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Article

The effect of molecular weights of chitosan on the synthesis and antifungal effect of copper chitosan

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SUMMARY

Pathogenic fungi such as *Alternaria alternata* (*A. alternata*) can decimate crop yields and severely limit food supplies when left untreated. Unfortunately, the chemical fungicides currently used to solve this issue create problems of their own in the form of high prices and environmental damage. Copper chitosan (CuCts) is a promising alternative fungicide for developing agricultural areas due to being inexpensive and nontoxic. Chitosan polymers' properties depend on their molecular weight, classified as high molecular weight chitosan (HMWc) and low molecular weight chitosan (LMWc). LMWc shows lower viscosity, greater solubility, and greater antimicrobial activity. However, LMWc is produced from HMWc through an expensive process. Therefore, we wanted to investigate the effect of HMWc and LMWc on producing CuCts as an antifungal agent. We hypothesized that LMWc CuCts would exhibit greater fungal inhibition due to the beneficial properties of LMWc. Using a unique bulk synthesis method, we produced HMWc CuCts and LMWc CuCts and then tested them on *A. alternata* with the poisoned food technique by comparing fungal growth and fungal inhibition. Indeed, LMWc CuCts showed significantly greater fungal inhibition. Our work serves as an important stage of the development of CuCts as a viable and available alternative to chemical fungicides in the future.

INTRODUCTION

Alternaria alternata (*A. alternata*) is one of many fungi that cause Alternaria leaf spot and blight, a plant pathology in various species that causes a loss of foliage and death (1). Alternaria leaf spot is most commonly seen in certain nightshades such as potatoes and tomatoes, and Brassica vegetables such as cauliflower and broccoli (1). *A. alternata* is a prevalent fungus that can ruin crops and stay in the soil for years (2). In South Africa, *A. alternata* leaf blights have caused an 80% reduction in the yield of Helianthus annuus sunflowers due to head rot and leaf damage, making it especially problematic (2). Available fungicides come in three forms: physical, biological, and chemical (3). Currently, most commercial fungicides used for dealing with Alternaria fungi are chemical (4). While they are effective, modern chemical fungicides are prohibitively expensive and environmentally damaging (4). These concerns render the majority of chemical fungicides unsustainable for long-term use in developing agricultural areas.

Due to these pressing issues, previous researchers have proposed that copper chitosan (CuCts) can be used as a chemical fungicide, and it may be an answer to the problems that current chemical fungicides present. CuCts is a complex of copper(II) ions and chitosan polymers. CuCts is an inexpensive alternative to chemical fungicides as chitosan is made from chitin, the second most commonly available biopolymer after cellulose (5). Chitin polymers are often derived from arthropod shells and then deacetylated and hydrolyzed to create chitosan (5). CuCts is known to have antibacterial and antifungal effects, and certain complexes with a 1:1 molar ratio of copper and chitosan almost entirely inhibit bacterial growth in vitro (6). In this complex, chitosan polymers wrap around the copper ion keeping it from reacting with pathogenic fungal cells until the chitosan is broken down or absorbed (7). Researchers have shown that CuCts is biocompatible and nontoxic, and that CuCts can remove certain pesticides and pollutants from water sources due to its adsorptive properties. While CuCts may still affect the environment, it is nevertheless a greener alternative to traditional chemical fungicides (8). However, a large drawback of CuCts is the production process. The standard synthesis process has historically resulted in less than or equal to 1 gram of yield from each synthesis due to issues such as agglomeration during synthesis and the use of specialized equipment for low volumes of materials (9). Agglomeration is a process when synthesized particles combine into a large mass instead of remaining separate. Even in these small quantities, standard synthesis processes can exceed 12 hours (10). Therefore, we developed a novel bulk synthesis to produce over 10 grams of CuCts at once in a shorter time period.

