## APPLICATION OF NANOPARTICLES FOR THE CONTROL OF Colletotrichum gloeosporioides CAUSING ANTHRACNOSE DISEASE OF CHILI (Capsicum frutescens L.)

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### SUMMARY

Chili (*Capsicum annum* L.) was one of the most important seasoning constituents of the cuisines of many countries in the world and it was ranked the fourth major crop cultivated globally. However, Anthracnose disease caused by *Colletotrichum gloeosporioides* has affected chili production worldwide, especially in tropical and subtropical regions. It is necessary to have greatly effective disease management. Therefore, this study had examined the antifungal effect of some nano solutions on *C. gloeosporioides* in both *in vitro* and *in vivo* conditions. The results clearly demonstrated that silver nano, copper nano, and silver-copper nano solutions had inhibited the spore germination and the growth of *C. gloeosporioides*. Inhibitory effects were 100% mycelial diameter at the solutions with concentrations of 125 ppm silver nano or 75 ppm copper nano or 50 ppm mixed silver-copper nano solution completely inhibited spore germination of *C. gloeosporioides*. In vivo experiment, the silver-copper solutions with a concentration of 50 ppm could control the growth of *C. gloeosporioides* in chili fruits the findings were that there were only 13.3% disease fruits, and the inhibition efficiency was 86.7%.

Keywords: Anthracnose, Colletotrichum gloeosporioides, chili, silver nano, copper nano.

### **1. INTRODUCTION**

Chili (Capsium frutescens L.) was a type of important spice and vegetable that has the most economic value in the world. Chili can reduce cholesterol in the blood, regulates the blood pressure of human being. improve cardiovascular condition, and enhance eyesight. Also, chili has capsaicin, which was highly thermogenic, stimulating the body to burn fat, therefore releasing calories effectively and quickly (Zheng et al., 2017). In addition, chili was also becoming an income-raising crop and an important export commodity for many countries around the world. It tends to bring high-value crops into cultivation, especially short-term crops, which are suitable for the rotational production method (Saidah et al., 2020).

*Colletotrichum* was one of the most serious plant pathogens worldwide causing anthracnose disease in a wide range of hosts including cereals, legumes, vegetables, perennial crops, and tree fruits (Bailey & Jeger, 1992). Specifically, *Colletotrichum* caused a devastating disease that occurs on many commercially important plants like chili, beans,

strawberries, mangos, and other crop plants. To various phytopathogenic fungi, control including Colletotrichum species, agrochemicals have been used for a long time (Meenakshi & Saurabh, 2015). However, the use of agrochemicals has certainly used the outbreak of fungal diseases, but at the same time promotes the development of resistant pathogens. Moreover, the use of pesticides was a matter of concern because the environment was polluted, natural enemies can be destroyed, and the balance of natural ecosystems was affected. On the other hand, nanotechnology has been developing and as result, many applications in agriculture are increasingly popular. Many nano inoculants have been used and proven to be highly effective against several plant diseases with a few adverse effects on the environment and consumer health (Marek et al., 2010; Lams al et al., 2011; Chowdappa et al., 2014; Ezzeldin et al., 2020). Therefore, this present study aimed to apply nano in controlling the Colletotrichum gloeosporioides causing disease anthracnose of chili (capsicum frutescens L.).

### 2. RESEARCH METHODOLOGY 2.1. Materials

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*C. gloeosporioides* strain was provided by the Tropical Plant Disease Center of the Vietnam National University of Agriculture. Silver nano, copper nano, and mixed silvercopper nano solutions were supported by the Faculty of Biotechnology, Vietnam National University of Agriculture.

### 2.2. Method

### \* Effect of Nano Silver, Nano Copper, and mixed Nano Silver-Copper on C. gloeosporioides colony in vitro

The mixture of PDA and nano solution was autoclaved at 121°C, poured into each  $90 \times 15$ mm Petri dish, and incubated at room temperature. Each kind of nano solution in the experiment ranged in different concentrations, such as silver nano solution (NS) with 25, 50, 75, 100, 125 ppm, with nano copper solution (NC) 12.5, 25, 37.5, 50, 62.5 ppm and mixed silver-copper nano (NS-NC) with 10, 20, 30, 40, 50 ppm as previously described (Lamsal *et al.*, 2011; Pandurang *et al.*, 2019). The control treatment was Score 250 EC (0.1%).

