DOI: https://doi.org/10.4038/sjhs.v3i1.50

Received: 20 October 2022 *Accepted: 20 January 2023*

In Vitro Evaluation of Antibacterial Activity of Copper and Sulfur Nanoparticles for Controlling Bacterial Blight Caused by Xanthomonas sp. in *Anthurium andraeanum* Lind.

Peiris, S. E.[1], Seneviratne, K. L.[2], Shashikala, R. P. A.[3], Peiris, Colin N.[4] , Imalka, M. I.[5] & Piumika, Y. P.[6]

sriyani.p@sliit.lk, kasunlaka@gmail.com, aruni.s@sliit.lk, colin.p@sliit.lk, imalka@sjp.ac.lk, piumikayapa@gmail.com

[1,3,4] *Department of Applied Science, Faculty of Humanities and Sciences, SLIIT, Malabe, Sri Lanka.*

[2] *Integrated Watershed and Water Resources Management Project, Ministry of Irrigation, Colombo, Sri Lanka.*

[5,6] *Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.*

Abstract

Bacterial blight in *Anthurium andraeanum* Lind. which is caused by *Xanthomonas sp*. is regarded as the most threatening disease in the anthurium industry worldwide. Therefore, the current study was carried out to determine whether the application of copper nano particles (CuNPs) and sulfur nanoparticles (SNPs) is a possible solution to control the bacterial blight in anthurium. The bacterium *Xanthomonas sp*. was isolated using standard methods and a single bacterial colony was grown in nutrient agar (NA). The colonies produced in cultures were identified as *Xanthomonas sp*. as they were Gram-negative, motile rods with yellow colour due to production of xanthin. The symptoms appeared in the pathogenicity test which was carried out by injecting purified *Xanthomonas sp*. into disease free anthurium plants confirmed the identification of the bacterial strain. Concentrations of 5, 15 and 25mg/100ml CuNPs and 50, 75 and 1000 mg in 100ml SNPs were mixed separately with isolated *Xanthomonas sp*. to investigate the behavior of two types of nano particles in destroying the bacterium. All three concentrations (5, 15 and 25 mg/100ml) of copper nanoparticles used in suspensions of the bacterium *Xanthomonas sp*. did not support any bacterial growth. In contrast, all three concentrations of SNPs in *Xanthomonas sp*. showed bacterial growth though it was less in 1000 mg /in 100ml compared to the control treatment. Hence it can be concluded that 5 mg/100 ml CuNPs is capable of destroying in vitro growth of *Xanthomonas sp*. bacterium which causes bacterial blight in *Anthurium andraeanum*.

Keywords: Anthurium, Bacterial blight, Copper nanoparticles, Sulfur nanoparticles, *Xanthomonas sp*.

Introduction

Anthurium andraeanum Lind. having second largest demand in tropical cut flowers, is cultivated throughout the tropics as well as in greenhouses in temperate countries. *Xanthomonas axonopodis* pv. *dieffenbachiae* (XAD), formerly known as *Xanthomonas campestris* pv. *dieffenbachiae*, has, however, had a substantial impact on anthurium production around the world which was first recorded in Brazil in 1960 and then in Hawaii in 1971 (Alvarez et al., 2006; Elibox and Umaharan, 2008). The disease became an epidemic in 1989, and Hawaiian anthurium industry dropped 50% within 10 years and continued to decline to 5% or less (Shehata,1992).

Bacterial blight in anthurium was controlled using chemicals with limited success (Nishijima and Chun, 1991). The reason why chemical control did not become popular was due to its inability to remove the bacterium completely from plants. Cultural practices, also applied to eliminate the disease by cultivating in pots rather than in beds and applying higher levels of ammonium fertilizer (Sakai, 1990). However, these applications also did not control the disease at the commercial level (Higaki et al., 1992). Attempts made to control the disease with the beneficial organisms, in foliar applications of microorganisms antagonistic to *X. axonopodis pv. dieffenbachiae* resulted in inconsistent and insignificant control of the disease (Fernandez et al., 1991)

