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Biofungicide potential of wood vinegar against anthracnose of bell pepper (*Capsicum anuum* L.) caused by *Colletotrichum* spp.

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ABSTRACT

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Bell pepper is an essential food ingredient used for home consumption, catering and in the food industry. However, bell pepper's shelf-life is reduced due to anthracnose caused by the fungus *Colletotrichum* spp. Control measures using wood vinegar as an alternative to commercial fungicides are explored to control bell pepper anthracnose. The study evaluated the efficacy of different concentrations of wood vinegar - 1%, 2%, 3%, 4% and, 5% against bell pepper anthracnose, laid out in a completely randomized design with triplicates. Spore germination assay and poisoned food technique revealed that 3% was the lowest concentration to completely inhibit the germination of conidia, while at 2%, the colony diameter and sporulation of *Colletotrichum* spp. was significantly reduced compared to the untreated control. In addition, treated fruits with 3% wood vinegar showed a significant decrease in disease incidence and lower disease severity ratings comparable with 0.0625% benomyl for both protective and eradicative treatments. The eradicative treatment was more effective than the protective treatment. Thus, wood vinegar was shown to have strong antifungal activity against *Colletotrichum* spp.

Keywords: Biofungicide, wood vinegar, anthracnose of bell pepper, *Colletotrichum* spp.

INTRODUCTION

Bell pepper (*Capsicum annuum* L.) is a solanaceous crop, and a year-round international vegetable used for home consumption, catering and in the food industry that is loaded with vitamins and minerals (Obidiebube et al 2012, Amarson

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2017). Bell pepper anthracnose caused by *Colletotrichum* spp. impose pre- and postharvest losses that can reach up to 50% and sometimes 80% (Pakdeevaraporn et al 2005, Mahasuk et al 2009).

The infected mature fruits have water-soaked, sunken lesions, reaching up to 4 cm in diameter, formed as dark concentric rings of fungal tissue (Boali 1991). In Brazil, *Colletotrichum acutatum, Colletotrichum gloeosporioides, Colletotrichum coccodes, Colletotrichum boninense,* and *Colletotrichum. capsici* were associated with anthracnose on bell pepper, where *C. acutatum* was the predominant species, accounting for more than 70% of cases (Alves et al 2015). In the Philippines, the disease incidence is reported to reach up to 80%, especially in Batangas, Cavite, La Union, Nueva Viscaya, and Laguna province (Opina 1993).

Chemical fungicides are being discouraged because of environmental and health threats, so researchers are eager to find alternative control measures, one of which is wood vinegar. Chuaboon et al (2016) revealed that wood vinegar controls phytopathogens such as *Bipolaris oryzae*, C*ercospora oryzae*, and *Alternaria padwickii*. It improves soil quality, and hastens the development of roots, stems, tubers, leaves, flowers, and fruits. According to Tiilikkala et al (2010) and, Theapparat et al (2018), the main organic components of wood vinegar with pesticide activity are methanol and acetic acid. Other components are acetone, methyl acetone, acetaldehyde, allyl alcohol, furan and furfural, formic, propionic, and butyric acids making the wood vinegar acidic that ranges from pH2–4. The fungicidal effect of the wood vinegar is poorly investigated against bell pepper anthracnose. Hence, this study was designed to (1) evaluate the efficacy of wood vinegar in controlling bell pepper anthracnose caused by *Colletotrichum* spp.; (2) know the lowest concentration of the wood vinegar for inhibiting pathogen growth in vitro; and (3) determine the in vivo efficacy of wood vinegar as a protectant and eradicant against anthracnose of bell pepper.

MATERIALS AND METHODS

Isolation and Mass Production of Colletotrichum spp.

Infected bell pepper fruits showing typical anthracnose symptoms were brought into the laboratory. The infected fruits were surface-sterilized, by dipping in a 1% sodium hypochlorite (NaOCl) for 15s. These were rinsed with three changes of sterile distilled water and blotted dry with sterile tissue paper. The advancing lesions were cut into sections (~5mm^2) and equidistantly placed on plated potato dextrose agar (PDA). After 72h incubation, the mycelial fragments were transferred to PDA slants to obtain pure cultures, maintained at room temperature. A pathogenicity test was done to verify the symptoms and causal organism and re-isolated to conduct the succeeding experiments.

Source of Woof Vinegar

The wood vinegar was obtained from Almeria Seafarer's Association in Almeria, Biliran, Philippines. This product is a common biopesticide used by the members of the said association. The raw materials for wood vinegar used in the study were a mixture of green residues, vegetables and trees present in the area

such as kakawate *(Gliricidia sepium)* and coconut. Wood vinegar is the by-product of the slow combustion of the raw materials to produce charcoal briquettes. According to Mela et al (2013) wood vinegar made from coconut has compounds such as phenol, 2 methoxy phenol, 2 methyl propyl ester butanoic acid, 2-lauro 1-3 dodecoin- C35H66O6, nitro 2 methyl 2 butane, 2, 6 dimethoxy phenol, 9-octadecenoic acid (Z)- tetradecyl ester (oleic acidtetradecyl ester)- C32H62O2, dodecanoid acid 1,2,3-propanetriyl ester (glyceryl tridodecanoate)- C39H74O6, octanoid acid 1,2,3, propanetriyl ester- C27H50O6, alkyl aril ether, and acetic acid. On the other hand, phytochemical examination of Kumar et al (2016) showed that the extracts of *G. sepium* contains glycosides, alkaloids, essential oils, sapponins, and flavonoids.

