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Article

Detection and Control of Spoilage Fungi in Refrigerated Vegetables and Fruits

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SUMMARY

Post-harvest infection of fruit and vegetable products is an ever-growing issue in the food industry. About 20% of all produced fruits and vegetables are lost each year due to spoilage. Some sources of spoilage are mycotoxin-producing fungi, which can cause serious health threats in humans, from acute poisoning to long-term effects such as cancer. Therefore, using preservatives to avoid contamination and the eventual production of mycotoxins in our food is essential. However, synthetic preservatives appear to be harmful to human health and the environment, making it favorable to replace them with natural and safer preservatives. Using the essential oils of aromatic plants as preservatives has been found to help prevent the deterioration of food. This work aimed to isolate and identify some of the filamentous fungi that contaminate refrigerated fruits and vegetables. We studied the effect of eugenol essential oil and its derivative, acetyl isoeugenol, at three different concentrations and temperatures on the fungal growth of two isolated fungal species. Our experiments revealed that eugenol was the better choice as an antifungal agent against the two tested fungi: Cladosporium cladosporioides and Penicillium italicum. The inhibition effect of both tested oils against fungal growth was concentration dependent, independent of any changes in temperature. However, further work on eugenol derivatives and their effects on both fungi and vegetables and fruits is still needed.

INTRODUCTION

Fruits and vegetables are an important source of nutrition for humans and animals as they contain vitamins, fats, and oils necessary for healthy growth and development. However, fruits and vegetables have a high-water content and pH hospitable for microorganismal growth, which can be harmful to humans and animals alike. Psychrotrophic fungi, a consistent problem for various fruit and vegetable species, are a group of molds that can survive in cold temperatures. They can change the chemical composition of the fruit or vegetable, making them toxic for consumption. Such changes are normally discovered in the form of taste, smell, appearance, and texture (1-3). Some examples of psychrotrophic fungi genera are *Penicillium*, *Alternaria*, and *Cladosporium*. Many of these molds can secrete mycotoxins and other harmful compounds.

Mycotoxins include a wide range of toxic secondary metabolites produced during fungal processes. Some mycotoxins, like patulin and ochratoxin A, are known to cause swelling in blood vessels and tissues and can also be carcinogenic when consumed in large quantities. These toxins can be teratogenic and have been observed to link between DNA molecules, which can interfere with the development of a fetus and cause birth defects in humans (4-7). As a result, various groups such as the World Health Organization (WHO) and the Food and Drug Administration (FDA) have placed restrictions on the amount of toxins that can be consumed or found in food products (8). In hopes of lowering levels of mycotoxins found in our food, researchers have looked at ecological factors that facilitate the growth of mycotoxin-producing fungi, including temperature, humidity, and the level of gases in the air (9).

To further understand the growth and spread of psychrotrophic fungi, the capacity for various fruits and vegetables to harbor fungal growth has been studied. For example, a group of fungi were isolated from mulberries stored in refrigerators, where temperatures are ideal for the growth of psychrotrophic fungi such as *Alternaria alternata*, *Aspergillus tenuissima*, *Phoma herbarum*, *Penicillium expansum*, and *Trichoderma atroviride* (10). In other fruits and vegetables, fungi of the genera *Penicillium*, *Monilinia*, *Cladosporium*, *Aspergillus*, *Rhizopus*, and *Fusarium* have been recovered (11).

In light of previous findings, it is clear that psychrotrophic fungi are a serious problem as they infest fruits and vegetables. Synthetic fungicides were found to reduce fungal growth and mycotoxin production. However, scientists found that using improper amounts of these fungicides led to increasing of the mycotoxin production. In addition, some fungal species have developed a resistance to these fungicides. Therefore, they suggested using other natural alternatives such as essential oils to help with these issues (12).

There is a high demand for using food preservatives as antimicrobial agents to prevent food spoilage. Artificial food additives like potassium sorbates and sodium benzoates have been used for a long time. However, some of these additives can cause unpleasant side effects such as skin rashes, breathing difficulty, and gastrointestinal upsets (13). Therefore, the use of natural preservatives has garnered interest. Eugenol ($C_{10}H_{12}O_2$) is a natural compound found in various essential oils, especially clove essential oil where it makes up roughly 70-90% of the entire oil composition (14). Eugenol has various commercial uses. It is used widely as a flavoring for foods and as an herbal oil used topically to treat toothache and to be taken orally to treat gastrointestinal and

respiratory complaints. However, ingestions of high doses of eugenol can cause severe liver injury (15). Interestingly, the oil's vapors were also seen to have fungistatic activity; when absent, fungi would continue to grow again (16). In one study, clove essential oil was fractionated through column chromatography, and only those fractions containing mainly eugenol actively worked against fungi (17). Currently, there are 17 known eugenol analogs, e.g., acetyl isoeugenol ($C_{12}H_{14}O_3$), isoeugenol, and safrole. Each derivative has a slightly different structure, which may have a different effect on fungi (18).

The information provided in the previous background explains how important it is to prevent fungal contamination to ensure a healthy food delivered to the consumers. Besides, there is a high demand to use natural compounds rather than using synthetic ones. Therefore, in our research, we studied the effect of eugenol and its analog, acetyl isoeugenol, on the growth of psychrotrophic fungi isolated from fruits and vegetables. We isolated and identified psychrotrophic fungi and treated them with eugenol and its derivative to suppress fungal growth. We identified eleven different fungal species out of the fourteen fruit and vegetable samples that we collected. Two species were selected to further study the effect of eugenol and acetyl isoeugenol on fungal growth as well as the impact of temperature differences. We concluded that eugenol had a better inhibitory effect than its derivative against the tested fungal species *Cladosporium cladosporioides* and *Penicillium italicum*. Eugenol completely inhibited the fungal growth at a concentration as low as 250` ppm.