Use of antibiotics such as streptomycin sulfate or oxytetracycline weekly for 6 to 8 weeks has been recommended in 1985 at the start of the Xanthomonas bacterial blight in Hawaii (Nishijima, and Fujiyama, 1985). However, the overuse of antibiotics and the development of resistance in bacterial populations have been reported more frequently as antibiotics were used on crops to eliminate bacterial diseases Copper included pesticides have been used heavily in integrated pest management specially to control bacterial diseases. However, frequent applications of copper pesticides have led to the emergence of copper-resistant strains of microorganisms in agriculture. Moreover, phytotoxicity, soil accumulation, and negative effects on soil fauna and flora as well as reduction of food quality parameters have reduced the use of copper based antimicrobial compounds (Lamichhane et al., 2018). Antimicrobial activity of CuNPs have been effective in destroying several bacterial species. Copper nano particles in concentrations of 100–250 mg/L have shown effective against bacterial species such as *Ralstonia, Pseudomonas* and *Xanthomonas*, in vitro as well as in plant applications (Chen et al., 2019; Strayer-Scherer et al., 2018). Growth of *Xanthomonas campestris* pv. *vesicatoria,* the causal agent of the bacterial blight disease, was significantly suppressed by two types of CuNPs, which had superior function compared to conventional commercial formulations of copper (Varympopi et al., 2020). Murthy et al., (2020) extracted green CuNPs from a medicinal plant in Ethiopia, Hagenia abyssinica (Brace) JF. Gmel., which showed positive degradation of two gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* and two gram-negative pathogenic bacteria *Escherichia coli* and *Pseudomonas aeruginosa* studied. They

recorded that there was a synergistic effect of bioactive compounds from medicinal plant coupled with CuNPs which was proved to be beneficial in destroying bacterial species evaluated. Therefore, CuNPs can be a possible solution to eliminate the bacterial diseases in plants.

Materials and Methods

Synthesis of copper nanoparticle (CuNPs)

A solution was prepared by mixing 1:1 volume ratio of TEOS and copper metal salt solution and few drops of 1M HNO_3 was added and stirred. Then a NH_3 solution was added dropwise to the mixture with vigorous stirring. The precipitate was separated and washed with excess water. The product was dried and calcined at 300 °C for 3 hours.

Synthesis of sulfur nanoparticle (SNPs)

A $Na_2S_2O_3.5H_2O$ (1M) solution was prepared using 62.04 g of $Na_2S_2O_3.5H_2O$ solid crystals dissolved in 250 mL of distilled water. The mixture was magnetically stirred at 500 rpm at 60 °C to dissolve crystals. The solution was then mixed with 125 mL of 1M HCl while being constantly stirring and heating. A white color turbidity was immediately formed with a few drops of 1M HCl and then turned into yellow particles, and the reaction was terminated after 40 minutes. The yellow precipitate which contains the SNP (Fig. 1) was collected, washed with distilled water (3-5 times) and dried at 70 °C for 2 hours (Shankar & Rhim, 2018; Suleiman et al., 2015). Ultimately ground the SNP into a fine powder using mortar and pestle.

Characterization of CuNPs and SNPs

X-ray Diffractometer (XRD) analysis was carried out using Rigaku Ultima-IV with Cu Kα radiation (λ=1.5405 Å, 30 mA, 40 kV), at the scanning rate of 4° min-1 within the range of 10-80° to determine the crystallographic characteristics of synthesized CuNPs and SNPs.

Morphological identification of *Xanthomonas* sp.

Colony morphologies of the isolates were Gram stained and pigmentation, growth, and colony features were noted through light microscopical observations. The Gram staining was done as per the previous studies (Moyes et al., 2009). Visual colony morphologies according to the standard descriptions, texture, color, and surface were recorded and compared with literature (Arshiya et al., 2014).

CuNPs and SNPs against *Xanthomonas* sp.

This experiment was carried out to investigate the bactericidal effect of CuNPs against *Xanthomanas* sp. About 5mm diameter bacterial growth from densely grown overnight cultures of *Xanthomanas* sp. were separately inoculated into the flasks containing sterilized deionized water supplemented with copper nano particles in 5, 15 and 25 mg/100m. These flasks were allowed to agitate at 400 rpm on magnetic stirrer for 30 minutes. After the respective time period, 15µl of bacterial CuNPs suspension of different concentrations were spread onto nutrient agar plates and the plates were incubated 48 hours at 37°C to observe the growth. The same procedure

was repeated for SNPs with the concertation of 50, 75 and 1000 mg/100ml. *Xanthomonas* suspended in the distilled water (without NPs) were used as the control treatment. All treatments were performed in triplicate.