Experiment 1. In Vitro Assay Of The Efficacy Of Wood Vinegar Against Colletotrichum spp.

Spore germination assay

The spore germination assay was adopted from Frick (1964) with modifications. A drop of the prepared concentrations of wood vinegar, namely 1%, 2%, 3%, 4%, and 5% was placed into a marked area of a clean glass slide. This was added with conidia of *Colletotrichum* spp. that were scraped directly from the 2-week-old pure culture and incubated on a V-shaped glass rod inside sterile Petri dishes lined with moistened tissue paper. For the control check, a drop of sterile distilled water was mixed thoroughly with the conidial suspension. After 48h of incubation, fifty conidia on the slides were observed in the field of vision under low power magnification and the number of germinated spores was determined. The basis to check whether a spore had germinated was the length of the germ tube which was half of the width of the spore.

The computations were as follows:

1. Percent spore germination (% SG)

$$= \frac{\text{number of spores germinated}}{\text{50 spores observed}} x100$$

2. Percent inhibition of spore germination (% ISG)

$$= \frac{\text{\% germination of control} - \text{\% germination of treatment}}{\text{\% germination of control}} x100$$

Poisoned Food Technique

The different concentrations of wood vinegar were further evaluated for mycelial inhibition of the pathogen. The poisoned food technique was adopted by Kiran et al (2010) with modifications. Upon cooling down to 45°C, the melted PDA in each test tube was added with different concentrations of wood vinegar to obtain 10mL per tube. The resulting amended medium was poured on a sterile Petri dish

labeled with 1%, 2%, 3%, 4%, and 5% wood vinegar. PDA alone served as the control. Upon solidification, the amended medium was inoculated with five mm diameter mycelia agar disc that was taken from plated seven-day old pure culture of the pathogen. Each treatment was replicated three times and the plates incubated at room temperature (25°C). The data gathered were the colony diameter, percent inhibition of colony diameter (%ICD), and sporulation for each treatment. Ten (10) mL sterile distilled water was poured into treated and non-treated plates with *Colletotrichum* spp. A wire loop was used to dislodge the spores. Afterwards, a loopful of the spore suspension was obtained for determination of spore count using hemocytometer expressed as spores/mL.

Percent Inhibition of Colony Diameter (%ICD)

$$= \frac{\text{colony diameter of control } (mm) - \text{colony diameter of treated } (mm)}{\text{colony diameter of control } (mm)} x100$$

Experiment 2. In Vitro Assay Of The Efficacy Of Wood Vinegar Against Colletotrichum spp.

The three lowest concentrations of the wood vinegar, which had a significant fungicidal effect, and were not phytotoxic were further evaluated for protective and eradicative effects on detached bell pepper fruits following Godoy and Canteri (2004) with modifications. The phytotoxic effect of the wood vinegar on sweet pepper fruits was determined through visual examination of the fruit surface (eg, wrinkled surface, presence lesion on the fruit surface).

Protective Treatment

Mature green bell pepper fruits were surfaced-sterilized with 1% NaOCl then rinsed three times with sterile distilled water. After air-drying, experimental fruits were surface sprayed to wetness with 1%, 2%, and 3% concentrations of wood vinegar. The positive control was sprayed with 0.0625% benomyl solution. Bell pepper fruits were placed inside a cabinet to avoid rat infestation and air-dried for one day. The following day, treated fruits were evenly sprayed with 1mL of spore suspension of *Colletotrichum* spp. ($1x10^6$ spores mL^{-1}), held at ambient temperature (25°C) inside polyethylene bags, loosely packed, and the mouth of the bag was closed to allow incubation for one day. Disease severity was recorded daily through visual rating until seven days after inoculation using a rating scale adopted from Britos (2001) see Figure 1 below.

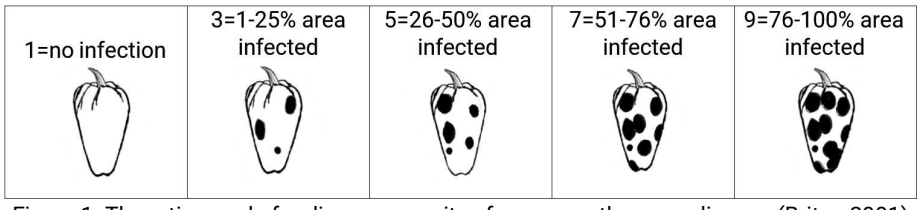


Figure 1. The rating scale for disease severity of pepper anthracose disease (Britos 2001)

The average disease severity rating was determined using the following formula:

$$\text{Average DS} = \frac{\text{Z (no. of fruits with a rating of (1,3,5,7,9) x rating (1,3,5,7,9)}}{\text{Total no. of fruits per replication}}$$