The properties of chitosan are reliant on the polymer length. Therefore, chitosan is classified by its molecular weight. High molecular weight chitosan (HMWc) is greater than 100 kDa and often above 300 kDa, and low molecular weight chitosan (LMWc) is generally less than 100 kDa but can range up to 200 kDa due to variability in production processes (5). Molecular weight is positively correlated with viscosity and negatively correlated with solubility, making the potential uses of HMWc less diverse (5). Chemically, researchers have shown that as molecular weight decreases, antimicrobial activity increases (11). However, LMWc has a variety of issues related to its production. Chitin polymers are naturally long and convert to HMWc when deacetylated. The HMWc must then be hydrolyzed into LMWc through chemical or enzymatic processes, which is environmentally problematic or expensive, respectively (5, 12). Even when it is converted to LMWc, the process is inconsistent and results in a variety of polymer lengths that all fall below approximately 100 kDa (5). While researchers are developing new methods of HMWc hydrolysis, no concrete process that is inexpensive, environmentally friendly, and time efficient currently exists.

Investigating the effects of utilizing HMWc and LMWc to

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synthesize CuCts is essential to optimizing the efficiency of CuCts as a widely used fungicide in growing agricultural regions. To the best of our knowledge, no studies so far have determined the difference in antifungal properties of CuCts made with chitosan of different molecular weights. By producing CuCts with a novel bulk synthesis method and testing it on *A. alternata* fungus with the poisoned food technique, we can easily compare the antifungal effects and then determine an ideal molecular weight. The poisoned food technique is an antimicrobial assay where one mixes treatments into a growth medium, "poisoning" the medium, and then measures growth of fungal disks on normal and poisoned mediums (3). One can then compare normal conditions to different treated conditions to determine the effectiveness of treatments. We hypothesized that LMWc CuCts would have greater fungal inhibition than HMWc CuCts when applied to *A. alternata* fungus due to the beneficial physical and antimicrobial properties of LMWc. After testing against a negative control, we observed that both HMWc CuCts and LMWc CuCts were effective antifungal agents, but LMWc CuCts had a significantly greater fungal inhibition. Our results support the idea that LMWc should be preferred over HMWc for the future industrial synthesis of CuCts, though further research is needed to find an ideal molecular weight of chitosan. Additionally, our observations supported the efficacy of the bulk synthesis process as yield increased while the CuCts retained antimicrobial properties.

RESULTS

The synthesis of CuCts

We synthesized CuCts particles through a unique bulk synthesis method based on the work of Usman et al. and Andal & Buvaneswari (**Figure 1**) (13, 14). A unique CuCts bulk synthesis method was used to increase the yield and speed up the process. The purpose of this method was to develop a scalable process that could move up to a factory level producing kilograms of CuCts at once. One notable observation during the bulk synthesis was the formation of hollow beads as copper(II) sulfate was added to dissolved chitosan (**Figure 2**). When copper(II) sulfate drops touch the chitosan solution, they aggregate. By dripping copper(II) sulfate into the solution at high stirring from high above, individual drops aggregated instead of mass aggregating into an unworkable single mass. CuCts particles were analyzed and characterized after synthesis. CuCts particles were massed, and the percent yield was calculated (**Table 1**). We observed a 9.79-fold for HMWc CuCts and 10.51-fold improvement for LMWc CuCts over literature values of 1 gram yield (9). Dissolving HMWc or LMWc in dilute acetic acid took approximately 5 hours. Completing synthesis after this step took 1 hour for HMWc CuCts (6 hours total) and 30 minutes for LMWc CuCts (5.5 hours total). This marks a 2-fold for HMWc CuCts and a 2.18-fold for LMWc CuCts improvement over literature values of 12 hours (10). CuCts synthesis was confirmed as the mixtures shifted from blue to yellow to red-brown as particles precipitated out and the viscosity rapidly reduced, marking copper(II) ions reducing to stable particles (13).

Figure 1: CuCts particles in suspension. Photo was taken immediately after CuCts synthesis. Red brown CuCts particles are suspended in water with dissolved byproducts.

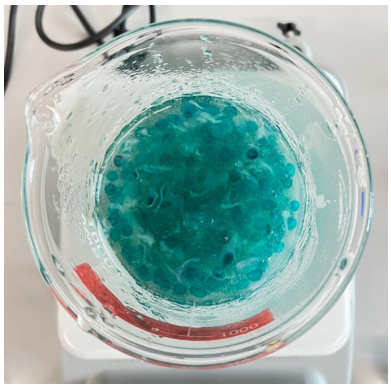

Figure 2: Drops of copper(II) sulfate in the chitosan mixture. This photo was taken midway through the synthesis process after the copper(II) sulfate and sodium hydroxide were added. The blue drops and blue coloration are from adding the copper(II) sulfate and the white wispy strands are from adding the sodium hydroxide.