*C. gloeosporioides* fungi were grown in the medium with potato dextrose agar (PDA) supplemented with NS or NCNC or mixed NS-NS at  $28 \pm 2^{\circ}$ C for 5 to 7 days.

From the active growing edge of 4-day-old cultures, a piece of fungi with a diameter of 2.5 mm was cut and put into the center of the Petri dishes containing a mixture of PDA-containing nano solutions. The petri dish was incubated at room temperature for 5 days. The effectiveness of inhibition to fungi growth was determined by the following formula,  $H (\%) = (D-d)/D \times 100$ . Among them, H refers to the percentage inhibition of NP on the diameter of the colony; D was the diameter of the colony on control, and d was the diameter of the colony on treatment. The test was repeated three and each treatment was replicated once times. The results were noted after 1, 3, and 5 days.

### \* Effect of Nano Silver, Nano Copper solution on C. gloeosporioides' spore germination

The 14-day-old spores were harvested and suspended in sterile distilled water. The spore suspension was filtered through two layers of sterile gauze and its concentration was adjusted to a density of 10<sup>6</sup> spores/ml. A total of 10 ml of spore suspension was dropped on potato dextrose (PA) liquid media supplemented with NP formulas. Each kind of nano in the experiment ranged with different concentration: 25, 37.5, 50, 62.5, 75 ppm NS; or 5, 10, 15, 20, 25 ppm NC; or 2.5, 5, 7.5, 10, 12.5 ppm mixed NS-NC.

The mixes were incubated for 24h and observed through a microscope at 40X magnification. The control treatment was done using a similar procedure, but the spore was treated with distilled water. To determine the number of germinated spores, conidia were considered the level of germination when the length of the germ tube equaled or exceeded the length of the conidia. The rate of spore germination was calculated using the following formula as G(%) = GS/TS (Yadi *et al.*, 2017). While "G" was germinated rate, "GS" was the number of germinated spores, and "TS" was the total number observed. The results were noted after 12, 24, and 48 hours. The degree of inhibition to spore germination was calculated by the following formula was that I (%) = (C-T)/T x 100. Among them, "I" refers to the percentage of conidial germination in the test with the pathogen, "C" was the number of germinated conidial in control, and "T" was the number of germinated conidial in treatment.

# \*Effect of Nano Solution on C. gloeosprioides in vivo

Red chili fruits were used for *in vivo* assay. The fruits were washed in running water and sterilized with alcohol 70% for 2 minutes and then washed with sterile distilled water twice and air-dried. After that, the fruits were wounded using a sterile needle, and inoculated by immersion fruits with the spore suspension solutions of *C. gloeosporioides* ( $10^{6}$ - $10^{7}$  spores per ml suspension) for 15 minutes and air-dried. The fruits were dipped for 15 minutes in sterile water. Then they were sprayed with a solution of nanoparticles for 60 minutes and air-dried. The fruits were stored on a sterile tray and wrapped to maintain suitable moisture (Yadi *et al.*, 2017). The experiment was arranged in a completely randomized design using three replications (consisting of 12 fruits in each treatment) (Chowdappa *et al.*, 2014). The infected fruits were followed for 3, 5, and 7 days. The effectiveness of nanoparticles inhibition was measured by the formula as "The rate of infected fruits = [(Number of fruits appear disease) / (total fruits)] \*100"; "The inhibition efficiency (%) = 100% - the rate of infected fruits".

\**Data analysis:* data of the works were collected and analyzed by a new version of Excell, XLSTAT 2019.1.2.