RESULTS

We started our investigation by isolating fungi from fourteen different refrigerated fruits and vegetables that showed a decay. After molecular identification, we eventually chose two species to further study the essential oil effect

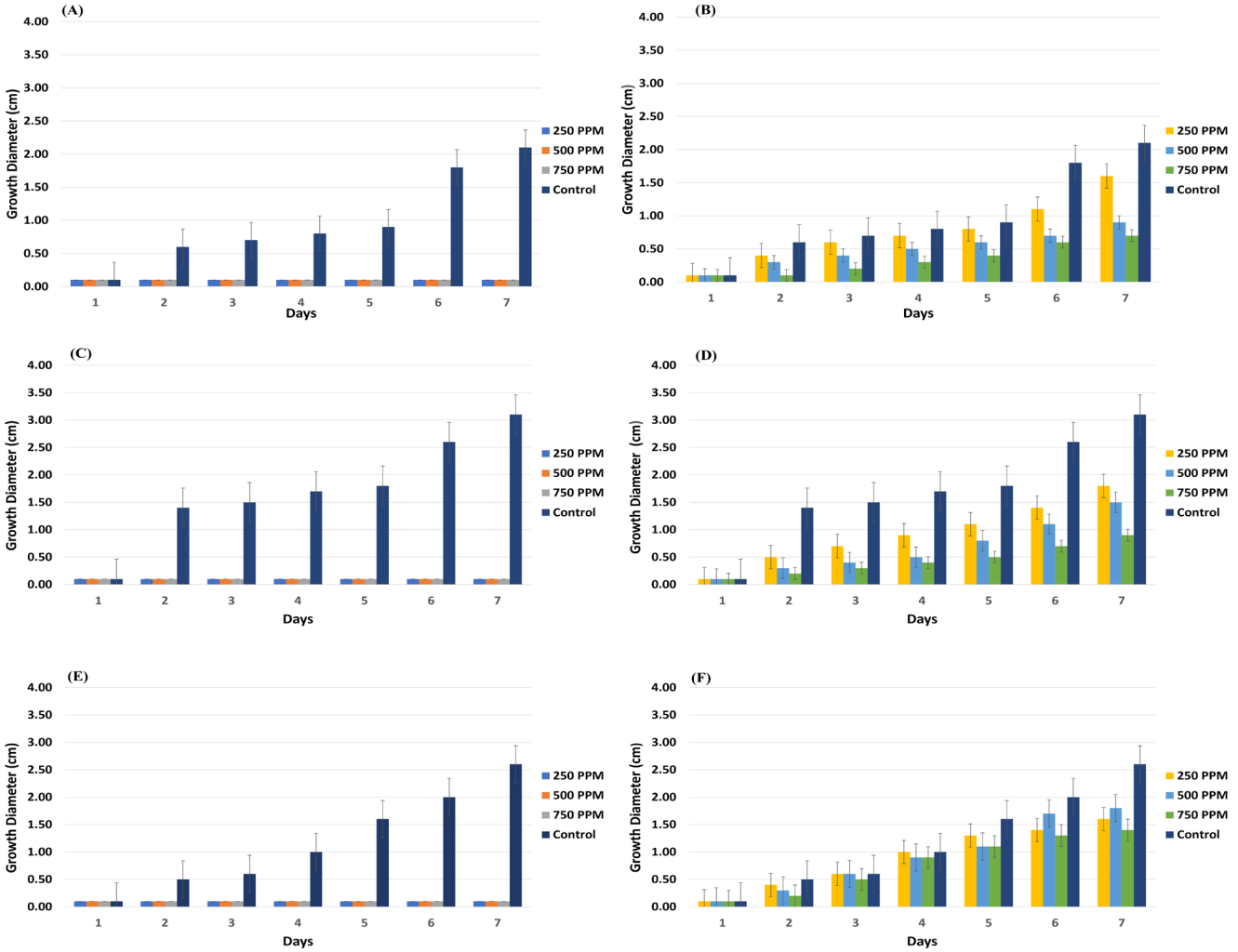

Figure 1. The daily growth of *P. italicum*. The plates were incubated with either (**A**) eugenol or (**B**) acetyl isoeugenol at 15 oC, either (**C**) eugenol or (**D**) acetyl isoeugenol at 20 oC, either (**E**) eugenol or (**F**) acetyl isoeugenol at 25 oC, compared with 0 ppm of either, as a control for 7 days. Legend (top to bottom) for subgraphs A, C, and E: eugenol concentrations of 250 ppm, 500 ppm, 750 ppm and control plates (no oil). For subgraphs **B**, **D**, and **F**: acetyl isoeugenol concentrations of 250 ppm, 500 ppm, 750 ppm and control plates (no oil). The data are expressed as the mean ± SEM (n = 3).