The antifungal effects of CuCts

To determine the antifungal effect of CuCts made with chitosan of different molecular weights, we employed the poisoned food technique. The poisoned food technique is an assay where one mixes an antimicrobial agent into agar to poison it and then measures if the antimicrobial agent decreases the growth of a fungus cultured on it (3). This method was selected as the CuCts particles easily dispersed in molten nutrient agar and the fungal mycelium was able to interact with the particles as it grew through the medium. This was carried out by first synthesizing approximately 10 grams of HMWc CuCts and LMWc CuCts particles (**Table 1**). The sourced HMWc was 310–375 kDa and the LMWc was 50–190 kDa according to the supplier. We prepared three sets of nutrient agar solution as a pure control, HMWc

	HMWc CuCts Measurements	LMWc CuCts Measurements
Yield (g)	9.79	10.51
Percent Yield (%)	82.16	88.21

Table 1: HMWc CuCts and LMWc CuCts yield data. HMWc CuCts and LMWc CuCts were massed after synthesis, and the percent yield of each was calculated.

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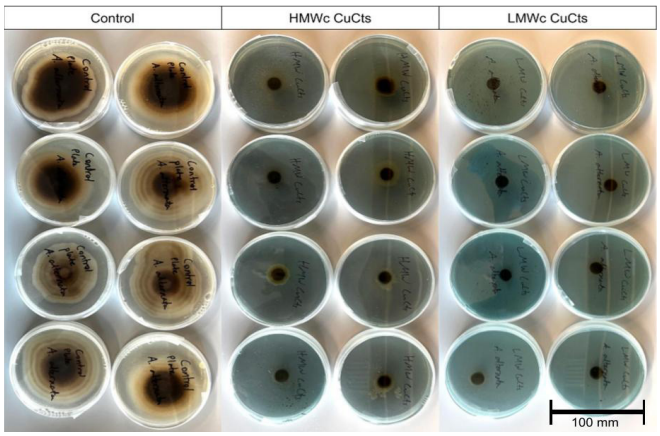

Figure 3: HMWc and LMWc CuCts inhibit fungal growth in a poisoned food model. Photos were taken after 8 days of incubation at room temperature following the placement of the mycelial disk. Control plates contained pure nutrient agar, and HMWc CuCts and LMWc CuCts plates contained nutrient agar mixed with the respective types of CuCts.

CuCts group, and LMWc CuCts group. Each CuCts group had approximately 45 g/L of CuCts particles in molten agar. Eight test plates were prepared for each group. *A. alternata* mycelial disks were cut and placed onto the center of each test plate face-down. The fungus was then allowed to grow for 8 days at room temperature (**Figure 3**). Disregarding the size of the initial mycelial disk, the growth of each fungal colony was measured with calipers as "fungal growth" (**Table 2**).

We observed fungal growth in all three test groups, which confirmed the viability of the *A. alternata*, the transferred mycelial disks, and the capability of nutrient agar to support fungal growth (**Figure 3**). Two control plates were potentially contaminated as we noticed small yellow growths that were most likely mold around the perimeter of the plate. Nevertheless, we still considered the contaminated plates in statistical analysis as the *A. alternata* colonies did not interact with the contamination growth in these plates. No contamination was noted in the CuCts plates.

Fungal growth in both HMWc CuCts and LMWc CuCts plates was significantly reduced compared to the control group (**Figure 4**, Control vs. HMWc CuCts $p < 0.05$, Control vs. LMWc CuCts $p < 0.001$). After demonstrating that both forms of CuCts had antifungal capabilities, we calculated the percent fungal inhibition of each plate (**Table 3**). By comparing the fungal inhibition of each type of CuCts, we observed that LMWc CuCts had significantly more inhibition than HMWc CuCts at 97.49% inhibition compared to 92.05% inhibition (**Figure 5**, $p < 0.05$). Additionally, three LMWc CuCts plates were the only plates from all three groups to exhibit no growth, indicating 100% inhibition (**Table 3**).