### **3. RESULTS AND DISCUSSION**

3.1. The Effect of Nano solution on *C. gloeosporioides* colony *in vitro* 

Silver nanoparticles could control *Colletotrichum* species *in vitro*. The results show that the application of nano silver solutions and nano copper solutions could maximize the inhibition of the growth of fungal hyphae in comparison to the control *in vitro* (Table 1).

Table 1. The inhibitor	v efficiency of nano o	on mycelial growth of	Colletotrichum	gloeosporioides

Kind	Concentration	The percentage inhibition of nano on <i>Colletotrichum gloeosporioides</i> (H) and the diameter of the colonies (d)						
of	(ppm)	Day 1	l	Day 3		Day 5		
nano	(bbm)	d (cm)	H (%)	d (cm)	H (%)	d (cm)	H (%)	
Fc (-)	0	1	0	$3.9\pm0{,}067$	0	$8.63\pm0.111$	0	
	25	$0.8 \pm 0.067$	20	$3.47\pm0.178$	11.11	$7.2\pm0.133$	16.60	
NG	50	$0.6 \pm 0.067$	40	$2.93\pm0.044$	24.79	$5.76\pm0.178$	33.20	
NS	75	0	100	$2.2\pm0.133$	43.59	$4.5\pm0.333$	47.87	
	100	0	100	$1.4\pm0.267$	64.10	$3.1\pm0.067$	64.09	
	12.5	0	100	0	100	0	100	
	25	$0.83 \pm 0.044$	16.67	$3.37 \pm 0.111$	13.67	$8.4\pm0.067$	2.7	
NG	37.5	$0.53 {\pm} 0.044$	46.67	$2.7\pm0.133$	30.77	$5.97 \pm 0.156$	30.88	
NC	50	0	100	$2.06\pm0,\!089$	47.01	$4.23\pm0.178$	50.96	
	62.5	0	100	$1.53 \pm 0.178$	60.68	$2.4\pm0.067$	72.21	
	75	0	100	0	100	0	100	
	10	$0.87\pm0.04$	13.33	$3.73 \pm 0.156$	4.27	$6.8\pm0.067$	21.23	
NS-	20	$0.6\pm0.067$	40	$3.07\pm0.089$	21.37	$5.63\pm0.111$	33.08	
NC	30	0	100	$1.8\pm0.067$	53.84	$4.2\pm0.067$	51.58	
	40	0	100	$0.6\pm0.067$	84.61	$2.7\pm0.067$	69.35	
	50	0	100	0	100	0	100	
Score 2	50 EC (0.1%)	0	100	0	100	0	100	

Specifically, NS inhibited the development of *C. gloeosporioides in vitro*. After 5-day culture, the diameter of the fungal colonies on the samples including the non-NS solution sample approached maximum levels, the mycelial growth was 8.63 cm. On the other hand, the highest inhibitory efficiency was observed in the case of treatment with 100% in the solution with a concentration of 125 ppm silver nano. The lowest inhibitory efficiency was 16.6% which was observed in the medium of 25 ppm NS. Similar to the finding of Lamsal *et al.*, (2011), a 50 ppm solution of silver nanoparticles stimulated the maximum inhibition of the growth of fungal hyphae as well as conidial germinations.

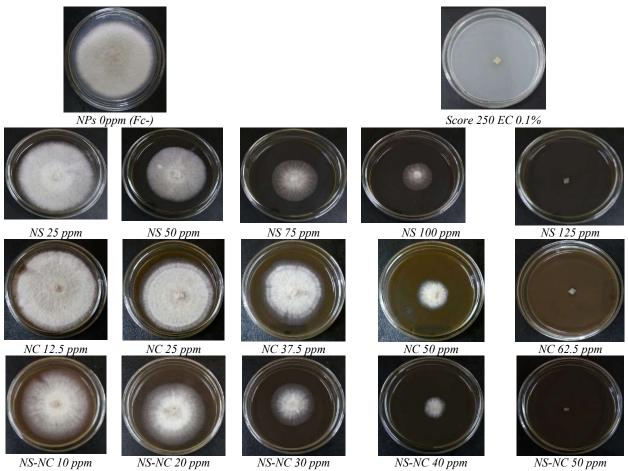


Figure 1. *Colletotrichum gloeosporioides* colonies at various concentrations nanoparticle solution after 5-day culture