Number of days to appearance of symptoms and its degree of progression were also determined. Likewise, disease incidence was computed based on the formula:

$$\text{Disease Incidence} = \frac{\text{no. of fruits showing anthracnose symptoms}}{\text{total no. of fruits observed}} x100$$

Eradicative Treatment

The surfaced-sterilized bell pepper fruits were sprayed using a hand sprayer with 1mL spore suspension of *Colletotrichum* spp. that was adjusted to $1x10^6$ spores/mL and incubated for one day. The prepared set-up was placed inside a cabinet to avoid rat infestation. On the following day, inoculated fruits were sprayed with 1%, 2%, and 3% concentrations of wood vinegar. The same data were gathered as in the protective treatment.

Experimental Design and Data Analysis

The experiments were conducted in a Completely Randomized Design (CRD) with three replications per treatment, in vitro and in vivo, with ten pepper fruits per replication. All data from the experiments were analyzed, and means were compared by Tukey's Honest Significant Difference (HSD) test at α=0.01.

RESULTS AND DISCUSSION

Effect of Different Concentrations of Wood Vinegar on the Spore Germination of Colletotrichum spp.

The initial response of *Colletotrichum* spp. to different concentrations of wood vinegar was evaluated using the spore germination assay in which the presence of germ tubes was recorded (Table 1). All concentrations had a significant inhibitory effect on the germtube formation of *Colletotrichum* spp. (*p*-value <0.01). After 48h, the treatments with 3%, 4%, and 5% concentration completely hindered the spore germination of *Colletotrichum* spp. whereas treatment with 1% and 2% wood vinegar reduced spore germination to 83.45% and 88.10% respectively compared to the untreated control which had 95.33% germination. Furthermore, results showed that the higher the concentration level of wood vinegar, the shorter were the germ tubes and lesser number of appressoria observed compared to the control. On the other hand, the ungerminated conidia treated with 3%, 4%, and 5% wood vinegar exhibited deformed shapes showing shrinkage of the conidia, which could be attributed to the low pH of the wood vinegar (Figure 2).

Table 1. Spore germination response of *Colletotrichum* spp. as affected by different concentrations of wood vinegar after 48h incubation under laboratory conditions

Wood Vinegar Concentration[1]	No. of Germinated Spores	%SG[2]	%ISG[3]
Distilled water (Control)	46.67a	93.34a	0.00d
1%	8.00b	16.00b	82.88c
2%	5.70b	11.40c	87.86b
3%	0.00c	0.00d	100.00a
4%	0.00c	0.00d	100.00a
5%	0.00c	0.00d	100.00a
CV (%)	10.82	1.71	0.55

[1]average of 3 replicates. Means followed by the same letter are not significantly different at α=0.01
[2]percent spore germination
[3]percent inhibition of spore germination