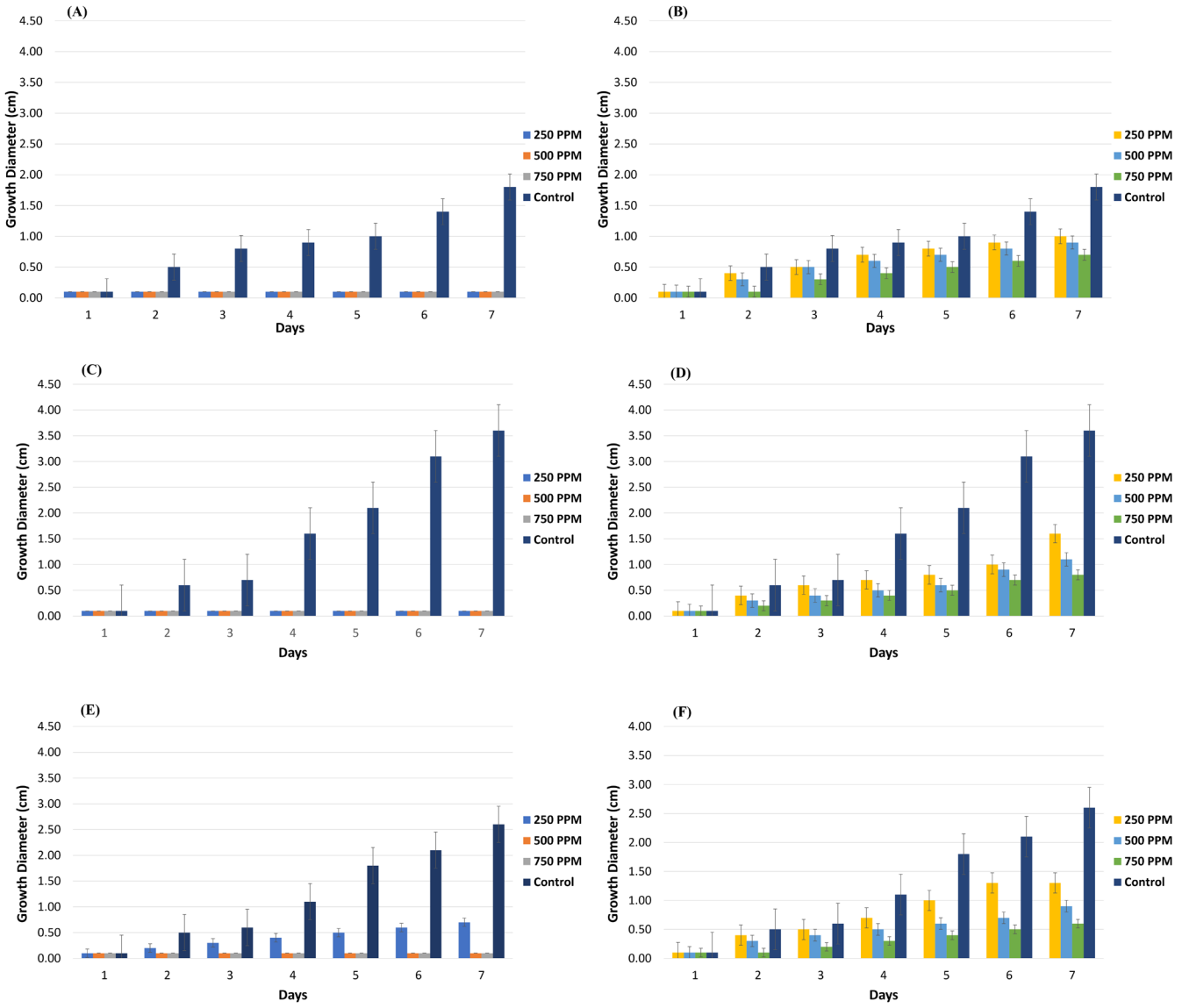

Figure 2. The daily growth of *C. cladosporioides*. The plates were incubated with either (**A**) eugenol or (**B**) acetyl isoeugenol at 15°C, either (**C**) eugenol or (**D**) acetyl isoeugenol at 20 oC, either (**E**) eugenol or (**F**) acetyl isoeugenol at 25°C, compared with 0 ppm of either, as a control for 7 days. Legend (top to bottom) for subgraphs **A**, **C**, and **E**: eugenol concentrations of 250 ppm, 500 ppm, 750 ppm and control plates (no oil). For subgraphs B, D, and F: acetyl isoeugenol concentrations of 250 ppm, 500 ppm, 750 ppm and control plates (no oil). The data are expressed as the mean ± SEM (n = 3).

as well as the temperature effect on their growth. Nineteen fungal isolates were selected and cultured on Czapex-Dox agar medium. Using molecular and morphological analyses, we were able to identify 11 different fungal isolates (**Table 1**).

The antifungal activity of eugenol and acetyl isoeugenol was tested on two of the isolated fungal species, *Penicillium italicum* and *Cladosporium cladosporioides*. Those two species were selected because they are known as psychrotrophic fungi and produce harmful mycotoxins at low temperature. We used the radial growth method to test the daily growth of each fungus at 3 different temperatures (15°C, 20°C, and 25°C) and in the presence or absence (control) of the essential oils at three different concentrations (250, 500, and 750 ppm). The diameter of the fungal disc was measured daily to observe the fungal growth for 7 days. The growth diameter of the control plates of both *Cladosporium cladosporioides* and *Penicillium italicum* was best at 20°C, suggesting that these fungi are psychotrophs (**Figure 1C, D**; **Figure 2 C, D**). All concentrations of both essential oils slowed down the growth of the fungal mycelia. Overall, eugenol appeared to be more effective against both fungal species than acetyl isoeugenol. Eugenol inhibited 100% of growth at all concentrations and temperatures for both species, except for *Cladosporium cladosporioides* at a concentration of 250 ppm and a temperature of 25°C (**Figures 3, 4**).

For *P. italicum*, acetyl isoeugenol was not as effective as the eugenol, but it did prevent the fungal growth by an average of 34.7%, 46.5%, and 61.3% at 250 ppm, 500 ppm, and

Fungal Species	Fruit/Vegetable
Alternaria alternata	Cucumber, Eggplant
Botrytis cinerea	Strawberry
Cladosporium cladosporioides	Blueberry, Raspberry, Strawberry
Colletotrichum gloeosporioides	Mango
Mucor racemosus	Cantaloupe, Peach, Strawberry
Myrothecium roridum	Bottle Gourd
Penicillium citrinum	Bitter Gourd, Blueberry
Penicillium digitatum	Orange
Penicillium expansum	Eggplant
Penicillium italicum	Bell Pepper, Lemon, Orange
Penicillium olsonii	Tomato

Table 1. Fungal species and their vegetables and fruits samples.

750 ppm, respectively, at all tested temperatures (**Figure 3B**). In comparison, the acetyl isoeugenol showed a better inhibition rate for *C. cladosporioides* (**Figure 4B**). The growth was inhibited by an average of 49.8%, 61.6%, and 71.9% at 250 ppm, 500 ppm, and 750 ppm, respectively, at all temperatures. Moreover, we noticed the strongest inhibition rate at 750 ppm acetyl isoeugenol and 20°C with 77.8% inhibition for *C. cladosporioides* (**Figure 4B**) and 71.1% inhibition for *P. Italicum* (**Figure 3B**). In general, the inhibition percentages of both fungal species in the presence of acetyl isoeugenol were higher at 20°C (**Figures 3, 4**). The fungal growth was inhibited at 750 ppm at 20°C by 77.8% and 71% for Cladosporium Cladosporioides and Penicillium italicum, respectively (**Figures 3, 4**).

DISCUSSION

Spoilage of fruits and vegetables are a common problem throughout the world since about 20% of the produced fruits and vegetables are lost each year. It is very important to find ways to decrease such food waste. To do that, we need first to detect the microorganisms that cause food spoilage. Then we propose deterring plans to combat the spoilage, using natural preservatives.

In our research, we detected 11 different fungal species that were able to grow on vegetables and fruits in our refrigerators. *Penicillium* was the dominant genus in our isolates. Eight out of the nineteen isolates were of the *Penicillium spp*. This is consistent with previous work which reported that *Penicillium* was among the most frequently occurring genera, along with *Aspergillus*, *Rhizopus*, *Alternaria*, and *Cladosporium* (22-26).