Table 1 also showed that nano copper affected significantly mycelial growth. Compared with samples including the non-NC solution, NPs in all the treatment formulas could inhibit Colletotrichum. The higher concentration of nano copper solution was, the higher percentage of the inhibition was. On the fifth day, the highest inhibition was observed in the solution of 75 ppm NC which illustrated the non-growth of any colony with 0 cm diameter in size, however, in the medium of 25 ppm concentration of NC, the colony still grew with  $8.4\pm0.067$  cm diameter of the colony. On other hand, we observed that 50 ppm concentration of NCs showed higher inhibition than 70 ppm concentration of NC. The nanoparticles of Cu showed antifungal activity against С. gloeosporioides different at concentrations with inhibitory efficiency. Pham Van Viet et al., (2016) reported that Copper Nanoparticles could be anti with Fusarium. The results show that the NC resulted in an average size of colony in the range of 20-50 nm with a spherical shape. In their research, NC were used

at 450 ppm concentration in 9-day incubation, and 93.98% of fungal growth was inhibited. Besides, Pandurang *et al.*, (2019) researched and demonstrated that these copper nanoparticles were synthesized and showed significant antifungal activity against a fungal pathogen like *C. capsici* at 75 and 100 ppm concentrations.

Moreover, the combination of NS and NC was used to inhibit the fungi. After 5-day-old culture, the completely inhibitory efficiency, 100%, was observed in the case of treatment of 50 ppm mixed NS-NS. Similarly, Raghavendra et al., (2019) reported that it had antifungal efficiency of copper oxychlorides-conjugated silver nano, against C. gloeosporiodes which caused anthracnose disease. The study illustrated that the inhibition of fungal growth was observed with both NS and COC-NS. The solution of 1% copper oxychloride (COC-NS) was significantly inhibited with an inhibition zone of 1.2 cm diameter which was 50% more than that of the silver nanoparticles, NS with an inhibition zone of the diameter of 0.8 cm. Further, the fungicide conjugated NS exhibited the highest growth inhibition of *C. gloeosporioides* (~187%) as compared to fungicide copper oxychloride alone with an inhibition zone of 2.3 cm.

**3.2.** The inhibitor efficiency of Nano Silver Solution on *C. gloeosporioides* spore germination

Based on data collected from the previous experiment, the spore germination of *C*. *gloeosporioides* on PDA mixed with nanoparticles was tested which was shown in table 2.

Nano concentration	1	2h	24	h	48	Sh
(ppm)	G (%)	I (%)	G (%)	I (%)	G (%)	I (%)
NS 0	100	0	100	0	100	0
NS 25	59.87	40.13	72.09	27.91	91.92	8.08
NS 37.5	35.95	64.05	48.27	51.73	83.9	16.1
NS 50	18.78	81.22	38.88	61.12	56.79	43.21
NS 62.5	0	100	16.57	83.43	27.7	72.3
NS 75	0	100	0	100	0	100
NC 5	47.86	52.14	68.76	31.24	88.14	11.86
NC 10	29.25	70.75	46.16	53.84	56.71	43.29
NC 15	14.93	85.07	33.67	66.33	68.85	31.15
NC 20	0	100	12.56	87.44	32.8	67.2
NC 25	0	100	0	100	0	100
NS-NC 2.5	36.8	63.2	47.71	52.29	78.67	21.33
NS-NC 5	23.7	76.3	34.86	65.14	53.78	46.22
NS-NC 7.5	7.77	92.23	23	77	37.05	62.95
NS-NC 10	0	100	6.7	93.3	22.14	77.86
NS-NC 12.5	0	100	0	100	0	100

Note: G\_germinated rate; I\_percentage inhibition of conidial germination in test pathogen

Table 2 was evidence that NS inhibited the development of *C. gloeosporioides* spore germination. The amount of germinated spore differed significantly between the control and NS formula treatments.