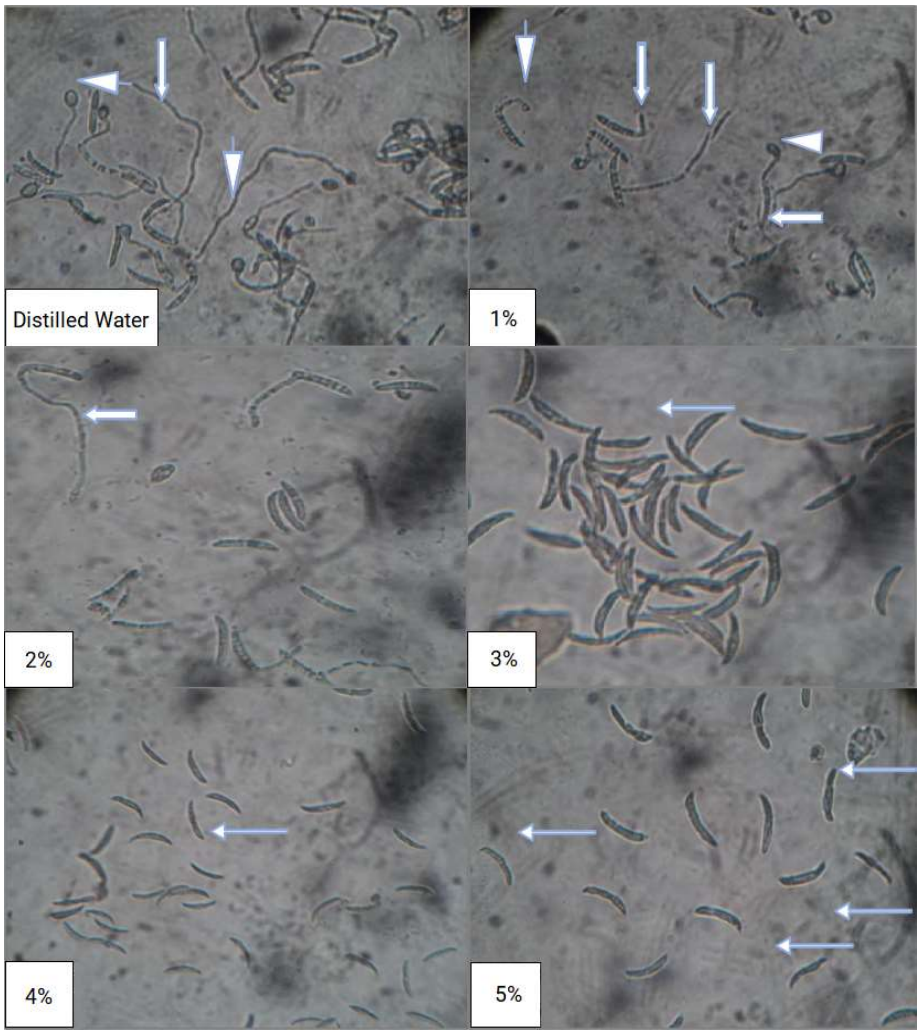


Figure 2. *Colletotrichum* spp. spores exposed to different wood vinegar treatments (400x) namely: 0%, 1 %,2%, 3%, 4%, and 5%; Note: reduced germ tube and appressorium development of the treated spores

According to Ibrahim et al (2013), the dichloromethane extracts of concentrated wood vinegar between 3.13 to 6.25mg mL^{-1} inhibited the morphogenesis of yeast cells, probably altering the cell membrane or plasmalemma structure and the permeability of the cells. This mechanism resulted in shrinkage of the cells, followed by accelerated pores or cavity formations and unusual cell morphogenesis.

Effect of the Different Concentrations of Wood Vinegar on the Mycelial Growth and Sporulation of Colletotrichum spp.

The mycelial growth of *Colletotrichum* spp. to different concentrations of wood vinegar was evaluated using the poisoned food technique (Table 2). The mycelial development of *Colletotrichum* spp. was completely inhibited on PDA plates amended with 2%, 3%, 4%, and 5% at seven days of incubation. The mycelial agar discs grown in wood vinegar concentrations quickly changed in color from a whitish appearance to brownish orange where the effect was dose dependent (Figure 3). For example, at 1% concentration, the growth of *Colletotrichum* spp. was visible on the 2nd day as whitish mycelia and later progressed at a slower rate with darker pigment compared to the control. The abnormal growth was evident on the third day. It could be attributed to the acidic nature of the wood vinegar that reacted with the mycelial disc being planted, resulting in growth retardation of the mycelia (Theapparat et al 2018). In the study of Oramahi et al (2018), Higher concentrations of total phenol and total acid were shown to have the highest antifungal activity of wood vinegar against *Trametes versicolor*. Also, *Colletotrichum* spp.- treated plates with 1% wood vinegar had a significantly smaller colony diameter (30.67mm) than the control (75.5mm), resulting in 59.28% inhibition of mycelial growth. The results of this experiment conformed with that of the studies made by Numata et al (1994) in which they reported a complete inhibition of many tested fungi at 3-4% wood vinegar on amended PDA.

Table 2. Growth response of *Colletotrichum* spp. in PDA plates amended with the different concentrations of wood vinegar 7 days of incubation

Wood vinegar concentration[1]	Colony Diameter (mm)	% ICD[2]	Sporulation (spores/mL)
distilled water	76.0a	0.0c	$1.48x10^{7}$a
1%	31.0b	59.0b	$4.17x10^{5}$b
2%	0.0c	100.0a	0.00c
3%	0.0c	100.0a	0.00c
4%	0.0c	100.0a	0.00c
5%	0.0c	100.0a	0.00c
CV (%)	3.71	1.13	21.31

[1]average of 3 replicates. Means followed by the same letter are not significantly different at α=0.01
[2] Percent Inhibition of Colony Diameter

The sporulation of *Colletotrichum* spp. significantly declined in the treated plates with the increasing concentration of wood vinegar. For instance, the sporulation was drastically decreased when *Colletotrichum* spp. was grown on PDA amended with a 1% concentration ($4.17x10^{5}$) compared to the control ($1.48x10^{7}$). No sporulation was observed at 2%, 3%, 4% and 5% concentration.

