*, *Curvularia lunata*, *F. oxysporum*, *F. solani*, *Monilia fructicola*, *Phoma destructiva*, and *Verticillium dahliae*[23,24] and the bacterial pathogens *Agrobacterium tumefaciens*, *Dickeya dadantii*, *Erwinia amylovora*, *Pectobacterium carotovorum*, *P. corrugata*, and *P. savastanoi* pv. *savastanoi*.[25] Interestingly, greater antimicrobial activity of CuNPs, when compared to their bulk counterparts, has been demonstrated.[24,25] In addition, copper material showed elicitor activity in plants. Copper ion induced resistance in Arabidopsis against *P. syringae* pv. *tomato* DC3000 at concentration lower than those required for antimicrobicity.[26] Treatment of CuNPs suppressed plant diseases, such as Fusarium wilt and late blight in tomatoes, and Verticillium wilt in eggplant.[22,27] CuNPs, which is expected to reduce copper usage, is attractive companion material to CNF for plant protection.

Here, we attempted to produce CuNPs-based antimicrobic CNF (CuNPs/CNF). We hypothesized that CuNPs/CNF exhibits high potency to plant disease protection, depending on the synergistic effect of induce resistance from CNF and antimicrobial activity from CuNPs. CuNPs were produced *via* the chemical reduction method and incorporated into CNF. The formation of CuNPs was confirmed by scanning electron microscopy. Thermogravimetric analysis determined that the amount of CuNPs on CNF was dose-dependent upon the precursor salt, copper acetate. CuNPs endowed CNF with strong antimicrobial activity against *A. brassicicola* and *P. carotovorum*. Moreover, CuNPs/CNF sufficiently reduced pathogen infection in cabbage. Antimicrobial activity and disease protection were greater with CuNPs/CNF treatment than with corresponding CNF or commercial agrochemical Bordeaux treatment. These results indicate that CuNPs conferred antimicrobial activity on CNF and increased the efficacy of plant disease protection, thus expanding the potential for using CNF and lowering copper usage in sustainable agriculture.

Materials and methods

1. Preparation of CuNPs/CNF composites

CNF homogeneous water dispersion without acetic acid was purchased from Marine Nano-fiber (Japan). It is manufactured according to previous papers, with modifications.[6] The raw material chitin is derived from crab shells. The preparation of the CuNPs/CNF composite was according to Phan *et al.*[17] A CNF dispersion containing 0.1 wt% chitin was mixed with copper acetate solution (FUJIFILM Wako Pure Chemical Co., Japan) at a concentration of 0.01, 0.05, or 0.1 M, in a ratio of 9 : 1 (v/v) and then gently agitated for 24 hr. Subsequently, a 0.1 M solution of sodium borohydride ($NaBH_4$; Sigma-Aldrich, USA) was added drop by drop with vigorous stirring for reducing Cu^{2+}. The color of the mixture was changed from blue to pale yellow indicating the formation of CuNPs, and addition of $NaBH_4$ was kept until the color change was complete. Samples of the CuNPs/CNF dispersion were centrifuged at 10,000 rpm for 5 min and the precipitate was resuspended in distilled water (DW). CuNPs/CNF was washed twice with DW by centrifugation at 10,000 rpm for 5 min, and finally three types of CuNPs-immobilized CNF (indicated as CuNPs/CNF 0.01, 0.05, and 0.1) were obtained.

2. Characterization of CuNPs/CNF composites

2.1. Scanning electron microscopy observation

For field-emission scanning electron microscopy (FE-SEM) evaluation, 10 mL of EtOH was added to 1.5 mL of the CuNPs/CNF dispersion and the dispersion was dried on Teflon petri dish in an oven at 60°C to obtain a cast film. The CuNPs/CNF

was further dried in a vacuum oven for 3 hr. Then, the samples were coated with a layer of platinum, approximately 2 nm thick, by an ion sputter coater (JFC-1600; JEOL Ltd., Japan), and the morphological structure of the CuNPs/CNF was observed using an FE-SEM (JSM-6701F; JEOL Ltd., Japan) operating at 2.0 kV.