Isolation of Xanthomonas sp. and Pathogenicity test

Diseased leaves of an anthurium plant showing typical bacterial blight symptoms were collected and surface sterilized with ethanol. Then the sections showing disease symptoms were crushed using sterilized scissors. The inoculum preparation followed by the serial dilution and pour plates techniques were carried out in the same manner as described by Collins et al. (2004). Then, a single bacterial colony was isolated and inoculated into a standard nutrient agar (NA) (HIMEDIA India), which was incubated for 48 hours at 36 °C. All experiments were conducted in triplicate. Individual colonies were then purified through two to three successive transfers and repeated streaking on respective media plates. Pathogenicity test was performed as described by Prior & Rott (1989). All isolates were evaluated for pathogenicity by injecting 1ml, 2ml and 3ml samples from a 1 X 108 CFU/mL culture into stems, juvenile and adult leaves of Anthurium andraeanum cv 'Tera' (Prior & Rott, 1989; Norman & Ali, 2012). The control anthurium plants were left uninoculated and kept far enough from inoculated plants provided with similar environmental condition to avoid spread of the disease to them.

Results and Discussion

XRD analysis of synthesized CuNPs and SNPs

The results of X-ray Diffraction (XRD) examination of the synthesized CuNPs and SNPs are shown in Figure 01 and 02. Figure 01 depicts the XRD pattern of synthesized CuNPs. The well-resolved diffraction peaks in the range of 30°–90° can be indexed to cubic copper structure (JCPDS file no. 48-1548). These peaks confirm the crystalline structure of CuNPs. SNPs matches with the Figure 02(PDF number 00-024-0733). According to the Debye–Scherrer formula (Seneviratne et al., 2021) the average crystallite size of the synthesized SNPs is around 5.9 nm and CuNPs is 6.2 nm.

Figure 1.
XRD pattern of synthesized CuNPs.

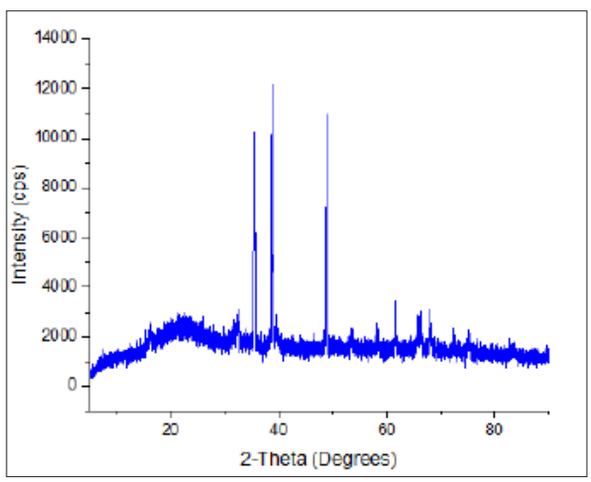


Figure 2.
XRD pattern of synthesized SNPs.

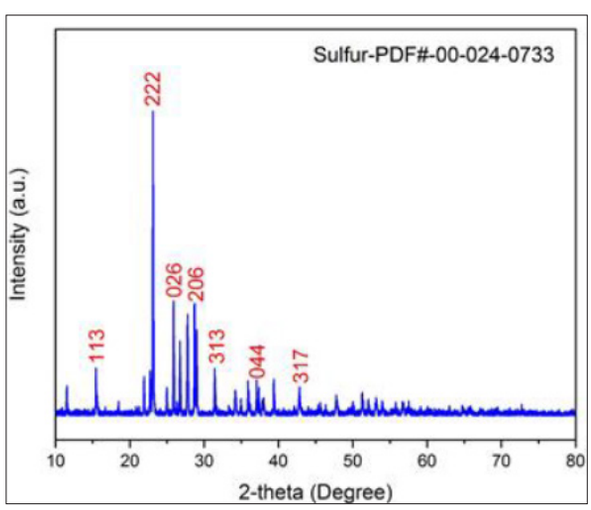
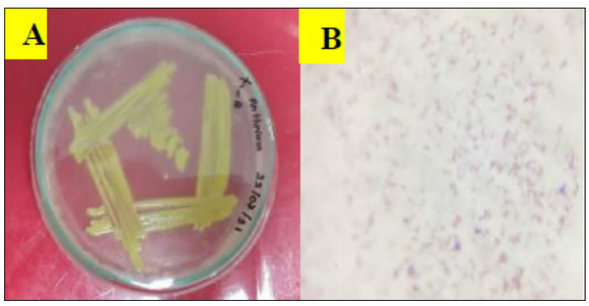


Figure 3.
(A) Purified Xanthomonas sp. on nutrient agar plates; (B) Gram staining of purified Xanthomonas sp.