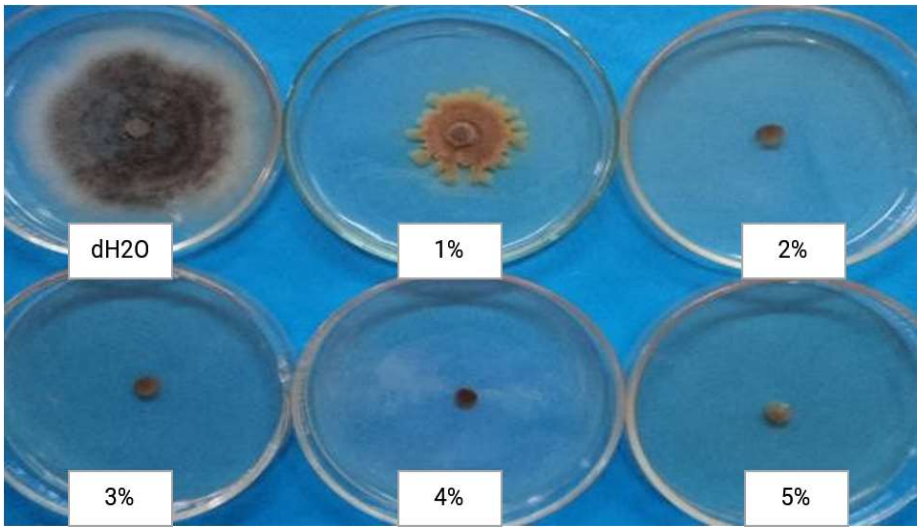
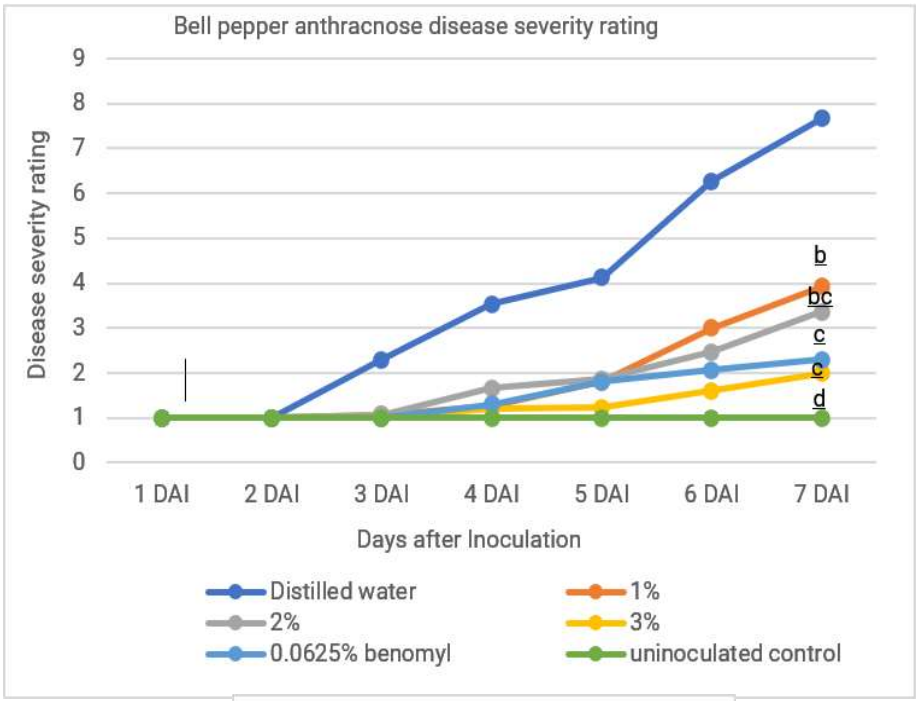


Figure 3. Mycelial growth of *Colletotrichum* sp. in PDA amended with varying concentrations of wood vinegar after seven days incubation.

Based on the two in vitro assays, 2% wood vinegar was the minimum concentration that caused 100% inhibition in the poisoned food technique, while in the sporegermination assay, the minimum 100% inhibitory concentration was at 3%. It implied that the mycelia were more sensitive and vulnerable than the spores to the effect of wood vinegar. The spores were the common source of inoculum to start the infection process, and these can be controlled using the 3% wood vinegar as post-harvest treatment against pepper anthracnose in consonance with the in vitro assays. To check the phytotoxicity of the compound to the fruits, lower concentrations at 1% and 2% wood vinegar were also tested in the succeeding in vivo experiments.

Protective Effect of Wood Vinegar Against Anthracnose Disease on Detached Bell Pepper Fruits

The disease progression of bell pepper anthracnose as affected by the increasing concentrations of wood vinegar was severely affected compared to the negative control (Figure 4). For instance, one, two and three percent wood vinegar significantly delayed the onset of symptom appearance compared to the control (Figure 4). The symptoms started to appear as tiny sunken whitish spots on the fruit surface. They later enlarged into blackish circular sunken spots with black acervuli in concentric rings in the advanced stage (especially the control). The initial symptoms of *Colletotrichum* spp. infection appeared 3 days after inoculation for 1% and 2% wood vinegar, while 3% wood vinegar and 0.0625% benomyl were observed after 4 days of inoculation, which had significantly lower disease severity ratings relative to the control. Moreover, 1% was comparable with 2% wood vinegar yet lower efficacy compared to the 3% and 0.0625% benomyl. The uninoculated and untreated control did not show typical symptoms of *Colletotrichum* spp. infection up to the seventh day, which indicated the absence of the latent infection.



