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To cite this article: EM De La Rama Jr. *et al* 2019 *J. Phys.: Conf. Ser.* **1310** 012003

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Synthesis, characterization, fabrication and application of electrospun nanofiber polymer composite: a potential bio-material for black mold rot (*Aspergillus niger*) of onions (*Allium cepa* L.)

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Abstract: Nanomaterial is widely researched because of its enormous potential. In this study, the anti-fungal activity of electrospun nanofiber polymer composites infused with malunggay extracts (ME) was investigated. Cellulose acetate (CA) from abaca fiber, nano silica (nSiO₂) from rice husks, and nano copper oxide nCuO from cavendish banana peel were synthesized, characterized and used as stabilizing agent. Electrospun nanofiber polymer composites infused with malunggay extracts was successfully fabricated. The inhibitory effects of the fabricated nanofiber were determined using different concentrations of ME. Results showed that 100 % concentration of malunggay extract (ME) mixed with 50 mg of nSiO₂ and 50 mg of nCuO had a presence of small crystal-like structures that indicates the successful infusion of the bio-materials. The nanofibers showed inhibitory effect against *A. niger* both in in vitro and in vivo. It displayed the highest zone of inhibition, lowest weight loss and disease severity index which is comparable to the fungicide. This study confirms the antifungal potential of the Electrospun nanofiber polymer composites infused with malunggay extracts on the test fungi and suggests the possibility of employing them in food preservation where spoilage is mainly caused by *A. niger*.

1. Introduction

Through nanotechnology, the innate physical and chemical nature of the base material changes including the changes in solubility, absorption, transport mechanism, excretion and importantly antagonisms (Gopi et al., 2017). One of the advanced products of nanotechnology is nanofiber which is produced by electrospinning method with maximum size of 100 nanometers (Agarwal et al., 2013). It exhibits unique properties, which are quite different than those of large particles. These fibers are potential because of so many advantages, they are abundantly available, low weight, biodegradable, cheaper, renewable, low abrasive nature, intersecting specific properties, and since these are waste biomass, they exhibit good mechanical properties (Bledzki, et. al, 1996; Hornsby et al., 1997; Oksman et. al, 2002).

Onions are high value crops in most part of the world, which are mainly grown for its bulbs. As a food material specifically as an ingredient and spice, they are indispensable because of their flavor that gives pungent aroma and sharp taste and also for their nutritional value that is rich in vitamins and minerals (Food and Agriculture Organization, 2003).

For the past 5 years, onion production declines at 7.89% (Philippine Statistics Authority, 2017). One of the main reasons is storage losses due to black mold rot brought about by *A. niger* which could cause 31% damage or loss (Yoo & Pike, 1995) during storage under tropical countries like Philippines. However, several treatments have been applied to solve the issues regarding postharvest losses of onion caused by *A. niger* and one of the technologies that could offer viable and alternative technique is nanotechnology. Storage facility owners can tap the technology of nanofiber. This study was conducted



to evaluate the antifungal effect of abaca-malunggay-nSiO₂-nCuO electrospun nanofiber polymer composite against black mold rot of onions. Indigenous materials were infused and successfully converted into high-valued nanofiber materials.

2. Experiment

2.1 Materials

Decorticated abaca fibers were obtained from Virac, Catanduanes, Bicol Region. Rice husks, Cavendish banana peel, and malunggay leaves samples were taken from CLSU, Science City of Muñoz, Nueva Ecija. PLA (poly lactic acid) in commercial grade with a molecular weight of 160,000 g / mole, low viscous chitosan from crab shell ($\leq 12\%$ loss on drying), Glacial Acetic Acid, dichloromethane ($>99.9\%$ purity, volatile and high toxicity), methanol (95% purity, volatile), and cellulose acetate with average Mn~50,000 by GPC and density of 1.3 g/ml were purchased from Sigma Aldrich Corporation, USA.