Moreover, bacterial colonies discovered were motile rods, small, medium, large and pink in color in the Gram staining under microscopic examination (Fig. 3B). The findings were consistent with previous research (Arshiya et al., 2014). Morphological data are given in the Table 1.

Pathogenicity test

Early leaf symptoms of anthurium bacterial blight were detected as leaf spots which were observed to be small, angular, oily spots on the abaxial surface around veins, leaf edges, and spathes (Fig. 4A). These lesions grew quickly, producing huge, black necrotic patches that aged to grey-black which made the leaves become deformed (Figure 04B). Necrotic patches had oily borders and narrow, brilliant chlorotic halos around them. Systemic infections caused a broad yellowing of the entire lamina as well as the usual black, necrotic lesions that develop from the leaf petioles into major veins (Fig. 4C). These observations were as same as which were reported by Prior & Rott (1989).

Figure 4.
Pathogenicity test: (A), (B) and (C) depict the progression of the Xanthomonas bacterial blight disease.

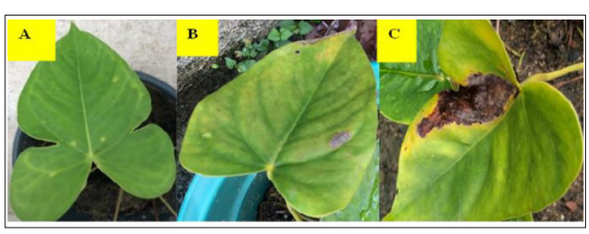


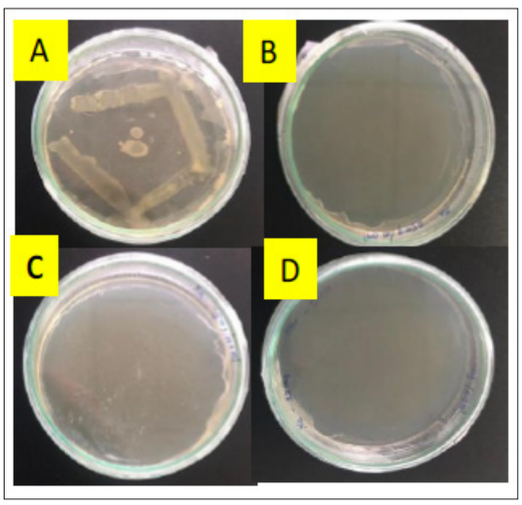
Table 1.
Morphological characterization of Xanthomonas sp. Colonies.

Isolates	Shape	Size	Grams (+/-)	Surface	Margin	Color	Elevation
X1-a	Rod	Small	+	Mucoidal	Even	Yellow	Convex
X2-b	Rod	Medium	+	Mucoidal	Even	Light yellow	Convex
X3-c	Rod	Medium	-	Mucoidal	Even	Light yellow	Convex
X4-d	Rod	Medium	-	Mucoidal	Even	Light yellow	Convex
X5-d	Rod	Medium	+	Mucoidal	Even	Yellow	Convex
X6-d	Rod	Small	-	Mucoidal	Even	Yellow	Convex
X7-d	Rod	Small	+	Mucoidal	Even	Light yellow	Convex

Antibacterial activity of CuNPs

Xanthomonas sp. did not show any growth in all replicates of the three concentrations of CuNPs included in the experiment (Fig. 5).

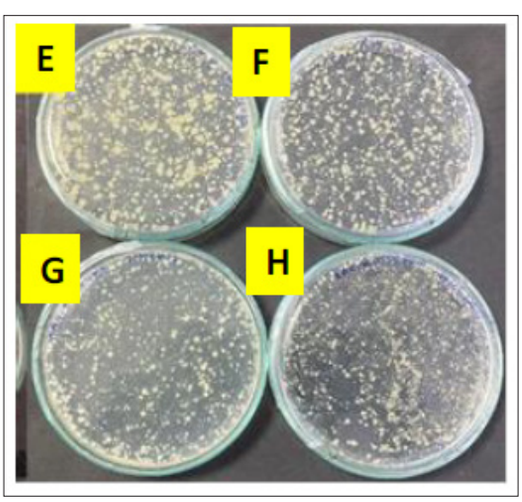
Figure 5.
Antibacterial activity of CuNPs in Xanthomonas sp. (A) Control. (B) 5 mg/100ml, (C) 15mg/100ml, (D) 25mg/100ml.