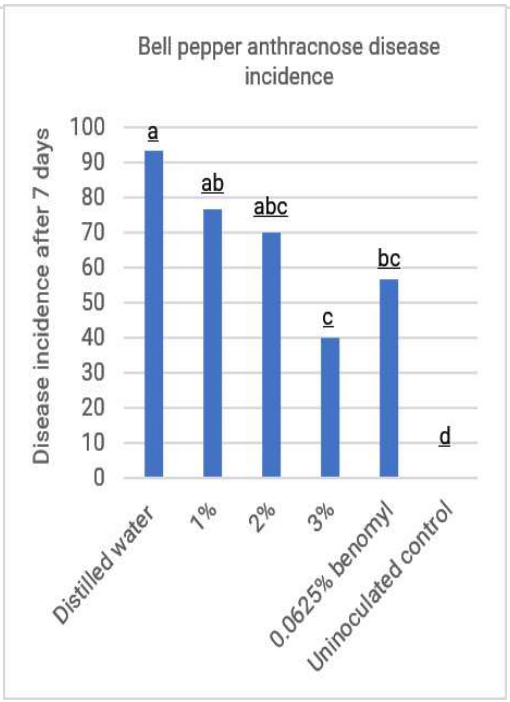


Figure 4. Daily anthracnose disease severity rating and disease incidence of bell pepper fruits as affected by protective treatment of wood vinegar concentrations at 7 days of incubation

Additionally, the progression of symptoms in the inoculated control fruits drastically increased from the first appearance of symptoms until seven days of incubation with a disease severity rating of 7.67. There was significant difference between treatments in fruits that were inoculated after wood vinegar application. Statistical analysis revealed that all the treatments significantly reduced the penetration and colonization of the *Colletotrichum* spp. that were observed with fewer sunken lesions compared to the control. Based on the parameter, the highest fungicidal effect was observed in bell pepper fruits treated with 3% concentration with 40% disease incidence and disease severity index of 2.0 after seven days of inoculation which was better than 0.0625% benomyl which had 56.67% disease incidence and disease severity index of 2.3 after seven days of incubation.

Likewise, the disease incidence was lowest at 3% wood vinegar, yet it was comparable with the disease incidence in 0.0625% benomyl yet significantly different from the control (Figure 4). Disease incidence appeared to be high because this reflected the number of fruits that showed visible symptoms regardless of the size and number of lesions present on the fruit surface. Results showed that although disease incidence was lower in fruits treated with 1% and 2% wood vinegar, the values (76.67% and 70.0%, respectively) were statistically comparable with that of the untreated fruits (93.33%). In terms of disease severity rating on the seventh day, however, the wood vinegar concentrations significantly provided better protection from anthracnose compared to the untreated fruits, i.e., 3.93 for 1% wood vinegar, 3.36 for 2% wood vinegar compared to 7.67 for the control. Results also revealed that the commercial fungicide benomyl at 0.0625% was better than the 1% and 2% wood vinegar in terms of protection of the treated fruits from anthracnose but was inferior to 3% wood vinegar which also gave slow progression of symptoms (Figure 5). Both benomyl and 3% wood vinegar, however, displayed shrinkage of the treated fruits which indicates slight phytotoxicity. Nevertheless, it is apparent that wood vinegar showed promising potential in protecting pepper fruits from anthracnose infection for up to seven days shelf life. However, phytotoxicity test is needed to determine the safe and acceptable concentration.

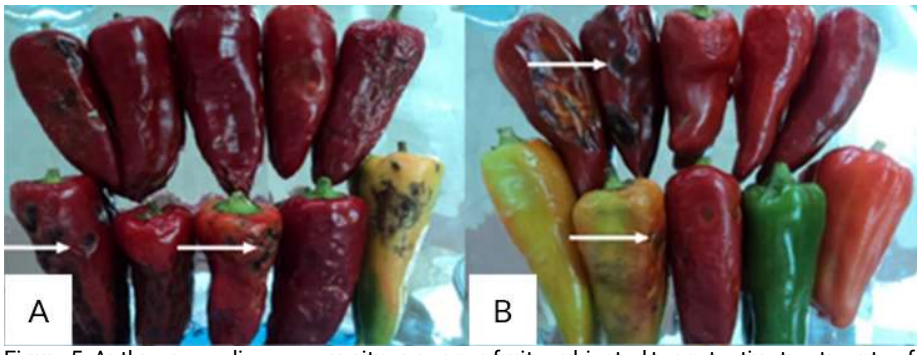


Figure 5. Anthracnose disease severity on pepper fruits subjected to protective treatments of wood vinegar seven days after inoculation: A) control, B) 1%, C) 2%, D) 3%, E) 0.0625% benomyl and F) Uninoculated control