We isolated *Penicillium italicum* and *Penicillium digitatum*, which are known as blue and green molds, respectively. These *Penicillium spp*. are the most common postharvest pathogens in citrus fruits which is consistent with our finding (25). We isolated P*enicillium italicum* from lemon, bell pepper, and orange samples, while *Penicillium digitatum* was isolated from only orange samples. However, *P. italicum* is also among the most common fungi that affect fresh vegetables and other foods worldwide (28). Sema *et al.* identified *Penicillium italicum* as 12.6 % of all their isolated fungi from

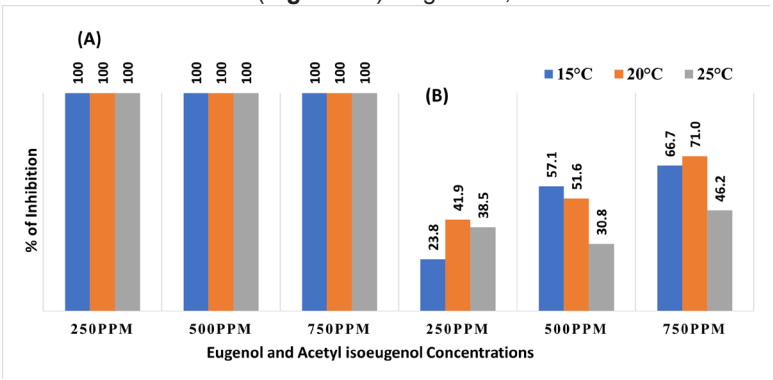

Figure 3. Percent inhibition of *P. italicum*. The fungus exposed to (**A**) 250, 500, and 750 ppm of eugenol at 15°C, 20°C, and 25°C and (**B**) 250, 500, and 750 ppm of acetyl isoeugenol at 15°C, 20°C, and 25°C.

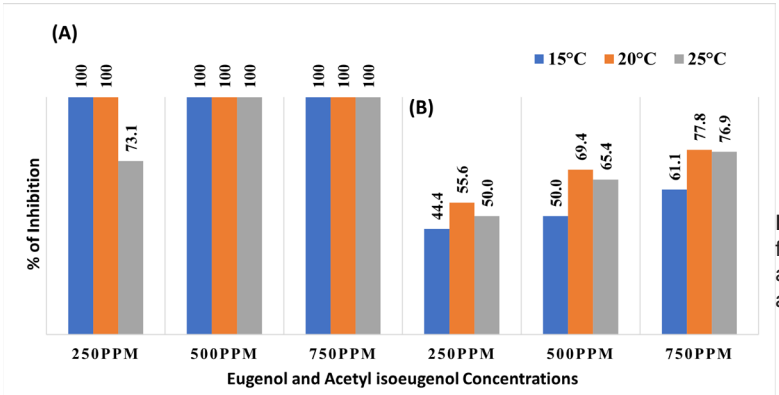

Figure 4. Percent inhibition of *C. cladosporioides*. The fungus exposed to (**A**) 250, 500, and 750 ppm of eugenol at 15°C, 20°C, and 25°C and (**B**) 250, 500, and 750 ppm of acetyl isoeugenol at 15°C, 20°C, and 25°C.

air refrigerators, making it their most common species (22). *Penicillium italicum* produces the mycotoxin 2,5-dihydro-4-methoxy-2H-pyran-2-one, while *Penicillium digitatum* has no known produced mycotoxin (29, 30). However, a case has been reported of a fatal pneumonia caused by *P. digitatum*, and the authors mentioned that this species was resistant to antimycotics which makes this species a hazard concern for human health although it does not produce any mycotoxins (31).

Cladosporium cladosporioides was isolated from the blueberry, strawberry, and raspberry samples. However, other scientists have isolated this fungus from cereal grains, peanuts, fruits, and refrigerated beef (32). The *Cladosporium* genus is known to produce mycotoxins such as cladosporin and emodin and is known to cause allergic reactions in humans. (33). *Cladosporium* cladosporioides is one of the fungi we used during our experiments with the essential oils.

We found *Colletotrichum* gloeosporioides on mango, while other scientists spotted this fungus on other fruits such as soursop and avocado and is known to cause anthracnose, a fungal disease (34).

The fifth fungus, *Mucor racemosus*, was identified on cantaloupe, peach, and strawberry samples. However, *Mucor racemosus* has been previously isolated from cheese, meat, and vegetables (22). So far, the *Mucor* genus has not been found to produce any mycotoxins (36). However, a report linked *M. racemosus* to zygomycosis in the blue tit and other garden birds. Zygomycosis is the broadest term used to refer to infections caused by bread mold fungi of the *Zygomycota* phylum. It is a life-threatening fungal infection that usually affects the face or oropharyngeal cavity (37).

We also found *Botrytis cinerea* on strawberry samples. This fungus is extremely common in many fruits and vegetables. *B. cinerea* causes grey mold and frequently contaminates berries and grapes which is consistent with our results (40). Although no known mycotoxins are produced by *Botrytis cinerea*, grey mold is a serious problem, as it is the most prominent cause for the rejection of fruit products (41).

We found *Penicillium olsonii* in the tomato sample. Our finding is persistent with other scientists as *Penicillium olsonii* has been reported to cause spoilage in tomatoes and in other foods such as grapes (40). *P. olsonii* has appeared to be pathogenic on the tomato fruit. In fact, healthy tomato fruits inoculated with a five-day-old culture of *P. olsonii* soon showed signs of mycelial growth (41). *P. olsonii* has been found to produce ochratoxin A in low amounts (42), but an alternative study argued that, unlike many other species in the Penicillium genus, *P. olsonii* does not produce any mycotoxins (43).

Alternaria alternata was identified on both cucumber and eggplant samples. It has been widely recognized that *A. alternata* causes diseases in many kinds of fruits and vegetables. In addition, it produces a variety of secondary metabolites including altemariol, alternariol monomethyl ether, tenuazonic acid, a derivative of tetramic acid, and altenuene, all of which have been reported to be found on certain foods in various concentrations (44).

Following this, we isolated *Penicillium citrinum*, a yellow-brown fungi, from bitter gourd and blueberry samples. However, other scientists detected this fungus in *Clerodendron cryptopyllum* (a tropical/temperate shrub) roots as well as in rotten orange and persian lime samples (45 - 47). *Penicillium citrinum* produces citrinin, a mycotoxin that, although still toxic, has antibiotic properties with the capacity to inhibit growth of various types of bacteria (48).

We discovered the fungus *Myrothecium roridum* in the bottle gourd samples. Nonetheless, this fungus has been found in the chili-pepper *Capsicum annuum*. This pathogen was discovered growing on the chili-pepper's leaves, indicating that the fungi are able to grow in a diverse range of environments (49). *Myrothecium roridum* is responsible for producing various trichothecene mycotoxins that can disrupt cellular processes (50).

Lastly, we found *Penicillium expansum* on eggplant samples. This blue-colored mold is extremely common, known to infest a variety of different fruits and vegetables. Previously, Kwon *et al*. studied common fungi in mulberries at low temperatures, and the authors found P*enicillium expansum* on mulberry samples (10). In an alternative study, *P. expansum* was found in nearly 50% of healthy-looking apples and 87% of rotten apples (51). This fungus has also been observed growing on onions and carrots (52). *P. expansum* is known to produce patulin, which, as mentioned earlier, can be extremely harmful (53).