Antibacterial activity of SNPs

The results of the antibacterial activity of SNPs are shown in the Figure 6. The SNPs particles in all three concentrations (50, 75 and 1000mg/100ml) tested did not show any effect on the growth of the purified gram-negative isolate (Fig. 6).

Figure 6.
Antibacterial activities of SNPs in Xanthomonas sp. (A) Control. (B) 50 mg/100ml, (C) 75mg/100ml, (D) 1000mg/100ml.



Copper nanoparticles show antibacterial properties mainly because its size and high surface to volume ratio. The close interaction of copper nano particles with bacterial membrane creates antibacterial activity induced by the copper ions which are in the bacterial suspension (Ramyadevi et al., 2012). Copper ions are released during the slow oxidation process of the nanoparticles. The copper ions released can create toxic hydroxyl free radicals which disassemble the lipids in bacterial cell membranes and degenerate it. This activity leads to secretion of the intracellular substances through the destructed membranes and cell death occurs (Wei et al, 2010). Copper ions also can damage the cell membrane by solidifying protein structure present in the cell membrane and altering enzyme function (Ohsumi et al., 1988; Dan et al., 2005). Furthermore, bacterial cells are inactivated by the presence of copper nanoparticles in the growth medium and their replication process is terminated which led to bacterial cell death (Hu & Xia, 2006).

Though SNPs are considered as sterilizing agents because of their strong antibacterial capabilities there have been several reports on their inconsistencies. Suleiman et al. (2015) reported that there was no antibacterial action of SNPs detected against Gram-negative bacteria, Escherichia coli and Pseudomonas aeruginosa, at concentrations ranging from 0.68 to 800 g/mL Also in another study (Saedi et al.(2020) showed that two types of SNPs did not have antibacterial activity against Gram-negative bacterium Escherichia coli but showed significant antibacterial activity against Gram-positive bacterium Listeria monocytogenes. According to Suleiman et al. (201) Gram-negative bacteria are less vulnerable to SNPs

because their outer membrane comprised of thicker lipopolysaccharides layer and they have observed that the cell wall of E. coli was shrinking without destruction. This explains the resistant behavior of Gram-negative bacteria to SNPs. Although the precise mechanism underlying SNPs antibacterial activity has not yet been identified, several likely antimicrobial mechanisms have been put forth, including interactions with biological molecules on the surface of bacteria that cause membrane rupture and lysis of the cell membrane, the production of toxic hydrogen sulfide gas through reactions with thiol groups in proteins and lipids, and interactions with DNA that cause denaturation of DNA and cell death (Rai et al., 2016).

Conclusions

Our results showed that the synthesized copper nanoparticles (CuNPs) completely destroyed the Xanthomonas bacteria at 5mg/100ml concentration. The results of the morphological and pathogenicity test confirmed that the isolates were Gram-negative and had a greater chance of being one of the strains of Xanthomonas sp. Therefore, copper nanoparticles can be used to investigate the possibility of controlling the bacterial blight disease in anthurium by experimenting with diseased plants.

Acknowledgement

This research was financially supported by the project of Accelerating Higher Education Expansion and Development (AHEAD) of the Ministry of Higher Education Sri Lanka funded by the World Bank with the grant number RIC No-01 provided to Sri Lanka Institute of Information Technology (SLIIT).

References

Abuyeva, B., Burkitbayeva, M., Muna, G., Uralbekova, B., Vorobyevaa, N., Zharlykasimova, D., & Urakaeva, F. (2018). Preparation of ointment materials based on sulfur nanoparticles in water-soluble polymers. *Materials Today: Proceedings*, 5(11), 22894-22899. https://doi.org/10.1016/j.matpr.2018.07.107.