2.2 Synthesis of Cellulose Acetate

The decorticated fibers were cut into smaller pieces (3 cm in length) using sterilized scissor. Twenty (20) grams of the prepared fibers was boiled with 10 % NaOH solution for 1 hour. The resulting mixture was filtered and washed with distilled water to free the fiber (defibring). The sample was bleached for 1 hour using 12.5 % bleaching agent solution and was air-dried, ready for acetylation.

For acetylation process, 8 grams of air-dried abaca fibers was used and placed in a 500 ml capacity flask. One hundred forty (140) ml of Glacial Acetic Acid (GAA) was added and heated into a double boiler water bath for 1 hour at 50 – 55 °C. After 1 hour of heating, 40 ml of Acetic Anhydride (AA) and 0.5 ml of Sulfuric Acid (H₂SO₄) was added and heated for 1 hour at 50 – 55 °C. After heating, 80 % of GAA was added and heated for another one hour and washed with 500 ml of water and filtered using Whatman number 1 filter paper. The precipitate was air dried up to 72% moisture content and ground.

2.3 Synthesis of Nano Silica from Rice Husk

Briefly, 10 grams of rice hull ash was refluxed with 2.5 N NaOH solution for 4 hours and then was filtered. The residue was washed with 40 mL of boiling distilled water. The viscous, transparent and colorless solution was allowed to cool down at room temperature and 5 N H₂SO₄ solution was added dropwise to the sodium silicate solution with constant stirring until it reaches a pH of 9. The solution was precipitated to gel after reaching pH 7 and the gel was aged for 12 hours at room temperature. After aging, the gel was washed with hot distilled water until the gel turns semi-transparent. The gel was dried in the oven for 48 hours at 80°C. The dried nSiO₂ gel was ground to obtain silica nanoparticles (nSiO₂) results.

2.4 Synthesis of Nano Copper Oxide

Cavendish banana peel served as a stabilizing agent in the synthesis of nCuO. Three thousand seven hundred fifty (3750) grams of fresh banana peel were cut into smaller pieces (4 cm in length) and air dried for 7 days that gave out 2000 grams with up to 47 % moisture content. After drying, the samples were pulverized using a multi-functional high-speed disintegrator and kept inside a zipper plastic bag.

One hundred fifty (150) grams of pulverized banana peel was placed in a 1000 ml capacity crystalline dish, added with 500 ml of distilled water and heated for 10 minutes, maintaining a temperature of 70 °C-80°C and was filtered. The filtrate was taken and heated for 10 minutes, adding 0.1 molar of Copper II Sulfate Pentahydrate oxide (CuSO₄.5H₂O) as source of copper oxide and the precipitate was taken and oven dried for 3 hours at 100 °C and subsequently fired using a furnace at 400 °C for 5 hours. The resulting particle turned into black ash and ground to obtain its nanoparticles.

2.5 Fabrication of Electrospun Nanofiber Polymer Composite

Three hundred fifty (350) grams of fresh malunggay leaves were washed three times by soaking and hand scrubbing in tap water and rinsed with distilled water. It was air dried for five days which gave out

an amount of 100 grams after air drying with up to 71.43 % moisture content. It was pulverized using a multi-functional high-speed disintegrator.

Methanol was added to the pulverized malunggay leaves, 10g: 50 ml; sample solvent ratio for 100% concentration, 7.5g: 50 ml for 75% concentration, 5g: 50 ml for 50% concentration after 48 hours. It was filtered using a Whatman filter paper number 1, then kept refrigerated until used.

Four (4) treatments were assigned in the nanofiber fabrication with the blending of cellulose acetate (CA), of Poly lactic acid (PLA), dichloromethane (DCM)/methanolic extract of malunggay leaves and nanoparticles. The preparation of these treatments was adapted from the methods established by PIMS-Nanoworks. For every 1 ml of methanolic extract of malunggay leaves, 3 ml of dichloromethane (DCM) was used. The combinations and the varying amounts of Cellulose Acetate (CA) and the Poly (lactic acid) (PLA) of the resulting solution for the electrospinning process is shown in Table 1.