In the concentration of 25 ppm NS, the germination rate was noted at 59.87%, inhibitor efficiency reached 40.13% after 12 hours. Besides, in a concentration of 37.5 ppm, the germination rate was reported as 35.95%, and the inhibitor efficiency was 64.05%. In the concentration of 50 ppm, the germination rate was 18.78%, and the inhibitor efficiency was 81.22%, 2 times higher than that of the medium with 25 ppm. In the mediums of 62.5 and 75 ppm NS, inhibitor efficiency reached 100%.

After 24 hours of culture, the medium of 25 ppm nano silver gave the lowest inhibitor efficiency (27.91%). In the medium of 62.5 ppm NS, the germination rate was 16.57% and the inhibitor efficiency reduced to 83.43%. In other

remaining formulas, while the germination rate increased, the inhibitor efficiency decreased.

After 48 hours of culturing, the germination rate was 91.92% at the medium of 25 ppm NS. The inhibitor efficiency at 37.5 ppm NS was 2 times higher than that of 25 ppm NS. The highest inhibition rate was 100% at 75 ppm of NS.

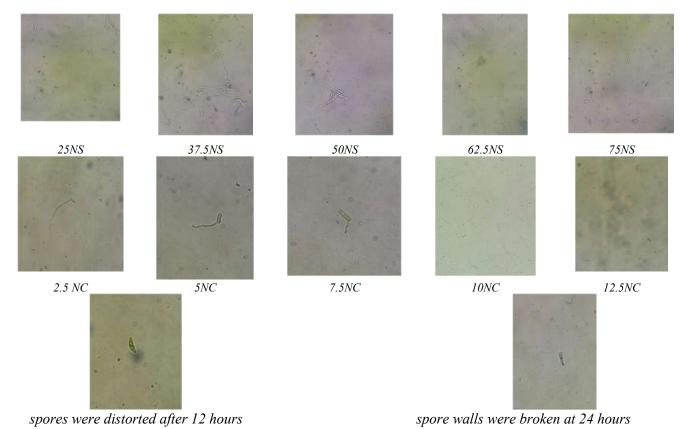
Marek *et al.*, (2010) reported that silver nanoparticles affected phytopathogenic spores of *Fusarium culmorum*. The data of sporulation test showed that the number of spores formed by mycelia increased in the medium after contact with silver nanoparticles, especially in the poor PDA medium. The 24-hour incubation of the spores with a 1.25 ppm solution of silver nanoparticles greatly reduced the number of germinating fragments and sprout length relative to the control.

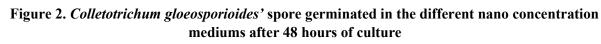
This experiment was performed to evaluate the inhibitor efficiency of NC on the spore germination of *C. gloeosporioides* at different concentrations. The results were obtained at different time intervals and were also shown in Table 2 and Fig 2.

It was undeniable that the amount of germination spore differed significantly between the control and copper nano treatments. Spore could germinate well in the control up to 100%, while less than half of spores treated with nano copper germinated. In the medium of 10 ppm NC solution, the germination rate approximately doubled (from 47.86% at 12 hours to 88.14% at 48 hours). In the medium of 20 ppm NC, the inhibitor efficiency reached 100% at 12 hours, then gradually decreased to 67.2% after 48 hours. 25 ppm nano copper inhibited the germination of spores and inhibitor efficiency reached 100%

The mechanism by which nano blocks the growth of *C. musae* was due to inhibition of germ tube length, hyphal growth, hyphal length, and width, as well as hyphal lyses. The decrease in the germination degree was suspected because of antifungal activity in nano copper oxygen. Zakharova *et al.*, (2018) reported that nano

copper (CuONPs) affected wheat seeds and seedlings and Alternaria solani fungi in vitro. The results of the in vitro study of the effect produced by CuONPs (0.001...0.1 g/L) on A. solani fungi. The most pronounced combined positive effect on the studied parameters had been observed at 0.01 g/L concentration of copper oxide nano. Improvement of germination capacity by 14.5 percent and a two-fold increase in the root and stem length compared to the control group have been recorded at this concentration. At higher NPs content in the dispersion, stimulation was combined with toxic effects (decrease of root length). Moreover, after 48 hours, the lowest inhibitor efficiency was 21.33% in the concentration of 2.5 ppm Ag-NC. When the concentration increased to 5 ppm, the inhibitory effect doubled, so the rate of germination also decreased (from 78.67% to 53.78%). Finally, the highest inhibitor efficiency of silver-copper nano solutions on spore 100% germination was in 12.5 ppm concentration.