Plate	Control Fungal Growth (cm)	HMWc CuCts Fungal Growth (cm)	LMWc CuCts Fungal Growth (cm)
1	5.49	1.35	0.07
2	4.68	0.18	0.52
3	5.19	0.48	0.00
4	5.59	0.45	0.20
5	4.65	0.91	0.00
6	5.50	0.12	0.30
7	5.36	0.45	0.00
8	5.68	0.46	0.30
Average	5.27	0.55	0.17

Table 2: Fungal growth data and averages. *A. alternata* was grown under either control conditions, with HMWc CuCts or LMWc CuCts, for 8 days. The most even diameter around the mycelial disk was measured, and the initial 1.65 cm disk was subtracted from measurements to show how much each fungal colony grew.

Figure 4: The effect of various treatments on the growth of *A. alternata*. Bar graph showing mean ± 2 Standard Error fungal growth (n = 8). Fungal growth is the colony diameter minus the 1.65 cm mycelial disk. Markers on each bar correspond to individual data points in ascending order. *A. alternata* was grown under control conditions, with HMWc CuCts, or with LMWc CuCts for 8 days. Kruskal-Wallis one-way ANOVA, $p < 0.001$. Dunn's post hoc comparisons, Control vs. HMWc CuCts $p < 0.05$, Control vs. LMWc CuCts $p < 0.001$, HMWc CuCts vs. LMWc CuCts $p > 0.05$.

DISCUSSION

The objective of our study was to determine what molecular weight of chitosan should be preferred for the mass production and use of CuCts. We hypothesized that LMWc CuCts would have greater fungal inhibition than HMWc CuCts would have on *A. alternata* due to the positive properties of LMWc. The results and observations supported this hypothesis, providing evidence that LMWc should be used over HMWc because LMWc CuCts is a stronger antifungal agent. Indeed, LMWc CuCts showed significantly greater fungal inhibition at an

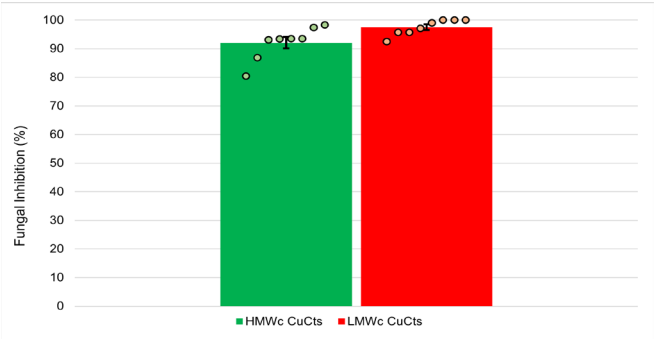

Figure 5: The effect CuCts made with chitosan of different molecular weights on percent fungal inhibition. Bar graph showing mean ± 2 Standard Error fungal inhibition (n = 8). Markers on each bar correspond to individual data points in ascending order. Percent fungal inhibition was calculated assuming the control fungal inhibition was 0% and control inhibition was therefore not shown. Mann-Whitney U test one-tailed, $p < 0.05$.

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Plate	HMWc CuCts Fungal Inhibition	LMWc CuCts Fungal Inhibition
1	80.48%	98.99%
2	97.40%	92.48%
3	93.06%	100.00%
4	93.49%	97.11%
5	86.84%	100.00%
6	98.27%	95.66%
7	93.49%	100.00%
8	93.35%	95.66%
Average	92.05%	97.49%

Table 3: Percent fungal inhibition and averages of HMWc CuCts and LMWc CuCts. Considering the average control fungal growth as 0% inhibition, percent fungal inhibition values were calculated for each CuCts plate and averaged.

average of 97.49% compared to an average HMWc CuCts inhibition of 92.05%. While there is evidence that using chitosan of lower molecular weights is preferable to higher molecular weights, specifically, the 50-190 kDa chitosan used here may not be the ideal molecular weight due to antifungal properties, synthesis issues, and crucially, price. Withstanding the molecular weights of chitosan, these results support the viability of the novel bulk synthesis process as both forms of CuCts effectively reduced fungal growth.