Table 1. Different Amount of Mixtures

Treatment	Volume of DCM and ME Leaves	Weight of CA g	Weight of PLA g	Weight of nSiO ₂ g	Weight of nCuO (dissolved in chitosan), g
T1	20 ml (50 %)	0.3	2.65	0.05	—
T2	20 ml (75 %)	0.6	2.35	0.05	—
T3	20 ml (100 %)	0.9	2.05	0.05	—
T4	20 ml (100 %)	0.9	2.05	0.05	0.05

ME = Malunggay extract; DCM = Dichloromethane; CA = Cellulose Acetate; nSiO₂ = Nano silica; nCuO = Nano copper oxide; PLA = Poly Lactic Acid;

2.6 Characterization of the electrospun nanofiber polymer composite

Synthesized nanomaterials and nanofiber polymer composites were characterized to describe the spectral and structural analysis, surface morphology and diameter size and particle size using the Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM)/Elemental Dispersive X-ray (EDX) and Zeta Potential Analyzer (Nanopartica), respectively.

2.7 In Vitro Analysis

2.7.1 Diffusion technique method. Filter paper discs were prepared using a paper puncher to have uniform circular shape and sterilized in an autoclave. Sterilized discs were placed in an oven drier set at 45 °C to remove moisture. The filter paper discs were soaked in the solutions used in the electrospinning. Fifteen (15) ml of potato dextrose agar (PDA) were poured per plate that was used as media. The plates were inoculated with *A. niger* and were settled and hardened in a laminar flow hood before assigning the different treatments. There were 8 discs placed equidistantly per plate. Finally, the disc seeded were placed in a room with a temperature of 28 °C – 30 °C.

2.7.2 Zone of inhibition. The diameter of the zone of inhibition was measured using a digital caliper in millimeters after 48, 72 and 96 hours. The different concentration of malunggay extract was based on the study of Arowora, et. al., (2014).

2.8 In Vivo Analysis

2.8.1 Onion sample collection and preparation. Healthy and mature onion bulb samples (Prajapati & Patil, 2016) were collected from Gabaldon, Nueva Ecija. The onion bulbs were weighed individually before inoculating the pure culture of black mold. Weight loss percentage was gathered after applying the electrospun nanofiber polymer composite to each onion bulbs both in cold and room storage.

2.8.2 Inoculation of *Aspergillus niger* on the onion bulb. Two (2) plates of pure culture of *A. niger* were dissolved in 200 ml of water and were sprayed to 108 pieces of onion bulbs used in the *in vivo* analysis. Approximately 2 ml of water inoculated with black mold was sprayed to each bulb, methods some with some modifications established by Prajapati & Patil (2016). The bulbs were kept in a box covered with a moist plastic bag to ensure the growth of the black mold. The symptoms that showed in the bulbs indicates infection with the black molds.

2.9 Application of nanofiber to onion bulbs

After two (2) days of growing the fungi, onion bulbs were weighed again individually. To validate the result of the *in vitro* analysis, the fabricated nanofibers were used to wrap the onions individually. They were divided into two: cold storage temperature and room storage temperature. Three (3) refrigerators were used in the cold storage temperature. The first refrigerator contained the treatments 1 (T1), 2 (T2) and 3 (T3), the second refrigerator contained the treatment 4 (T4) that has nCuO, while the third refrigerator contained the positive and negative control. The temperature and relative humidity were controlled and monitored using a hygrometer. The temperature (T) was maintained to 0 °C- 4 °C and a relative humidity (RH) of 65 % - 70 % (Halkema, 2017).