Marek *et al.*, (2010) reported that silver nanoparticles affected phytopathogenic spores of *F. culmorum* as well. The sporulation test showed that, relative to control samples, the number of spores formed by mycelia increased in the culture after contact with silver nanoparticles, especially in the nutrient-poor PDA medium. The 24 h incubation of control formula spores with a 1.25 ppm solution of silver nanoparticles greatly reduced the number of germinating fragments and sprout length relative to the control.

In this experiment, in the medium of 20 ppm copper nano, the inhibitor efficiency reached 100% at 12 hours, then gradually decreased to 67.2% after 48 hours.

Ezzeldin Ibrahim *et al.*, (2019) researched Green-Synthesization of silver nanoparticles using endophytic bacteria isolated from garlic and its antifungal activity against wheat *Fusarium* Head Blight Pathogen. The results showed that the *in vitro* antifungal activity of the biosynthesized NS was the best with strong inhibition in the mycelium growth, spore germination, the length of the germ tubes, and the mycotoxin production of the wheat *Fusarium* head blight pathogen *F*. graminearum. Furthermore, the microscopic examination showed that the morphological mycelia had deformities and been collapsed when treated with NS, causing DNA and proteins to leak outside cells. The results of this study indicated that the biosynthesized NS at four different concentrations were able to effectively suppress the spore germination and germ tube growth of F. graminearum. The inhibitory effect increased with the increase of the concentration of the biosynthesized NSs. Indeed, the spore germination rate was 98.00%, while the length of the germ tubes was  $76.14 \,\mu m$ in absence of the biosynthesized NS. In the presence of NS at 2.5, 5, 7.5, and 10  $\mu$ g/mL, the spore germination rates were 85.00%, 67.67%, 44.00%, and 24.33%, respectively, and the germ tube lengths were 57.86, 48.57, 31.86, and 21.14 µm.

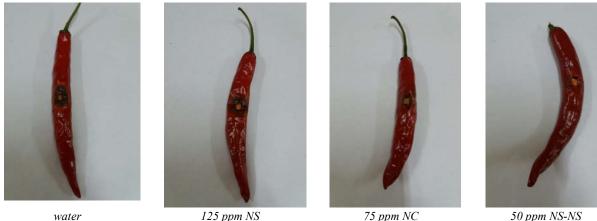
# **3.3.** The Effect of Nano on *C. gloeosporioides in vivo*

The purpose of assessing the ability of the nano to prevent and treat diseases caused by the fungus *C. gloeosporioides* is to bring practical plant production. It was carried out fungal infection with a density of  $10^6$  spores/ml. The results are evaluated according to table 3.

Treatment	Total of fruits	The number of fruits appear disease	The rate of fruits (%)	The inhibition efficiency (%)
Water (control)	36	36	100.00	0.00
125 ppm NS	36	18	50.00	50.00
75 ppm NC	36	10	27.28	72.72
50 ppm NS-NC	36	3	13.30	86.70

Table 3. F	Effect of	nano on	C	glaeasn	orio	ides	in	vivo
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In all the treatments, nano-silver expressed the ability to prevent and treat disease. With 125 ppm NS treatment, the rate of infected fruit was 50%, and the inhibition efficiency was 50%. Also, 75 ppm NCs could prevent the disease with the rate of affected fruit being only 27.28%, and the inhibition efficiency reaching 72.72%. Furthermore, with the 50 ppm NS-NS treatment, there were only 13.3% disease fruits, and the inhibition efficiency was 86.7%.