2.2. X-ray diffraction

X-ray diffraction (XRD) profiles of the CuNPs/CNF were obtained in an X-ray generator (Ultima IV; Rigaku, Japan) operating at 40 kV and 40 mA. The diffraction profile was detected using an X-ray goniometer with a scanning range of 5° to 80°.

2.3. Thermogravimetric analysis

The copper content of CuNPs/CNF was determined by thermogravimetric analysis (TGA) (Thermo Plus EVO II; Rigaku, Japan). Samples were heated at 20°C/min from 19 to 1000°C under an air atmosphere.

3. Plant material and plant pathogen cultivation

Cabbage (*Brassica oleracea*) cv. Shoshu (Takii Seed, Japan) was used in this study. Seeds were grown in commercial garden soil for flowers and vegetables (Green Grow, Japan) under controlled environmental conditions with 14-hr light/8-hr dark cycles at 24°C. Plants were nurtured with HYPONeX (N–P–K=6–10–5) (Hyponex Japan, Japan) at a concentration of 0.1% (v/v) every week. After five weeks, young leaves were detached and used for experiments. The fungus *A. brassicicola* strain O-264, the causal agent of black leaf spot of *Brassica* plants, was maintained on a potato dextrose agar (Kyokuto Seiyaku, Japan) slant at 4°C. Spores of O-264 were prepared as described previously.[8] The bacterium *P. carotovorum* ssp. *carotovorum* strain EC1, which causes soft rot disease in various crops, was stored in 30% glycerol at −80°C. For inoculum preparation, the bacterial strain was precultured in Luria Bertani (LB) broth at 25°C for 24 hr. Cells were harvested by centrifugation 15,000 rpm for 3 min, washed with sterile DW, and resuspended in 0.9% NaCl for use in inoculation. The OD_{600} value was measured using a spectrophotometer (DeNovix DS-11; DeNovix Inc., USA).

4. Antimicrobial activity test

Both CNF and CuNPs/CNF were diluted to a final chitin concentration of 0.01%. For the antifungal activity test, spores of *A. brassicicola* strain O-264 were suspended in CNF, CuNPs/CNF, or Bordeaux (San Bordeaux; Sumitomo Chemical Garden, Japan) with final concentrations of copper at 2, 10, and 880 ppm. The concentration of Bordeaux at 2 and 10 ppm was corresponding with a copper level in CuNPs/CNF 0.01 and 0.1, respectively, and the concentration of Bordeaux at 880 ppm was the usual concentration following the instruction manual. The spore suspension was placed on a microscope slide and kept in a moist chamber. After 24 hr, spore germination was observed under a light microscope. (BX-53; Olympus, Japan). This test was conducted more than six times. For the antibacterial activity test, 100 μL of precultured *P. carotovorum* strain EC1 at OD_{600} of 0.1 was added to 5 mL of CNF, CuNPs/CNF, or Bordeaux solutions and incubated in a reciprocal shaker at 22°C. After 24 hr, 100 μL sample solutions were transferred into 5 mL of LB media and cultured under the same condition for 24 hr. The OD_{600} value was measured before (0 hr) and after (24 hr) cultivation in LB media. Bacterial growth was determined by subtracting the OD_{600} value at 0 hr from 24 hr. This test was conducted six times.

5. Inoculation test

CNF and CuNPs/CNF were used at a chitin concentration of 0.01%. For the inoculation test, young cabbage leaves were detached and washed with tap water to remove the wax layer. Leaves were pre-treated with CNF, CuNPs/CNF, or Bordeaux solution by spray. After 24 hr, the spore suspension of *A. brassicicola* strain O-264 (1.5×10^4 spores/mL) was inoculated by spray. Inoculated leaves were kept in a moist dark chamber at 25°C and the number of lesions was counted after two days of inoculation. For bacteria inoculation, 10 μL of inoculum at OD_{600} of 0.01, prepared *via* the above method, was dropped on the wound leaf, which was poked with a micropipette tip. Inoculated leaves were kept under high humidity conditions in a moist chamber with a 14 hr photoperiod at 25°C and the lesion diameter was measured after 24 hr of inoculation.