3. Results and Discussion

3.1 Cellulose acetate

FTIR spectrum for the cellulose acetate is shown in Figure 1 for the verification of the functional groups present in the cellulose acetate sample which corresponds to different peaks and wavenumber (cm^{-1}). A weak absorption peak was observed in the region from 3200 cm^{-1} to 3700 cm^{-1} and assigned as the O-H stretching. The hydroxyl group (O-H) present in the sample was due to its own characteristics as cellulose. Since cellulose acetate is an ester polymer, the spectra would be expected to have C=O, C-O, C-H, and C-C peaks which were the functional groups present in ester. The strong absorption peak of carbonyl (C=O) group was observed between 1700 cm^{-1} – 1800 cm^{-1} and assigned as the C=O stretching. C-O bending has strong absorption peak between 1000 cm^{-1} – 1120 cm^{-1} . The carbon to carbon (C-C) bonding was observed ranging from 1200 cm^{-1} - 1320 cm^{-1} which were assigned as the C-C bending. The weak absorption peak of C-H bending was also observed between 1330 cm^{-1} – 1400 cm^{-1} . In addition to that, two different peaks were observed for the R-group (CH_2 rocking and CH_2 scissoring) of the cellulose acetate sample, the absorption peak between 800 cm^{-1} - 960 cm^{-1} for CH_2 rocking and ranging from 1400 cm^{-1} - 1520 cm^{-1} for CH_2 scissoring, this observable peaks might be due to the presence of R-group (CH_3) in the ester.

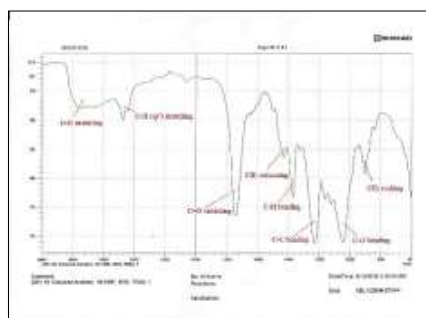


Figure 1. FTIR Spectra of Cellulose Acetate

3.2 Nano Silica

The particle size analysis reveals that the nSiO_2 has a mean average size of 7.8 nm which is within the required range of nanoparticles size (1-100 nm) as shown in Figure 2a. The morphology of the nSiO_2 is shown in Figure 2a, the SEM image gives a cloudy-like structure which means that the nano silica produced was amorphous. This is due to the aggregation of Si-O-Si and surface Si-OH molecular gel

network (Ke et al., 2016). SEM image also reveals that the particle diameter size of the nSiO₂ has values ranging from 31 nm to 48 nm which are also within the range of a nanoparticle.

Figure 2b shows strong absorption peaks between 1000 and 1500 cm⁻¹ were observed in the FTIR spectra of silica nanoparticle sample due to the stretching vibration peak of Si-O bond. The shoulder of Si-O peak was due to the presence of the Si-O-Si which had observed vibrational peak between 1000 - 1500 cm⁻¹. The weak absorption peak of C=O stretching was also observed in region ranges from 1500-2000 cm⁻¹ is due to the carbon residue after firing.

The strong and broad absorption peak was also observed in the region from 3000 cm⁻¹ to 3600 cm⁻¹ due to the presence of hydroxyl group (O-H stretching). The peak between 2000 cm⁻¹ - 2500 cm⁻¹ was due to the carbon dioxide in the atmosphere. The results suggest that the synthesis of silica nanoparticles is successful due to the presence of the Si-O and OH bonds, however, there are still impurities presence in the sample that cannot be avoided during the experiment.

X-ray diffraction image of the synthesized nSiO₂ is shown in Figure 2c. It is used to determine the crystallinity of the nSiO₂. However, the matrix on the figure shows that the silica is amorphous that confirms the absence of the crystalline structure. Rice husk is amorphous in nature, thus it belongs to non-crystalline silica (Ke et al., 2016)