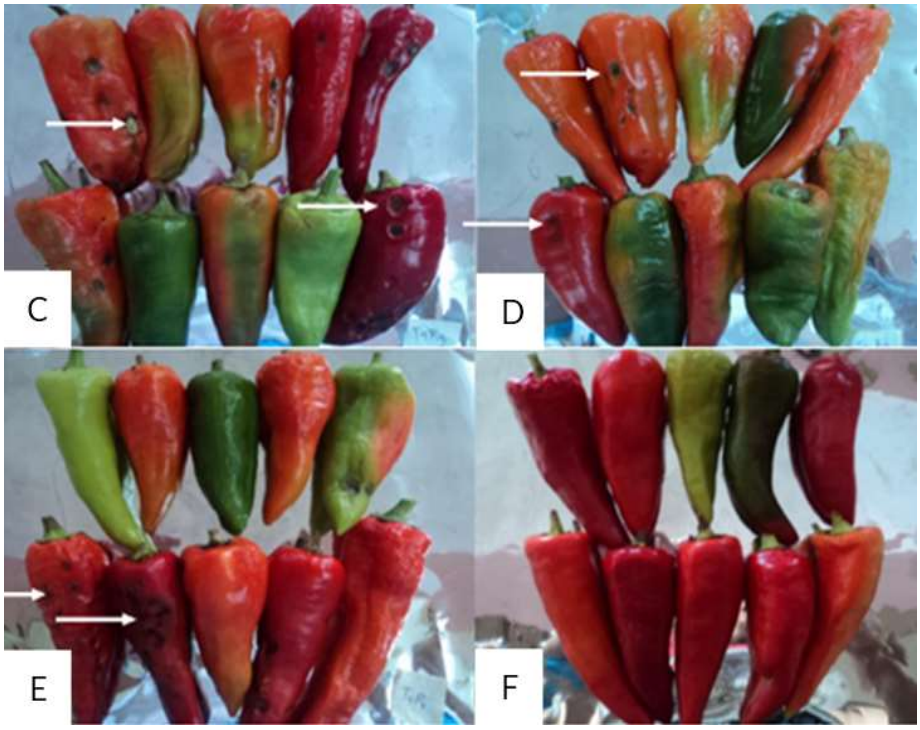


Figure 5.continued

Eradicative Effect of Wood Vinegar Against Anthracnose Disease on Detached Bell Pepper Fruits

Based on the disease severity and incidence data, all concentrations of wood vinegar displayed eradicative efficacy against bell pepper anthracnose compared to the control (Figure 6). Three days after inoculation, initial symptoms were already visible on the control treatments as well as on the fruits treated with 1%, and 2% wood vinegar, but less than the control. On the other hand, 3% wood vinegar and 0.0625% benomyl-treated fruits started to develop initial symptoms four days after inoculation but displayed fewer and smaller lesions. The delayed rate of disease progression for all treatments with wood vinegar may be due to the inhibition of spore germination and the reduction of the vegetative growth of the pathogen resulting in the late establishment, colonization, and appearance of symptoms. It is evident that wood vinegar and 0.0625% benomyl significantly reduced colonization of *Colletotrichum* spp. compared to the inoculated control.