6. Statistical analysis

Antifungal and antibacterial activity tests were repeated at least

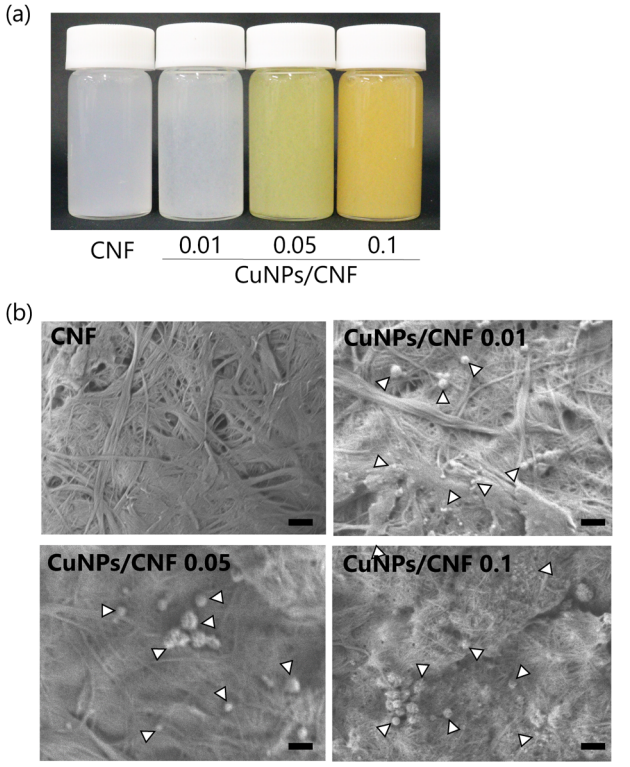

Fig. 1. CuNPs/CNF composites. (a) Color of CuNPs/CNF composites. CNF was mixed with different concentrations of copper acetate at 0.01, 0.05, and 0.1 M. (b) Field-emission scanning electron microscopy micrographs of CNF and CuNPs/CNF composites. Arrowheads indicate copper particles. Bars indicate 500 nm.

six times with two technical replicates for each treatment. The inoculation test was carried out three times with four biological replicates. The data were compared using Tukey's test with R (version 3.6.1).

Results and discussion

1. Preparation and characterization of CuNPs/CNF

In this study, different concentrations of copper acetate, 0.01, 0.05, and 0.1 M, were mixed with 0.1% CNF, and three types of CuNPs-immobilized CNF were obtained (Fig. 1). The appearances of CuNPs/CNF became pale yellow after chemical reduction, darkening as the concentration of precursor salt, copper acetate, increased (Fig. 1a). The observed colors (gold, pale yellow, ocher, or black-brown) are characteristic of CuNPs and the color variation is dependent on particle size.[28] Figure 1b shows FE-SEM images of the CNF and CuNPs/CNF. CNF consists of submicron and nanosized chitin fibers and CuNPs of around 50 to 500 nm were detected on all CuNPs/CNF surface. There were no distinct differences in the Cu particle size distribution among 0.01, 0.05, and 0.1 of CuNPs/CNF. The size of the nanoparticles depends on several factors, such as temperature, reaction time, precursor salt, reductant type, and concentration.[28–31]

Figure 2 shows the XRD profiles of CuNPs/CNF and CNF. The XRD patterns of CNF and CuNPs/CNF show three diffraction peaks at around 2θ values of 9.2°, 19.2°, and 23.2°, which can be assigned to the (020), (110), and (130) planes, respectively. These typically correspond to the crystal structure of α-chitin.[32] Besides chitin peaks, several other diffraction peaks appeared in CuNPs/CNF 0.1 at 36.5°, 42.4°, and 61.5°, corresponding to the (111), (200), and (220) planes of crystalline Cu_2O, respectively.[33] This suggests that copper acetate was converted to copper(I) oxide in the presence of a reducing agent. XRD patterns assigned to CuNPs could not be observed in CuNPs/CNF 0.01 and 0.05. This is probably because the number of CuNP crystals produced was low when compared with chitin, so their diffraction patterns were not observed.