There were a few key limitations in this study. Standard CuCts synthesis processes include hydrazine as a reductant instead of ascorbic acid as ascorbic acid can produce byproducts. We used ascorbic acid as hydrazine is highly flammable and carcinogenic and the hazards would be amplified in a bulk synthesis method. The issue of byproducts was mitigated through repeatedly washing the CuCts particles, which should have removed soluble byproducts. Another issue was the loss of reactants as they were physically stuck to the various tools and containers. The chitosan solutions were highly viscous, and parts of them were lost as it was transferred from beakers or mixed with stir rods or an immersion blender. The loss of reactants here may have contributed to the reported synthesis issues, and we could have avoided this issue with greater care. Being an in vitro study inherently limits the scope of this study. While previous research has shown that CuCts is effectively antifungal in infected tomato plants, the effects of different molecular weights of chitosan on CuCts in living plants is unknown (14). Additionally, it is unknown whether CuCts negatively interacts with nutrient agar. If the CuCts deteriorated the nutrient agar instead of directly affecting the fungus, the drawn conclusions would be skewed as the control would no longer be appropriate. An important limitation of this study was not knowing the exact amount of CuCts in each plate, only approximating it to 0.9 g per plate, or 45 g/L. If different plates had different amounts of CuCts, that would influence the fungal inhibition and impact the results. This was mitigated by continuously mixing the CuCts nutrient agar mixture to ensure that the CuCts was evenly distributed.

Future research will be required to solve the objective of this study and find an ideal molecular weight of chitosan to use in CuCts production. Besides accounting for the issues above, two angles should be investigated: environmental effects and a wider scale of molecular weights. Previous research has indicated that LMWc is linked to various environmental issues (5, 12). For example, a study examining the effects of LMWc on zebrafish as a model freshwater organism found that LMWc damaged cell membranes and was toxic to eggs, larvae, and adults (12). Therefore, finding the environmental effects of CuCts made with chitosan of different molecular weights will allow for using CuCts in the most sustainable way. A wide scale of chitosan molecular weights in CuCts should be investigated. Using chitosan polymers in the < 50 kDa range, 100 kDa, 150 kDa, 200 kDa, 225 kDa, 250 kDa, 275 kDa, and 300 kDa for example would reveal at what point chitosan of lower molecular weight has diminishing returns. As producing LMWc is an expensive process, the highest molecular weight of chitosan that is still a highly effective antifungal agent with as few as possible synthesis issues would be an ideal molecular weight for CuCts synthesis. If CuCts is to be used in developing agricultural regions, minimizing price is key. Unfortunately, LMWc synthesis is currently a variable process that produces multiple weights at once, meaning that LMWc synthesis issues would have to be solved first so that testing the example set of molecular weights can be feasible (5).

Changing the molecular weight of the chitosan used in CuCts can also be applied to prior research and the real-world use of CuCts. It has been shown that CuCts is an effective fungicide and preventative agent when applied to tomato plants in vivo using a foliar spray (15). A foliar spray is a spray that is applied directly to plant leaves and is a common method of applying fungicides. Therefore, testing HMWc CuCts and LMWc CuCts on live plants with a real-world approach such as a foliar spray is the next step to support our conclusions. When CuCts is applied to plants, it also has biostimulant properties shown by increased yield and stimulated existing plant defense responses (16). Researchers can also evaluate these responses on live plants with HMWc CuCts and LMWc CuCts to study whether the responses are different. LMWc CuCts seems to be a stronger antifungal than HMWc CuCts, but possible differences in biostimulant effects could offset that when tested on live plants. While a foliar spray is widely applicable to most crops, it is not usable for large crops and trees (15). CuCts would have to be tested with new methods including soil additives, water additives, and irrigation for certain plants.

To conclude, using low molecular weight chitosan in the bulk synthesis and antifungal use of CuCts resulted in high fungal inhibition. This serves as an important steppingstone for the future development of CuCts as a mass-produced product for real-world use. Reducing the damage that fungi such as *A. alternata* cause can increase crop yields in vulnerable and growing areas, and CuCts is a highly promising solution that will still require further refining.