Alvarez, A. M., Toves, P. J., & Vowell, T. S. (2006). Bacterial blight of Anthuriums: Hawaii's Experience with a Global Disease. Online. APS net Features. doi: 10.1094/APSnetFeature-2006-0206.

Arshiya, M., Suryawanshi, A., More, D., & Baig, M. M. V., (2014). Repetitive PCR based detection of genetic diversity in *Xanthomonas axonopodis pv. citri* Strains. *J. Appl. Biology. Biotech*., 2(1), 17- 22.

Breed, R. S., Murry, E. G. D., & Smith, N. R. (1989). Bergey's Manual of Systemic Bacteriology. Eds. S. T. Williums CABIJOURNALCABI Agriculture and Bioscience. doi:10.1186/s43170-020-00001-y.

Chen, J., Mao, S., Xu Z., & Ding, W. (2019). Various antibacterial mechanisms of biosynthesized copper oxide nanoparticles against soilborne *Ralstonia solanacearum*. RSC Adv. 9, 3788-3799. doi: 10.1 039/C8RA09186B.

Collins, C. H., Lyne, P. M., & Grange, J. M. (2004). Microbiological methods. 5th. Arnold, a member of the holder

headline Group. London. Commercial Greenhouse Operations. UF/IFAS Extension. 292-298. ISBN 0 340 80896 9 1 2 3 4 5 6 7 8 9 10.

Dan, Z. G., Ni, H., & Xu, B. F. (2005). Microstructure and antibacterial properties of AISI 420 stainless steel implanted by copper ions. *Thin Solid Films*, 492, 93-100.

Elibox, W., & Umaharan, P. (2008). A quantitative screening method for the detection of foliar resistance to *Xanthomonas axonopodis* pv. *dieffenbachiae* in anthurium. *European Journal of Plant Pathology,* 121, 35-42. https://doi.org/10.1007/s10658-007-9239-0.

Fernandez, J. A., Tanabe, M. J., Wolff, W. J., & Moriyasu, P. (1991). Biological control. Pages 28-30 in: Proc. Anthurium Blight Conf., 4th. A. M. Alvarez, D. C. Deardorff, and K. B. Wadsworth, eds. Hawaii Inst. *Trop. Agric. Human Res*., University of Hawaii, Honolulu.

Higaki, T., Imamura, J. S., & Moniz, D. (1992). Nutritional and cultural effects on bacterial blight of anthurium. Proc. Anthurium Blight Conf., 5th. K. M. Delate, and C. H. M. Tome, eds. Hawaii Inst. *Trop. Agric. Human Res*., *University of Hawaii, Honolulu*. 44-45.

Hu, C. H., & Xia, M. S. (2006). Adsorption and antibacterial effect of copper-exchanged montmorillonite on *Escherichia coli* K88. *Appl Clay Sci,* 31, 180-184.

Lamichhane, J. R., Osdaghi, E., Behlau, F.,Köhl J., Jones, J. B., & Aubertotet, J., (2018). Thirteen decades of antimicrobial copper compounds applied in agriculture. A review. Agron. Sustain. Dev. 38, 28 (2018). https://doi.org/10.1007/s13593-018-0503-9.

Murthy, H. C. A., Desalegn, T., Kassa, M., Abeb, B., & Assefa, T. (2020). Synthesis of Green Copper Nanoparticles Using Medicinal Plant *Hagenia abyssinica* (Brace) JF. Gmel. Leaf Extract: Antimicrobial Properties, Journal of Nanomaterials. Article ID 3924081/ https://doi.org/10.1155/2020/39240812.

Moyes, R., Reynolds, J., & Breakwell, D. (2009). Differential Staining of Bacteria: Gram Stain. *Current Protocols in Microbiology*, 15(1). https://doi.org/10.1002/9780471729259.mca03cs15.

Nishijima, W., & Chun, M. (1991). Chemical control of anthurium blight. in: Proc. Anthurium Blight Conf., 4th. A. M. Alvarez, D. C. Deardorff, and K. B. Wadsworth, eds. Hawaii Inst. *Trop. Agric. Human Res., University of Hawaii, Honolulu*. 21- 23.

Norman, D. J., & Ali, S. G. (2012). Anthurium Diseases: Identification and Control in in of anthurium. Commodity-Fact-Sheet, Co-operative. Publication #PP292. Date: 7/27/2018 UF/ IFAS Extension, University of Florida.