The copper content of CuNPs/CNF was determined by using thermogravimetric weight-loss curves (Fig. 3). The TGA of the

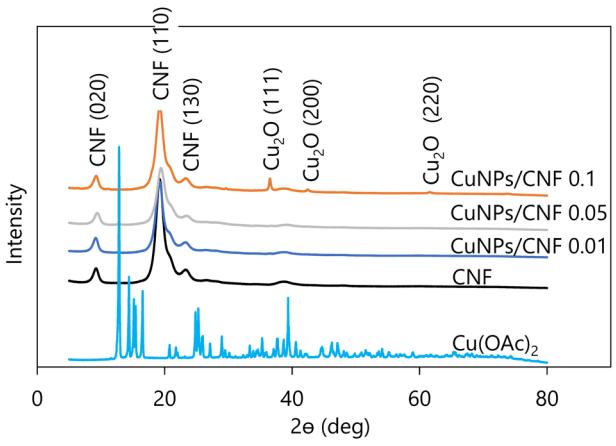

Fig. 2. XRD profiles of CuNPs/CNF composites.

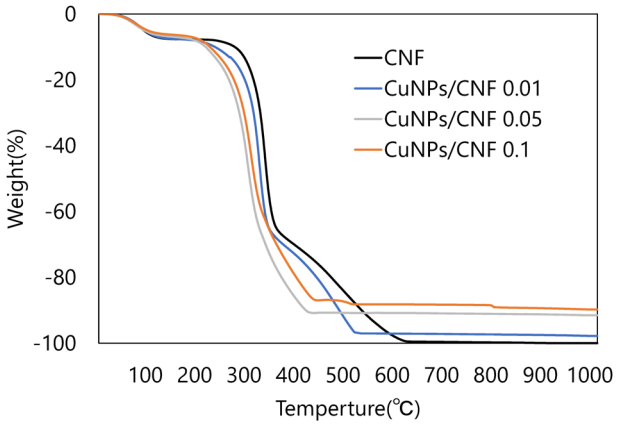

Fig. 3. Thermogravimetric analysis curve of CuNPs/CNF composites.

CuNPs/CNF was conducted from ambient temperature up to 1000°C. Generally, CNF show two main weight-loss regions. During the first step, at a temperature of 80–120°C, all samples exhibited a weight loss that can be attributed to water loss due to evaporation. During the second stage, a more significant weight loss was observed at 250–400°C, which could be attributed to the degradation of the saccharide structure of the molecules, dehydration of saccharide rings, or the decomposition of acetylated and deacetylated units of chitin.[32,34] At higher temperatures of more than 400°C, other materials were gradually degraded until copper was the sole remaining residue at 1000°C. The total weight loss in those ranges was 97.8%, 91.5%, and 89.8% for CuNPs/CNF 0.01, 0.05, and 0.1 respectively, whereas it was 100% for CNF. The amount of copper was estimated to be 23, 93, and 114 ppm for CuNPs/CNF 0.01, 0.05, and 0.1, respectively, which is reflected by the dosage of copper acetate. These results indicated that CuNPs were produced *via* the chemical reduction method and incorporated into CNF.

2. Antimicrobial activity of CuNPs/CNF

Antifungal activity of CuNPs/CNF or Bordeaux solutions was determined by inhibition of spore germination. CNF did not have antifungal activity and the rate of spore germination was comparable with control DW. In contrast, all CuNPs/CNF varieties had a strong antifungal effect on spore germination (Fig. 4a). The concentration of copper at 880 ppm in Bordeaux was the usual concentration, per the instruction manual. Spore germination was strongly inhibited in Bordeaux 880 and 10 ppm and moderately in 2 ppm. The concentration of copper in Bordeaux 10 and 2 ppm was the same level as in CuNPs/CNF 0.1 and 0.01, respectively. Interestingly, spore germination in CuNPs/CNF 0.01 and 0.1 was inhibited more than in the corresponding Bordeaux 2 and 10 ppm. Greater antimicrobial activity of CuNPs, when compared to their bulk counterparts, has been demonstrated.[24,25] Although the possible action modes of both bulk and nanosized copper materials are explained by the same method, with the disintegration of the cell wall or membrane and oxidative stress,[35,36] size, shape, and surface charge of cop-