According to our results, some of our isolated fungal species were found in different food sources than other researchers did. This might be explained by considering the locality where the samples were collected. Another reason might be related to the storage facilities used in the experiments. We are planning to investigate these possibilities in our future work.

In our experiments with eugenol and acetyl isoeugenol, we found that both temperature and the essential oils had considerable effect to reduce or inhibit the growth rate of the two selected fungal species, *Cladosporium cladosporioides* and *Penicillium italicum*. Those species were selected because of their vast postharvest decay that they cause to our foods. *Penicillium italicum* was reported to be the most economically decaying fungus to the citrus fruit. The losses by this fungus can reach up to 80% of the total postharvest pathogen-related wastage and it produces mycotoxin, 2,5-dihydro-4-methoxy-2H-pyran-2-one (29). *Cladosporium cladosporioides* is considered a psychrotroph because it can grow at freezing temperatures; it can grow at a slower pace at temperatures between -10°C and -3°C (30). Interestingly, *C. cladosporioides* has also been found to produce higher amounts of mycotoxins at lower temperatures of 10-15°C compared to cultures incubated at higher temperatures between 20°C and 30°C. It was found to decay a number of crops such as grapes, strawberries, peas and spinach (31).

The used concentrations of the essential oils in our

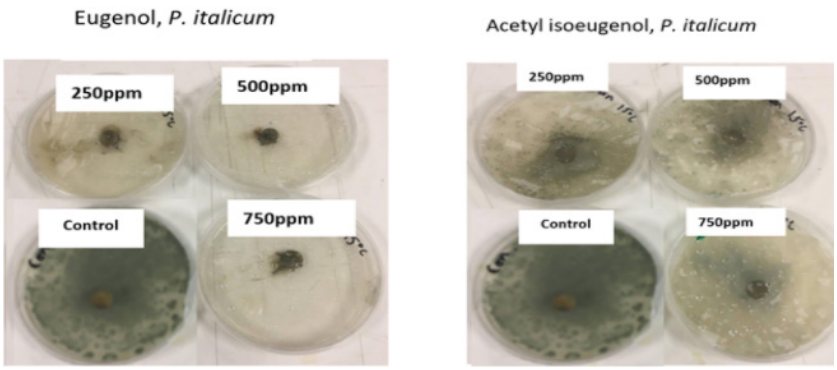

Figure 5. Example Plates of *P. italicum*. Plates were kept at 15°C with either 250, 500, and 750 ppm of eugenol and acetyl isoeugenol or 0 ppm of either, as a control.

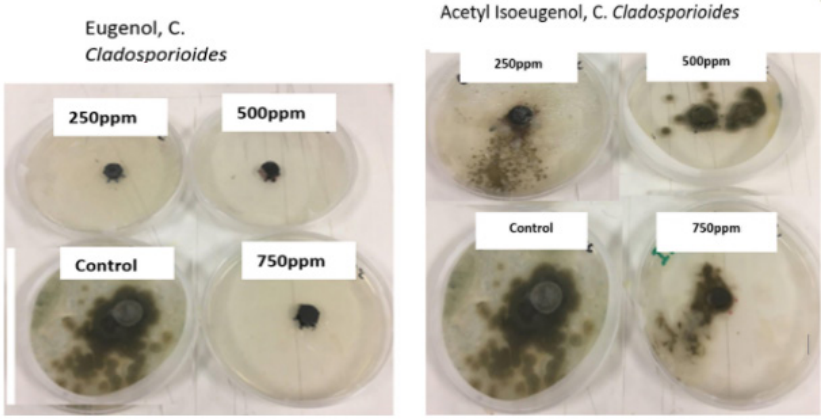

Figure 6. Example Plates of *C. cladosporioides*. Plates kept at 15oC with either 250, 500, and 750 ppm of eugenol and acetyl isoeugenol or 0 ppm of either, as a control.

experiments were ranging from 250 to 750 ppm because the FDA recommends using less than 1500 ppm of eugenol. Although eugenol is considered safe in this concentration range, higher concentrations can lead to fatal conditions such as acute respiratory distress syndrome, fulminant hepatic failure, and central nervous system depression (54, 55). The use of clove essential oils as antimicrobial agents in place of synthetics has increased with time because they are labelled as generally recognized as safe (GRAS) by the FDA (55). The oils were dissolved in 5% dimethylsulfoxide (DMSO). However, we have not tested the effect of DMSO alone on the fungal growth. This step is necessary to be done in the future to study the effect of this solvent on the fungal growth.

Both of our fungal species were more responsive to eugenol than acetyl isoeugenol considering that all of the eugenol plates, with one exception, completely inhibited fungal growth (**Figures 5, 6**). The *Cladosporium cladosporioides* was able to grow in 250 ppm eugenol at 25°C (**Figure 2E**). Although the eugenol was able to completely suppress *C. cladosporioides* at concentrations of 500 ppm and 750 ppm, the fungal growth rate was still very slow in the presence of acetyl isoeugenol compared to the control plates at the same temperatures (**Figure 2B,D,F**). In addition, the highest concentration of acetyl isoeugenol (750 ppm) was able to slow down the growth of both fungal species more than 500 and 250 ppm, showing that as the concentration increased, the fungal growth decreased. Penicillium italicum was not able to grow in all the tested concentrations of eugenol (**Figure 1A,C,E**). However, the fungus slowly grew in the presence of acetyl isoeugenol compared to the control plates at all tested temperatures (**Figure 1B,D,F**). The 750 ppm acetyl isoeugenol had the most suppressive effect on the *P. italicum* and *C. cladosporioides* fungal growth, which was inhibited by an average of 61.3% and 71.9%, respectively, at all temperatures (**Figure 3, 4**). We did not find any previous research using acetyl isoeugenol as an antifungal agent against filamentous fungi. Campaniello *et al*. stated that 100 ppm eugenol represented a critical value for *P. expansum*, *P. glabrum*, *P. italicum*, *A. niger*, and *E. nidulans* because higher concentrations of eugenol resulted in fungistatic activity (58). We noticed that other researchers who used acetyl eugenol also reported a lower effect on fungi in comparison to eugenol itself (17). The change of a hydroxyl group into an acetyl group may be what reduces the potency of such eugenol derivatives (18).