Ohsumi, Y., Kitamoto K., & Anraku, Y. (1988). Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion. *J Bacterial*, 170:2676-2682.

Prior, P., & Rott, P. (1989). Bacterial Leaf Spot of Anthurium (Anthurium sp.) Caused by a *Pseudomonas* sp. in the French West Indies. *Journal Of Phytopathology*, 124(3), 215-224. https://doi.org/10.1111/j.1439-0434.1989.tb04917.x.

Rai, M., Ingle, A., & Paralikar, P. (2016). Sulfur and sulfur nanoparticles as potential antimicrobials: from traditional medicine to nanomedicine. *Expert Review of Anti-Infective Therapy*, 14(10), 969-978. https://doi.org/10.1080/14787210.2016.1221340.

Ramyadevi, J., Jeyasubramanian, K. Marikani, A. Rajakumar, G., & Rahuman, A. A. (2012). Synthesis and antimicrobial activity of copper nanoparticles. Mater. Lett. 71, 114-116. doi:10.1016/j.matlet.2011.12.055.

Rao, K. J., & Paria, S.(2013). Use of sulfur nanoparticles as a green pesticide on *fusarium solani* and *Venturia inaequalis* phytopathogens. RSC Advances, 3(26), 10471. https://doi.org/10.1039/C3RA40500A.

Saedi, S., Shokri, M., & Rhim, J. (2020). Antimicrobial activity of sulfur nanoparticles: Effect of preparation methods. *Arabian Journal of Chemistry*, 13(8), 6580-6588. https://doi.org/10.1016/j.arabjc.2020.06.014.

Sakai, D. S. (1990). The effect of nitrate and ammonium fertilizer on the contents of anthurium guttation fluid. In: Proc. Anthurium Blight Conf., 3rd. A. M. Alvarez, ed. Hawaii Inst. *Trop. Agric. Human Res., University of Hawaii*, *Honolulu*. 18-19.

Seneviratne, K. L., Munaweera, I., Peiris, S. E., Peiris, C. N., & Kottegoda, N., (2021). Recent Progress in Visible-Light Active (VLA) TiO2 Nano-Structures for Enhanced Photocatalytic Activity (PCA) and Antibacterial Properties: A Review. Iran. J. Catal. 11, 217-245.

Shankar, S., & Rhim, J. W. (2018). Preparation of sulfur nanoparticle-incorporated antimicrobial chitosan films. *Food Hydrocolloids*, 82, 116-123.

Shehata, S. (1992). Supply-demand and market analysis of the cut-flower industry: a focus on the Hawaiian anthurium industry. In: Proc. Anthurium Blight Conf., 5th. K. M. Delate and C. H. M. Tome, eds. Hawaii Inst. *Trop. Agric. Human Res., University of Hawaii, Honolulu, 35-38.*

Strayer-Scherer, A., Liao, Y. Y., Young, M., Ritchie, L., Vallad, G. E., Santra S., Freeman J. H., Clark, D., Jones, J. B., & Paret, M. L. (2018) Advanced copper composites against copper-tolerant *xanthomonas perforans* and tomato bacterial spot. Phytopathology. 108, 196-205. doi: 10.1094/PHYTO-06-17-0221-R.

Suleiman, M., Al-Masri, M., Al Ali, A., Aref, D., Hussein, A., Saadeddin, I., & Warad, I. (2015). Synthesis of

nano-sized sulfur nanoparticles and their antibacterial activities. *J Mater Environ Sci*, 6(2), 513-518.

Varympopi, A., Dimopoulou, A., Theologidis, I., Karamanidou, T., Kaldeli, K., A., Vlachou, A., Karfaridis, D., Papafotis, D., Hatzinikolaou, D.,G., Tsouknidas, A. & Skandalis, N. (2020). Bactericides Based on Copper Nanoparticles Restrain Growth of Important Plant Pathogens. Pathogens. 59(12), 1024. doi: 10.3390/pathogens9121024. PMID: 33291381; PMCID: PMC7762092.

Wei, Y., Chen, S., Kowalczyk, B., Huda, S., Gray, T. P., & Grzybowski, B. A. (2010). Synthesis of Stable, Low-Dispersity Copper Nanoparticles and Nanorods and Their Antifungal and Catalytic Properties. *J. Phys. Chem*. 114 (37), 15612-15616. doi:10.1021/jp1055683.