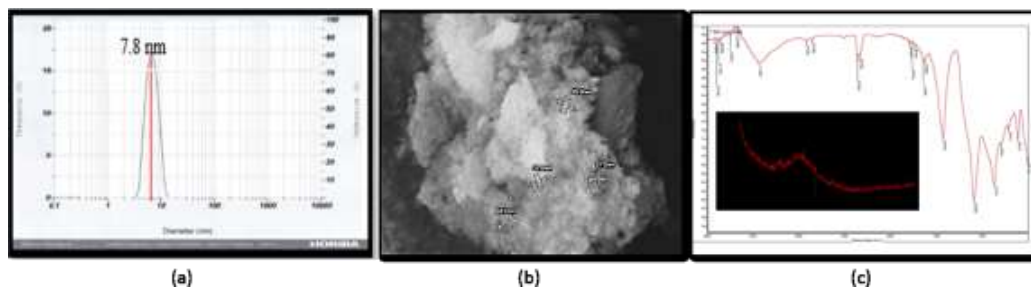


Figure 2. (a) Particle size of nSiO₂, (b) SEM image of the synthesized nSiO₂, (c) FTIR and XRD Analysis of nSiO₂

3.3 Nano Copper Oxide

The synthesized nCuO as shown in Figure 3a, have a diameter size of 19.5 nm; this confirms that the copper oxide is a nanoparticle. Figure 3b shows the SEM image for the nCuO appears cloudy-like structure that validates the nCuO is amorphous.

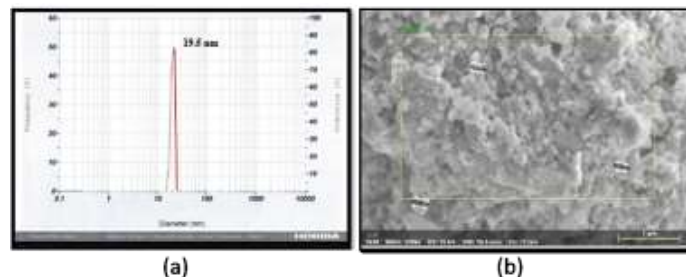


Figure 3. (a) Particle size of nCuO; (b) SEM image of nCuO

3.4 Nanofiber Polymer Composite

Nanofibers were fabricated using the modified electrospinning method established by PIMS-Nanoworks. The nanofibers produced from the electrospinning process were received by an Aluminum (Al) sheet as a ground collector resulted into randomly oriented fiber mats (Leach, et al., 2011).

Figure 4 shows the SEM images of the nanofibers produced. Treatment 1, 2 and 3 have no differences from each other in terms of morphology because of the reinforcements used such as cellulose acetate, and poly lactic acid with varying amounts used in the treatments as well as the antifungal agents such as the malunggay extracts combined with nano particles using the ratio of dichloromethane and methanol as solvents shows the resemblance of a web-like structures in the SEM image. In treatment 4, it was observed that the presence of small crystal-like and its web-like structure is evident. This is due to the addition of nCuO dissolved in chitosan. SEM morphology revealed an agglomerated crystalline structure that confirms nCuO exfoliated in the fiber matrix.

The particle size (nm) distributions of treatment 1 to 4 are shown in Figure 5. The mean size of the diameter of the nanofiber ranged from 0.20 μm (20 nm) to 0.90 μm (90 nm). Treatments 1 to 3 have a mean particle size near from each other which suggest that the three treatments have minimal effect to the size of nanofiber because they have the same reinforcements. On the other hand, treatment 4 was found to have a significant effect on the particle size of the fiber. This is may be due to the addition of nano copper oxide dissolved in chitosan. The mode size of the diameter of the nanofibers showed that the most frequent size ranged from 0.08 μm (8 nm) to 0.6 μm (60 nm) which confirms that the most fiber produced were in nanometer size which is 1-100 nm.

Figure 4. SEM image of the nanofibers produced

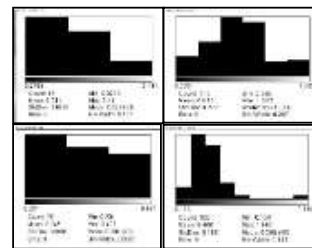


Figure 5. Histogram of particle size for treatment 1(a), treatment 2 (b), treatment 3(c), and treatment 4 (d)

As shown in Table 2, particle size analysis using 100 bins from ImageJ application reveals that T4 is significantly different and have the highest diameter size compared to all other treatments with which treatments 1 to 3 have the same reinforcements infused in the fiber although varying amount of other component such as PLA and CA, those 3 treatments are comparable and way far from the T4 which has the complete combination of the infused nanomaterials. The combination of different nanomaterials, such as nSiO₂ and nCuO as well as the malunggay extracts infused have increased the diameter size of the nanofibers.