MATERIALS AND METHODS

CuCts synthesis

The work of Usman et al. was used as a basis for the general synthesis process with the work of Andal & Buvaneswari being used in the reduction process (13, 14). Both HMWc CuCts and LMWc CuCts followed the same overall process.

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The LMWc CuCts bulk synthesis began by dissolving 6.7 mL of 6M acetic acid in 300 mL of distilled water. We gradually dissolved 11.5 g of 50–190 kDa LMWc (Millipore Sigma, Cat# 448869) into solution under high stirring and heated at 95 °C until completely dissolved. Then, 0.5 g of solid ascorbic acid was dissolved in 30 mL of distilled water and added to the mixture. Four g of copper(II) sulfate pentahydrate was dissolved in 50 mL of distilled water and dripped into the chitosan solution to prevent mass aggregation. The copper(II) sulfate was dripped from 2 feet above the chitosan to ensure the drops would fully submerge into the mixture instead of sitting on the surface and combining with other drops. Each drop formed a distinct light blue bead floating in the mixture. Twenty mL of 0.6 M sodium hydroxide was then added to the mixture. Two hundred mL of distilled water was added, and the mixture was blended until particles were below 1 mm in size. Next, 3.5 g of ascorbic acid were dissolved in 50 mL of distilled water and added through a burette to begin the reduction process to form the LMWc CuCts particles. The solution rapidly changed from dark blue to yellow to red brown in approximately 10 minutes. This process was repeated with 310-375 kDa HMWc (Millipore Sigma, Cat# 419419) instead of LMWc in equal quantities. Instead of stirring the HMWc to dissolve it in the acetic acid, blending was required. Additional water was added to both setups as needed to reduce viscosity.

Separation and Drying

CuCts particles needed to be isolated from the supernatant liquid. The mixture was covered, and the particles were allowed to settle for 2 days. The liquid was then aspirated with a pipette, being careful not to disturb the particles. Three hundred mL of distilled water was added to wash the particles and the process was repeated two additional times. After air drying any remaining water, distinct LMWc CuCts and HMWc CuCts particles were obtained. However, the molecular weight of the final particles was not characterized. The LMWc CuCts showed slightly greater yield.

Poisoned Food Technique

A. alternata (Carolina, Cat# 155922) was spread onto 8 sterile 100 mL petri dishes with 20 mL of nutrient agar in a lawn pattern. This was allowed to grow for one week. The LMWc CuCts and HMWc CuCts particles were added to separate beakers of molten nutrient agar and mixed. The HMWc CuCts had slightly less molten nutrient agar to adjust for the difference in yield and ensure the concentration of CuCts was equal in both beakers at approximately 45 g/L. 20 mL of LMWc CuCts nutrient agar was then added to 8 sterile 100 mL petri dishes and the same was done for the HMWc CuCts. Approximately 0.9 g of CuCts was in each petri dish. 8 sterile petri dishes with 20 mL of pure nutrient agar were poured as the control group. Using a cork bore with a 1.65 cm inner diameter, mycelial disks were cut from the outer edge of the actively growing region of the viable lawn plates and placed upside down onto the center of each CuCts and control plate. They were placed upside down to ensure direct contact of the fungus and the testing plate. These plates were allowed to grow for 8 days at room temperature before data collection.

Data Collection

After the 8-day growth period, physical calipers ± 0.05 cm were used to measure the diameter of the fungal colony. The initial 1.65 cm mycelial disk was subtracted from the total colony size to determine the amount of fungal growth. The testing plates and lawn plates were then bleach killed and disposed of.

Determining Percent Fungal Inhibition

Each experimental fungal inhibition value was calculated as

$$PI = \frac{D_c - D_s}{D_c} \times 100\%$$

where D_c is the control fungal growth mean, D_s is the sample fungal growth value, and PI is the percent fungal inhibition.

Statistical Analysis

Statistical analysis was performed with GraphPad InStat 2 software. We used a Kruskal-Wallis one-way ANOVA to analyze the fungal growth data from the control, HMWc CuCts, and LMWc CuCts and a Mann-Whitney U test one tailed to analyze the fungal inhibition values from the HMWc CuCts and LMWc CuCts.

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