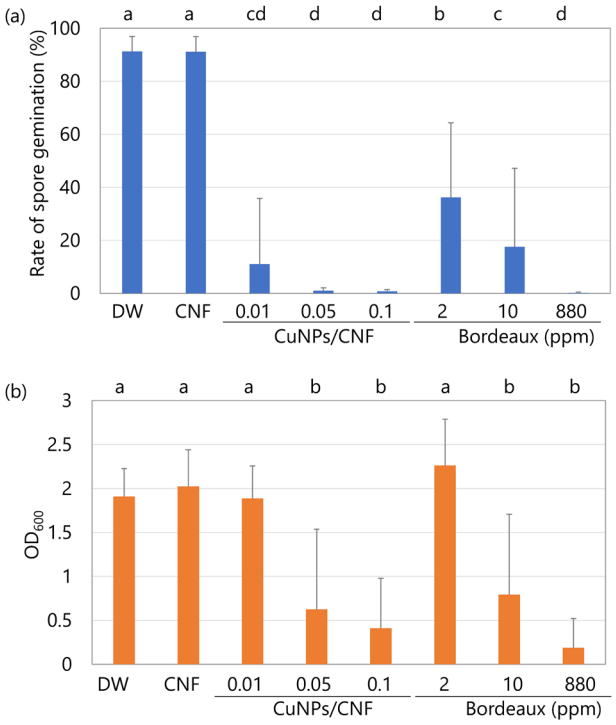

Fig. 4. Antimicrobial activity of CuNPs/CNF composites. (a) The rate of spore germination of *A. brassicicola*. Spores of *A. brassicicola* were suspended in CNF, CuNPs-immobilized CNF (CuNPs/CNF; 0.01, 0.05, 0.1), Bordeaux (2, 10, 880 ppm) solutions or control DW. After 24 hr, spore germination was observed. (b) The growth of *P. carotovorum*. *P. carotovorum* was pre-incubated in CNF, CuNPs/CNF, or Bordeaux solutions and subsequently post-cultivated in LB medium. After 24 hr, the optical density was measured at 600 nm. Data represent the means and standard deviation. Bars with letters indicate statistically significant differences according to Tukey's test ($p<0.05$).

per are considered to have a great influence on the antimicrobial activity.[37] Besides antifungal activity, CuNPs/CNF showed antibacterial activity against *P. carotovorum* strain EC1. The bacterial suspension was pre-incubated with CuNPs/CNF or Bordeaux and then a portion of the culture solution was post-cultivated in LB medium. Bacterial growth was lower for CuNPs/CNF 0.05 and 0.1 and Bordeaux 10 and 880 ppm than for control DW, indicating that bacteria were killed during incubation in these solutions. Unlike the effect on spore germination of filamentous fungi, CuNPs/CNF 0.01 and Bordeaux 2 ppm had no antibacterial activity. Differences in efficacy between antifungal and antibacterial activity could be attributed to the variation in chemical composition between the fungal cell wall, comprising predominantly chitin and glucan, and the bacterial cell wall, composed of peptidoglycan.[38] These results indicate that CuNPs conferred strong antimicrobial activity to CNF.

3. *Protective effects of CuNPs/CNF against plant disease*

All concentrations of CuNPs/CNF significantly reduced the number of lesions by *A. brassicicola* infection on the cabbage leaves compared with control DW (Fig. 5a). Similarly, pretreatment with Bordeaux 10 and 880 ppm decreased disease symptoms (Fig. 5a). In addition, CuNPs/CNF treatment prevented infection by the bacterial pathogen *P. carotovorum*. Lesion diameter was significantly reduced in CuNPs/CNF 0.05 and 0.1 treated leaves (Fig. 5b). Disease suppression by CuNPs/CNF 0.1 was higher than Bordeaux 10 ppm, albeit with the same copper concentration (Fig. 5b). Overall, the protective effect of CuNPs/CNF was higher than the corresponding Bordeaux (Fig. 5a and 5b). CuNPs were reported to have a greater effect on disease suppression than reference agrochemicals in tomato plant against *P. infestans*.[27] Moreover, CuNPs/CNF worked comparably to or better than CNF alone (Fig. 5a and 5b). The mechanisms of disease suppression by CuNPs/CNF were presumed to have two contributing factors: antimicrobial activity of copper and elicitor activity of CNF and copper. Although it has been reported that CuNPs-based chitin and chitosan nanomaterial show antimicrobial activity and protective effect against plant disease, elicitor activity of chitosan and nanoparticle form of chitin remain poorly defined.[15,39–41] Besides CNF, copper has also been reported to elicit defense responses in Arabidopsis and potato.[26,42] The results herein indicate that CuNPs/CNF show a protective effect