Based on our results and other research, eugenol appears to be especially potent against various fungal species. When tested against *Trichophyton rubrum*, eugenol was found to affect the fungi's cell wall and membranes (57). The fungus also started growing distorted hyphae that were twisted and smaller than in the absence of eugenol. Upon seeing this, researchers concluded that eugenol inhibits the ergosterol biosynthesis in the fungal cells, which is necessary for permeability in the cell's membranes. The lower ergosterol content interferes with the integrity and functionality of the cell membrane (57).

A study recognized that the minimum inhibitory concentration (MIC) of eugenol in *C. cladosporioides* was 350

ppm (58); however, in our study, eugenol concentration of 250 ppm was able to stop fungal growth of the tested species, except for *C. cladosporioides* at 25°C. Šimović *et al*. observed that the MIC of eugenol against *Aspergillus carbonarius* was 2000 ppm, while the MIC against *Penicillium roqueforti* was 1000 ppm (59). Vazquez *et al.* concluded that 200 ug/ml of eugenol increased the lag time of growth of *Penicillium citrinum* up to 9 days and decreased the rate of colony growth (60).

The essential oils were most effective at inhibiting fungal growth of both *Cladosporium cladosporioides* and *Penicillium italicum* at 20°C, which was the optimum growth temperature of the control plates for both fungi (**Figures 3, 4**). Our experiments suggest that eugenol is the best essential oil that can be used as an antifungal agent against the tested fungal species, *C. cladosporium*, and *P. italicum*. However, further work is still needed in order to study more effects of a wider range of eugenol derivatives on both fungi and on the vegetables and fruits. Food decay is an ever-growing issue in the food industry. Spoilage of our food with mycotoxin-producing fungi can cause serious health problems in humans. Therefore, using preservatives to avoid contamination and the eventual production of mycotoxins in our food is essential. Since synthetic preservatives have appeared to be harmful to human health and the environment, the use of natural and safer preservatives is currently recommended. Using essential oils of aromatic plants as preservatives is suggested to help prevent the deterioration of food.

METHODS

Samples

Fourteen different fruits and vegetables were collected and stored at home refrigerators until they showed signs of spoilage. They then kept in a Ziploc bag until it was transferred to ASDRP laboratory. These samples include spoiled bottle gourd, cucumber, peach, cantaloupe, tomato, bitter gourd, eggplant, mango, orange, lemon, raspberry, blueberry, strawberry, and bell pepper. After allowing the microorganisms to grow for about five days in their respective conditions, we transferred the samples to the ASDRP laboratory for further investigation.

Preparing CDA Medium

To isolate and identify fungi growing on the various samples, we prepared Czapek-Dox agar (CDA) (25). When preparing the Czapek-Dox agar to inoculate our plates with food homogenate suspension, we added 100 mg/L chloramphenicol and 50 mg/L rose bengal dye in order to restrict the bacterial growth while still allowing the fungi to grow. Previously, rose bengal and light have been shown to inhibit the growth of unwanted microorganisms, specifically *Escherichia coli*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae*, in agar media (19). Chloramphenicol has antibiotic properties and can withstand enough heat to be added to the media before sterilization (20). Because of this, chloramphenicol is regularly added into media prepared for fungal detection (23).

Preparing Food Homogenates

We blended 10 g of each food sample with distilled water in a sterilized blender, homogenizing the samples. To further dilute the food samples, we transferred 1ml of the mixtures into test tubes containing 9ml of 0.1% peptone diluent. Using the diluted mixture, we inoculated the plates by 1 ml of the tube mixture in each. Two plates were prepared from each isolate. We did an additional direct inoculation by adding a tiny piece of the contaminated food samples into our plates for each fruit or vegetable, leading to three plates from each food sample.

Isolating Fungi

The plates were incubated at 20°C and examined regularly until fungal colonies were distinctive in the plates. Consequently, we differentiated the fungal species based on their morphological characteristics. The selected colonies were re-inoculated into new CDA plates and incubated for 5 days at 20°C.

Morphological Identification of Fungi

We performed macroscopic and microscopic examinations of the isolated fungi using the identification books (61, 62).

DNA Extraction

After our fungi grew, we first used morphological evidence to classify fungi into 11 different species. The mycelia of each species were scraped off and ground into a fine powder with liquid nitrogen (12). For genomic DNA (gDNA) extraction, 5 mg of mycelial powder were transferred into a container with Zymo Research Bashing Beads along with its buffer to lyse cells. We continued to follow the procedure provided with the Zymo Quick-DNATM Fungal/Bacterial Miniprep Kit.

PCR Amplification

Before starting PCR, the gDNA concentration of each sample was measured using a spectrophotometer. Each concentration helped us to determine the volume of template DNA necessary for PCR reaction of each fungal species (63). We amplified the internal transcribed spacer (ITS) region. The ITS region is the most recommended universal fungal barcode sequence that is used because of its highest probability of successful identification of fungi. We used DreamTaq Hot Start PCR Master Mix (2X) with primers ITS1F (CTT GGT CAT TTA GAG GAA GTA A) Forward and ITS4 (TCC TCC GCT TAT TGA TAT GC) Reverse to amplify the ITS region (64). Each PCR reaction was done in a 50 ul solution. DreamTaq hot start PCR master mix (2X) 25 µL, forward primer 2.5 µL, reverse primer 2.5 µL, template DNA 10 pg–1 µg. We placed all the tubes in the thermal cycler along with a negative control to test for any contamination. The following thermocycling parameters were used: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C

for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 8 min.

Agarose Gel Electrophoresis

Detection of PCR-amplified products was performed by electrophoresis on an ethidium bromide-stained 1% agarose gel. We used a 1 kb DNA ladder to compare the lengths of the DNA bands and stained all DNA samples with a loading dye. After filling each well with a specific sample, we ran electrophoresis for fifteen minutes at 110 volts.

DNA Purification & Sequencing

PCR products were purified using the procedure provided with the DNA Clean & ConcentratorTM-5 kit. We added DNA binding buffer to each DNA sample in a ratio of 5:1. Then we transferred the mixture to a provided Zymo-SpinTM Column in a collection tube and centrifuged for 30 seconds. We discarded the flow-through and added 200 µl DNA wash buffer to the column followed by centrifugation. We repeated the wash step and then added ≥ 6 µl DNA elution buffer directly to the column matrix. We then transferred the column to a 1.5 ml microcentrifuge tube and centrifuged for 30 seconds to elute the DNA. The concentration of each sample was measured using a fluorimeter. All the data was recorded and sent to Elim Biopharmaceuticals, who sequenced each DNA sample through Sanger sequencing. The consensus sequences of the ITS region were submitted for a BLAST search using the NCBI GenBank database to obtain species-level information.