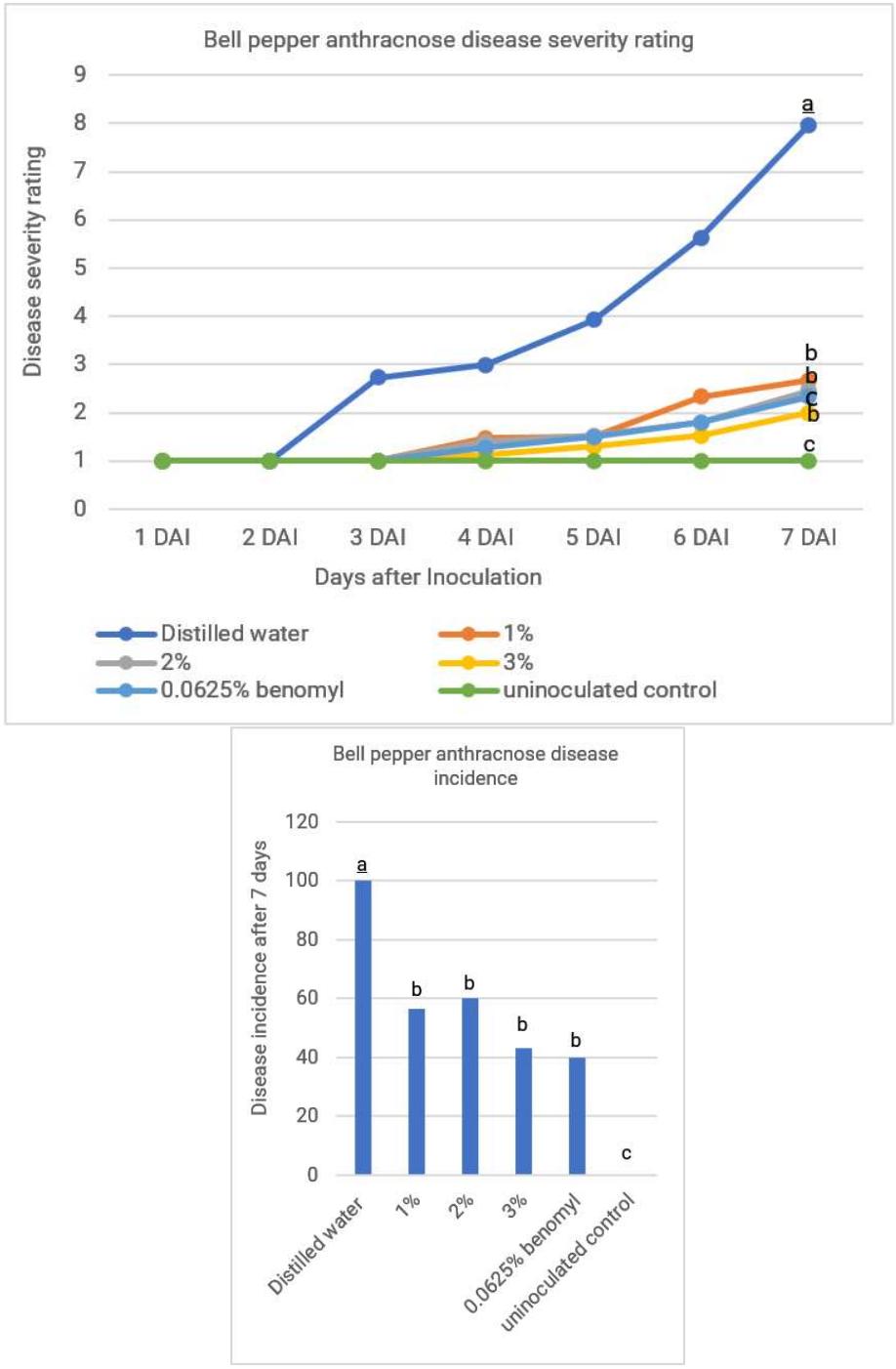


Figure 6. Daily anthracnose disease severity rating and disease incidence of bell pepper fruits as affected by eradicative treatment of wood vinegar concentrations at 7 days of incubation

All wood vinegar concentrations used as eradicant were statistically as effective to control anthracnose infection as 0.0625% benomyl in terms of assessing the disease severity and disease incidence (Figure 7). In addition, disease severity rating taken after 7 days revealed that 3% wood vinegar showed the lowest disease severity rating of 2.0 and 43.33% infection and was comparable to 0.0625% benomyl with a severity rating of 2.33 and 40%, respectively. Both treatments, however, showed shrinkage of the treated fruit, which is a sign of phytotoxicity. For the disease incidence, the lowest occurrence of the disease was observed in the bell pepper fruits applied with 0.0625% benomyl yet it was not statistically different than 1%, 2% and 3% wood vinegar. The result implied that 1% wood vinegar was already effective in delaying the symptom development and can be used as an alternative to chemical fungicides. Results also revealed that the disease incidence for all treatments were significantly lower than the negative control. The group of compounds present in wood vinegar was likely responsible for fungitoxicity against *Colletotrichum* spp. According to Tiilikkala et al (2010), the main organic components of wood vinegar with pesticide activity are methanol and acetic acid. Other components are acetone, methyl acetone, acetaldehyde, allyl alcohol, furan and furfural, formic, propionic, and butyric acids. Velmurugan et al (2008) also stated that the main inhibitory compound present in wood vinegar effective against fungi are the phenolic compounds. These studies may explain the mechanism of the slower progression of symptoms of the treated fruits compared to the inoculated control.

Based on the data at seven days after inoculation, the selected wood vinegar concentrations were more efficient when applied as eradicative treatments to control the progression of anthracnose symptoms compared to protective treatments. It was evident in both experiments that 3% wood vinegar afforded inhibitory effect, as shown by the least disease severity rating (2.0) in protective and eradicative treatments. Moreover, in terms of disease incidence, 3% of wood vinegar gave 40% and 43.33% infection in fruits, respectively. Benomyl gave a disease incidence of 56.67% and 40% for protective and eradicative treatments, respectively, which were comparable to those of 3% wood vinegar.

Figure 7. Anthracnose disease severity on pepper fruits subjected to eradicative treatments of wood vinegar at seven days after inoculation A) control, B) 1%, C) 2%, D) 3%, E) 0.0625% benomyl and F) Uninoculated control Note: reduced disease severity of symptoms of the treated bell pepper fruits as affected by varying wood vinegar concentrations.

Figure 7. continued

Wood vinegar applied as a protective treatment did not completely protect the fruits from anthracnose. This may be due to the volatile compounds present in wood vinegar not giving lasting protection against upcoming inoculum. Volatile compounds such as methanol, acetone, and some volatile acids of wood vinegar may have decreased in concentration prior to inoculation, hence, the reduced protective effect. According to Loo et al (2007), wood vinegar contains many volatile acids (8-10%) which contribute to its acidity of pH 2-3.

It was observed that the fruits treated with varying concentrations of wood vinegar in both protective and eradicative treatments had improved shelf life compared to the control seven days after inoculation. Most of the treated fruits had at least maintained their green color and exhibited a desirable appearance, especially at 3% wood vinegar compared to the control. According to Ohira (2012), this may be due to the antioxidant property of wood vinegar that prevents lipid oxidation through the action of the phenolic compounds.

CONCLUSION

Wood vinegar had significant antifungal activity against *Colletotrichum* spp. The lowest inhibitory concentration of wood vinegar effective against spore germination and mycelial growth of *Colletotrichum* spp. were 2% and 3%, respectively. Wood vinegar at 3% concentration was effective against *Colletotrichum* spp. when applied as protective and eradicative treatments and comparable with 0.0625% benomyl. Regardless of concentration, wood vinegar effectively reduced anthracnose disease severity, disease incidence, and development of symptoms on treated fruits when applied as eradicative treatments. Further study on phytotoxicity and chemical analysis of wood vinegar from different sources is recommended.

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