Table 2. Particle size of the Nanofiber Composite

Treatments	Particle Size (diameter)
T ₁ – 50 % C ME with nSiO ₂	6.60 ^b
T ₂ – 75 % C ME with nSiO ₂	6.20 ^b
T ₃ – 100% C ME with nSiO ₂	6.20 ^b
T ₄ – 100% ME with nSiO ₂ and nCuO	27.40 ^a
Tukey's HSD value (1%)	2.42
cv %	15.54

Means followed by common letter are not significantly different at 1% level, HSD

C = Concentration; ME = Malunggay extract; nSiO₂ = Nano silica; nCuO = Nano copper oxide

3.5 In vitro analysis

Zone of Inhibition (ZOI) was highly significantly affected by the treatments T4, T7 (+) and T5, as shown in Table 3 had the highest inhibitory effect on the growth of black mold rot for 48 hours. A similar but slightly more pronounced trend was observed in 72 and 96 hours. The results in the in vitro analysis

were similar to the studies of El-Newehy et. al. (2012) and Qin et. al. (2017) where nanofiber produced from electrospinning had remarkable antifungal effect on black mold rot. Moreover, treatments with silica have better inhibitory capacity to black mold rot compared to treatments with no silica (negative control and T6 – 100% ME). Janatova et al. (2015), assert that silica from plant materials like rice husk have essential oils – thymol, eugenol and thymoquinone which have inhibitory properties against black mold rot.

Table 3. Inhibition of black mold rot as affected by different treatments observed in 48, 72, and 96 hours

Treatments	Zone of Inhibition (mm)		
	48h	72h	96h
T ₁ – 50 % C ME with nSiO ₂	4.46 ^b	4.40 ^b	4.56 ^b
T ₂ – 75 % C ME with nSiO ₂	5.17 ^b	5.12 ^b	4.94 ^b
T ₃ – 100% C ME with nSiO ₂	5.19 ^b	5.11 ^b	4.99 ^b
T ₄ – 100% C ME with nSiO ₂ and nCuO	14.96 ^a	15.33 ^a	15.44 ^a
T ₅ – 100% C ME and nCuO	12.27 ^a	12.28 ^{bc}	12.38 ^b
T ₆ – 100% C ME	2.29 ^{bc}	2.19 ^c	1.98 ^{bc}
T ₇₍₊₎ – Positive Control (Ny)	15.09 ^a	14.84 ^a	14.61 ^a
T ₈₍₋₎ – Negative Control (DW)	0.55 ^c	0.42 ^c	0.26 ^d
Tukey's HSD value (1%)	3.64	3.66	0.95
cv %	13.83	13.97	13.65

Means followed by common letter are not significantly different at 1% level, HSD

Means with the same letter are not significantly different at 1% level

ME = Malunggay extract; C = Concentration; nSiO₂ = Nano silica; nCuO = Nano copper oxide;

Ny = Nystatin; DW = Distilled water

Irrespective on the hour of inhibition, T₄ have the highest zone of inhibition. Combination capacity of nCuO dissolved in chitosan, nSiO₂ and 100 % concentration of malunggay extracts, enhances the overall antifungal capacity of T₄. Negative control (T₈), which is distilled water, and T₆ with malunggay extract alone had the lowest inhibitory effects, this is similar to the results of Busani et al. (2012) wherein both acetone and aqueous extracts with malunggay did not exhibit antifungal activity against black mold rot even at the highest concentration of 10 mg/ml. As compared to other microbial organism, black mold rot is one of the most difficult to control because it is an invasive fungus once penetrated (Sugita et al., 2004).