125 ppm NS 75 ppm NC Figure 3. Nano treated chili against anthracnose after 7 days

50 ppm NS-NS

The obtained results of the antifungal activity reveal that the growth of Colletotrichum species including C. gloeosporioides was at different concentrations inhibited of nanoparticles. Based on the comparison of results obtained before and after the disease outbreak treatments, the results indicate that the inhibition of fungus can be achieved when it was applied before disease symptoms occur on plants. The highest antifungal properties were observed in the case of treatment with 50 ppm nano silver-copper complex solution in vivo. Therefore, the results demonstrate that the nano solution would have the potential to inhibit species of the fungal pathogen Colletotrichum in field conditions as well. According to Kabir et al., (2011) silver nanoparticles can control Colletotrichum species in vitro and pepper anthracnose disease in the field. Chowdappa et al., (2014) also reported that the chitosan-NS composite, at 0.5 and 1% concentration could reduce anthracnose by 45.75 and 71.3% respectively. Chitosan at 0.5 and 1% concentration showed 35.5 and 41.85 reductions in the disease. Moreover, the combination of chitosan-NS with 0.1% Tween-80 reduced the disease by 75.8% at 0.5% and 84.6% at 1% concentration.

### 4. CONCLUSIONS

Nano solution could be used as a protective agent to clean and protect chili after harvest to prevent and treat diseases caused by the C. gloeosporioides fungus. In vitro experiments showed that the best concentration for the inhibition of growth mycelial was 125 ppm NS, 75 ppm NC, and 50 ppm Ag-NC. As for the spore germination inhibition experiments, the concentration of 75 ppm of silver nano, 25 ppm of copper nano, and 12.5 ppm of silver nano copper showed complete inhibition of spore growth. For in vivo experiments, the results also confirmed that nano solutions were completely effective in the treatment of anthracnose caused by the C. gloeosporioides fungus in chili. Nano silver-copper solution has the best inhibitory effect compared to nanosilver and nano copper. REFERENCES

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### ÚNG DỤNG NANO TRONG ĐIỀU TRỊ NẤM Colletotrichum gloeosporioides GÂY BỆNH THÁN THƯ Ở ỚT (Capsicum frutescens L.)

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#### TÓM TẮT

Ćt (*Capsicum annum* L.) là một trong những thành phần gia vị quan trọng nhất của các món ăn trên nhiều quốc gia và được xếp hạng thứ tư trong loại cây trồng chính được trồng trên toàn cầu. Tuy nhiên, bệnh thán thư đã ảnh hưởng đến sản xuất ớt trên toàn thế giới, đặc biệt là ở các vùng nhiệt đới và cận nhiệt đới. Do đó, người nông dân và nhà khoa học đang rất cần có những nghiên cứu và áp dụng biện pháp quản lý dịch bệnh này một cách hiệu quả. Do đó, nghiên cứu này đã kiểm tra tác dụng chống nấm của một số dung dịch nano đối với *Colletotrichum gloeosporioides* cả điều kiện *in vitro* và *in vivo*. Kết quả đã chứng minh rõ ràng rằng các dung dịch nano bạc, nano đồng và nano bạc đồng đã ức chế sự nảy mầm của bào tử và sự phát triển của *C. gloeosporioides*. Hiệu ứng ức chế là 100% khi đường kính sợi nấm ở các dung dịch có nồng độ 125 ppm nano bạc 75 ppm, hoặc nano đồng 25 ppm, hoặc dung dịch nano bạc đồng 12.5 ppm ức chế hoàn toàn sự nảy mầm của bào tử *C. gloeosporioides*. Ở thí nghiệm *in vivo*, dung dịch bạc đồng với nồng độ 50 ppm có thể kiểm soát sự phát triển của *C. gloeosporioides* trong quả ớt, kết quả là chỉ có 13,3% quả bị bệnh và hiệu quả ức chế là 86,7% khi ớt bị lây nhiễm với nấm này.

Từ khóa: Colletotrichum gloeosporioides, ót, nano đồng, nano bạc, thán thư.

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