Essential oils and Temperature Effect

The antifungal activity of eugenol and acetyl isoeugenol was tested on two of the isolated fungal species, *Penicillium italicum* and *Cladosporium cladosporioides*, which were isolated from lemon and berry samples respectively. Eugenol (concentrations used: 250, 500, and 750 ppm) and acetyl isoeugenol (concentrations used: 250, 500, and 750 ppm) were dissolved in 5% dimethyl sulfoxide solution (DMSO). We chose to use concentrations ranging from 250 to 750 ppm of the essential oils because the FDA recommends using less than 1500 ppm of eugenol. CDA was used as a growth medium for radial growth measurements to test the inhibitory effect of both essential oils at three different concentrations and temperatures: 15, 20, and 25°C. We also made control plates at each temperature to compare the fungal growth rates. In those plates, we did not add any essential oils or DMSO to the culture media. Eugenol and acetyl isoeugenol were added to the medium after sterilization. The fungal radial growth was measured and recorded daily for 7 days. Three measurements of each treatment were recorded daily (n = 3). The percentage of inhibition was calculated by subtracting the radial growth of the control plate on day 7 from the radial growth at each oil's concentration on day 7 divided by the radial growth of the control on day 7*100.

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REFERENCES

1. Amusa, Nojimu A., *et al.* "Bio-deterioration of breadfruit (Artocarpus communis) in storage and its effects on the nutrient composition." A*frican Journal of Biotechnology*, vol. 1, no. 2, 2002, pp. 57-60.
2. Tournas, V. H. "Spoilage of vegetable crops by bacteria and fungi and related health hazards." *Critical reviews in microbiology*, 2005, vol. 31, no. 1, pp. 33-44.
3. Singleton, Paul, and Sainsbury D. "Dictionary of microbiology and molecular biology". No. Ed. 2. J*ohn Wiley & Sons*, 1987.
4. Ciegler, A., *et al.* "Patulin, penicillic acid, and other carcinogenic lactones." *Microbial toxins*, 1971, vol. 6, pp. 409-434.
5. Dickens, Frank, and Jones H. E. H. "Carcinogenic activity of a series of reactive lactones and related substances." *British Journal of Cancer*, 1961, vol. 15, no. 1, pp. 85.
6. El Khoury, André, and Ali Atoui. "Ochratoxin A: general overview and actual molecular status." *Toxins*, 2010, vol. 2, no. 4, pp. 461-493.
7. Schumacher, *et al.* "DNA–DNA cross-links contribute to the mutagenic potential of the mycotoxin patulin." *Toxicology letters*, 2006, vol. 166, no. 3, pp. 268-275.
8. Dolan L. C., Matulka R. A., and Burdock G. A. "Naturally occurring food toxins." *Toxins* (Basel). 2010 Sep; 2(9): 2289–2332.
9. Andersen, Birgitte, and Frisvad J. C. "Characterization of Alternaria and Penicillium species from similar substrata based on growth at different temperature, pH and water activity." *Systematic and Applied Microbiology*, 2002, vol. 25, no. 1, pp. 162-172.
10. Kwon, *et al.* "Isolation and Identification of Postharvest Spoilage Fungi from Mulberry Fruit in Korea." *Korean Journal of Environmental Agriculture*, 2018, vol. 37, no. 3, pp. 221-228.
11. Alwakeel, Suaad S. "Molecular identification of isolated fungi from stored apples in Riyadh, Saudi Arabia." *Saudi journal of biological sciences*, 2013, vol. 20, no. 4, pp. 311-317.
12. Sanzani, Simona M., *et al.* "Mycotoxins in harvested fruits and vegetables: Insights in producing fungi, biological role, conducive conditions, and tools to manage postharvest contamination." *Postharvest Biology and Technology*, 2016. vol. 122, pp. 95-105.
13. Silva, Manuela M., and Lindon F. "Food preservatives–An overview on applications and side effects." *Emirates Journal of Food and Agriculture*, 2016, vol. 28, no. 6, pp. 366-373.