3.6 In vivo analysis

3.6.1 Weight Loss. Onion bulbs were weighed individually prior to storage experiments. Physiological weight loss is presented in Table 4 where treatments are highly significant different from each other. Onion after harvest still respire so it is expected to lose weight during storage (Kukanor, 2005). Regardless of storage type, negative control has the highest percentage weight loss. Onions stored at room temperature have lose weight more compared to those stored in cold condition as shown in Table 4. This is due to the uncontrolled temperature and relative humidity. After five days of storing the onion in cold and room temperature, it was weighed individually and physically inspected.

Table 4. Effect the different treatment on weight loss (%) of the onions

Treatments	Storage Type	
	Cold	Room
T ₁ – 50 % C ME with nSiO ₂	2.40 ^{abc}	1.91 ^b
T ₂ – 75 % C ME with nSiO ₂	2.035 ^b	2.12 ^b
T ₃ – 100% C ME with nSiO ₂	1.62 ^c	2.52 ^b
T ₄ – 100% C ME with nSiO ₂ and nCuO	0.99 ^c	1.89 ^b
T ₅₍₋₎ – Negative Control (DW)	3.32 ^a	4.32 ^a
T _{6 (+)} – Positive Control (Ch)	2.69 ^{ab}	1.92 ^b
Tukey's HSD value _(1%)	1.67	2.95
cv %	21.84	16.85

Means with the same letter are not significantly different at 1% level

ME = Malunggay extract; C = Concentration; nSiO₂ = Nano silica; nCuO = Nano copper oxide;

DW = Distilled water; Ch = Chlorothalonil

4.1.1 Disease severity index. Disease severity index (DSI) was significantly affected by the treatment combinations. Under cold storage, negative control has the highest disease index. This is expected since the onions were deep only in distilled water. Treatment 4 was the most effective among treatments applied. This is because of the combination of nanoparticles and high concentration of malunggay leaves extract that possess an antifungal property that can inhibit black mold rot.

Under room storage, positive control (T₆) have the lowest disease severity index as shown in Table 5. It is expected because a commercialized fungicide (Chlorothalonil) was used to act as positive control to compare to other treatments.

Table 5. Effect the different treatment on disease severity index of the onions

Treatments	Storage Type	
	Cold	Room
T ₁ – 50 % C ME with nSiO ₂	53.15 ^b	79.45 ^{ab}
T ₂ – 75 % C ME with nSiO ₂	52.85 ^b	74.11 ^b
T ₃ – 100% C ME with nSiO ₂	49.07 ^b	74.45 ^b
T ₄ – 100% C ME with nSiO ₂ and nCuO	44.19 ^c	68.38 ^b
T ₅₍₋₎ – Negative Control (DW)	74.67 ^a	80.67 ^a
T _{6 (+)} – Positive Control (Ch)	48.89 ^b	50.34 ^c
Tukey's HSD value _(1%)	5.38	5.69
cv %	3.63	2.88

Means with the same letter are not significantly different at 1% level

ME = Malunggay extract; C = Concentration; nSiO₂ = Nano silica; nCuO = Nano copper oxide; DW = Distilled water;

Ch = Chlorothalonil

4 Conclusion

Synthesis of cellulose acetate from abaca, and infusion of nSiO₂ and nCuO, electrospun into nanofiber composite were successful using the method established by PIMS-Nanoworks.

The electrospun nanofiber abaca polymer composite infused with 100% extract from malunggay leaves, nanosilica and copper oxide have the highest zone of inhibition under *in vitro* analysis against black mold rot (*A. niger*). Further assessment of these nanofiber composite in field conditions is needed to uncover their effectiveness at field level.

Acknowledgment

This study was ably conducted and completed through the auspices of the DOST-SEI under the Engineering Research and Development for technology (ERDT) scholarship grant and accepted for oral presentation during the 2018 Applied Nanoscience and Nanotechnology International Conference (ANNIC2018) last October 22-24, 2018, Berlin, Germany through the sponsorship of DOST-PCIEERD.

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