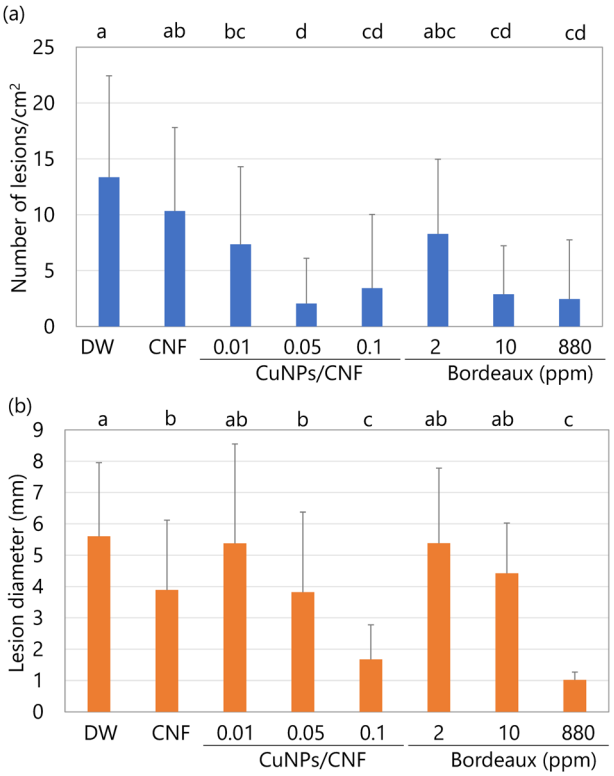

Fig. 5. Protective effect of CuNPs/CNF against cabbage disease. Detached cabbage leaves were sprayed with CNF, CuNPs-immobilized CNF (CuNPs/CNF; 0.01, 0.05, 0.1), Bordeaux (2, 10, 880 ppm) solutions, or control DW 24 hr before pathogen inoculation. (a) Pre-treated cabbage leaves were inoculated with the fungal pathogen *A. brassicicola*. The number of lesions was investigated 48 hr after inoculation. (b) Pre-treated cabbage leaves were inoculated with the bacterial pathogen *P. carotovorum*. Lesion diameter was measured after 24 hr inoculation. Data represent the means and standard deviation. Bars with letters indicate statistically significant differences according to Tukey's test ($p<0.05$).

against plant pathogen infection and that this is achieved by antimicrobial activity and elicitor activity of CuNPs/CNF.

Conclusion

In this study, CuNPs-based antimicrobic CNF was successfully prepared by the chemical reduction method. FT-IR and TGA analyses showed the presence of CuNPs on the CNF. The amount of CuNPs on CNF increased in a dose-dependent manner as the amount of precursor salt, copper acetate, increased. CuNPs endowed CNF with both antifungal and antibacterial activity. Moreover, CuNPs/CNF treatment was able to sufficiently reduce both fungal and bacterial pathogen infection in cabbage. Antimicrobial activity and disease prevention were favorably addressed by CuNPs/CNF when compared with corresponding Bordeaux and CNF treatments. These results indicate that CuNPs on CNF increased the efficiency of the antimicrobial effect and plant disease protection, which could help reduce copper usage in the future. CNF, whose features resolve the difficulties involved with handling chitins, is expected to be useful in agriculture, yet its applications could be greatly broadened by enhancement with CuNPs. Taken together, we conclude that CuNPs/CNF could be a promising environmentally friendly material to use in the control of plant disease.

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