14. Hu Q., Zhou M., and Wei S. "Progress on the Antimicrobial Activity Research of Clove Oil and Eugenol in the Food Antisepsis Field" J*ournal of food science*, June 2018 , vol. 83, no. 6.
15. National Institutes of Health. "LiverTox: clinical and research information on drug-induced liver injury." Nih. gov https://livertox. nih. gov, 2017.
17. Chee, Youn H., and Lee M. H. "Antifungal activity of clove essential oil and its volatile vapour against dermatophytic fungi." *Mycobiology*, 2007, vol. 35, no. 4, pp. 241-243.
18. Singh, *et al*. "Evaluation of antifungal activity in essential oil of the Syzygium aromaticum (L.) by extraction, purification and analysis of its main component eugenol." *Brazilian Journal of Microbiology*, 2011, vol. 42, no. 4, pp. 1269-1277.
19. Andrés O. F., *et al.* "Antifungal activity of eugenol derivatives against Botrytis cinerea." *Molecules*, 2019, vol. 24, no. 7, pp. 1239.
19. Chilvers K. F., *et al*. "Phototoxicity of Rose Bengal in mycological media – implications for laboratory practice." *Letters in applied microbiology*, 1999, vol. 28, no. 2, pp. 103-107.
20. King D., *et al*. "Dichloran-rose bengal medium for enumeration and isolation of molds from foods." *Applied and environmental microbiology*, 1979, vol. 37, no. 5, pp. 959-964.
21. Roberts, Diane, and Melody Greenwood. Practical food microbiology. *John Wiley & Sons*, 2008.
22. Altunatmaz S., *et al*. "Detection of airborne psychrotrophic bacteria and fungi in food storage refrigerators." *Brazilian Journal of Microbiology*, 2012, vol. 43, no. 4, pp. 1436-1443.
23. Akinro E. B., *et al.* "Isolation and identification of fungal species associated with the spoilage of some selected edible fruits in Iree town of Boripe Local Government, Osun State, Nigeria." *Journal of Research in Pharmaceutical Science*, 2015, vol. 2, no. 7, pp. 7-10.
24. Aydogdu, Halide, and Asan A. "Airborne fungi in child day care centers in Edirne City, Turkey." *Environmental monitoring and assessment*, 2008, vol. 147, no. 1-3, pp. 423-444.
25. Chaudhary L. "Isolation and identification of fungi from some spoiled vegetables" *Plant Archives*, 2019, vol. 19, no. 2, pp. 2596-2600.
26. Saleh, Iman, and Al-Thani R. "Fungal food spoilage of supermarkets' displayed fruits." *Veterinary World*, 2019, vol. 12, no. 11, pp. 1877-1883.
27. Nunes C., *et al.* "Postharvest biological control of citrus fruit." Tree and Forestry Science and Biotechnology, 2009, vol. 3, no. 2, pp. 116-126.
28. Long, Chao-An, *et al.* "Biological control of Penicillium italicum of citrus and Botrytis cinerea of grape by strain 34--9 of Kloeckera apiculata." *European Food Research and Technology*, 2005, vol. 221, no. 1-2, pp. 197-201.
29. Gorst-Allman, C. P., and Steyn P. S. "Biosynthesis of 5,6-dihydro-4-methoxy-2H-pyran-2-one in Penicillium italicum." *South African Journal of Chemistry*, 1983, vol. 36, no. 3, pp. 83-84.
30. Ariza M. R., *et al*. "Penicillium digitatum metabolites on synthetic media and citrus fruits." *Journal of Agricultural and Food Chemistry*, 2002, vol. 50, no. 22, pp. 6361-6365.
31. Oshikata, C., *et al*. "Fatal pneumonia caused by Penicillium digitatum: a case report." *BMC Pulm Med*, 2013, vol. 13, no. 16, pp. 1-4.
32. Bullerman L. B. "Spoilage Fungi in Food - An Overview." Encyclopedia of Food Sciences and Nutrition, Second Edition, *Academic Press*, 2003, pp. 5511-5522
33. Alwatban, Mohannad Abdullah, *et al*. "Mycotoxin production in Cladosporium species influenced by temperature regimes." J*ournal of Pure and Applied Microbiology*, 2014, vol. 8, no. 5, pp. 4061-4069.
34. Guardado-Valdivia L., *et al*. "Identification and characterization of a new Bacillus atrophaeus strain B5 as biocontrol agent of postharvest anthracnose disease in soursop (Annona muricata) and avocado (Persea americana)." *Microbiological research*, 2018, vol. 210, pp. 26-32.
35. Marin P., *et al.* "Effect of solute and matric potential on growth rate of fungal species isolated from cheese." *International Dairy Journal*, 2014, vol. 36, no. 2, pp. 89-94.
36. Botes A., and Alfred B. "Mucor." Encyclopedia of Food Microbiology, 2014, vol. 2, *Academic Press,* pp. 834-840.
37. Schmitz, A., *et al*. "Zygomycosis due to Mucor racemosus Associated with a Circovirus Infection in a Blue Tit (*Cyanistes caeruleus*)." Journal of comparative pathology, 2018, vol. 165, pp. 67-71.
38. Rupp S., *et al*. "Spread of Botrytis cinerea strains with multiple fungicide resistance in german horticulture." *Frontiers in microbiology*, 2017, vol. 7, pp. 2075.
39. Petrasch S., *et al*. "Grey mould of strawberry, a devastating disease caused by the ubiquitous necrotrophic fungal pathogen Botrytis cinerea." *Molecular plant pathology*, 2019, vol. 20, no. 6, pp. 877-892.
40. Serra R. and Peterson S. W. "Penicillium astrolabium and Penicillium neocrassum, two new species isolated from grapes and their phylogenetic placement in the P. olsonii and P. brevicompactum clade." *Mycologia*, 2007, vol. 99, no. 1, pp. 78-87.
41. Anjum, N., *et al*. "First report of postharvest fruit rot of tomato (Lycopersicum esculentum Mill.) caused by Penicillium olsonii in Pakistan." *Plant Disease*, 2018, vol. 102, no. 2, pp. 451.
42. Wang Y., *et al*. "Ochratoxin A producing fungi, biosynthetic pathway and regulatory mechanisms." *Toxins*, 2016, vol. 8, no. 3, pp. 83.
43. Frisvad, J. C., *et al*. "Mycotoxins, drugs and other extrolites produced by species in Penicillium subgenus Penicillium." *Stud Mycol*, 2004, vol. 49, no. 201, pp. 41.
44. Magan N., *et al*. "Effect of water activity and temperature on mycotoxin production by Alternaria alternata in culture and on wheat grain." *Applied and environmental microbiology*, 1984, vol. 47, no. 5, 1984, pp. 1113-1117.
45. Doan, Duy Tien, *et al.* "Isolation of Penicillium citrinum from Roots of Clerodendron cyrtophyllum and Application in Biosynthesis of Aglycone Isoflavones from Soybean Waste

Fermentation." *Foods*, 2019, vol. 8, no. 11, pp. 554.
46. Coutinho T. C., *et al*. "Penicillium citrinum and Penicillium mallochii: New phytopathogens of orange fruit and their control using chitosan." *Carbohydrate Polymers*, vol. 234, 2020, pp. 115918.
47. González-Estrada, Ramón R., *et al*. "Use of a marine yeast as a biocontrol agent of the novel pathogen Penicillium citrinum on Persian lime." *Emirates Journal of Food and Agriculture*, vol. 29, no. 2, 2017, pp. 114-122. doi:10.9755/ejfa.2016-09-1273
48. Mazumder P. M., *et al*. "Antimicrobial activity of the mycotoxin citrinin obtained from the fungus Penicillium citrinum." *Ancient science of life*, 2002, vol. 21, no. 3, pp. 191-197.
49. Jordan B., *et al*. "First report of Myrothecium leaf spot caused by Myrothecium roridum on pepper in the United States." *Plant Disease*, 2018, vol. 102, no. 1, pp. 246.
50. Ye Wei, *et al*. "Two trichothecene mycotoxins from Myrothecium roridum induce apoptosis of HepG-2 cells via caspase activation and disruption of mitochondrial membrane potential." *Molecules*, 2016, vol. 21, no. 6, pp. 781.
51. Valiuškaitė, Alma, *et al*. "Ecological aspects of distribution of potential toxin-producing micromycetes on stored apple fruit." *Ekologija*, 2006, vol. 3, pp. 60-63.
52. Lugauskas, Albinas, *et al*. "Micromycetes, producers of toxins, detected on stored vegetables." *Annals of agricultural and environmental medicine*, 2005, vol. 12, no. 2, pp. 253-260.
53. Garcia D., *et al*. "Intraspecific variability of growth and patulin production of 79 Penicillium expansum isolates at two temperatures." *International journal of food microbiology*, 2011, vol. 151, no. 2, pp. 195-200.
54. Kegley, S., *et al*. "Chapter 6 Clove Oil (Eugenol)." Marin Municipal Water District Herbicide Risk Assessment, 2010, *Pesticide Research Institute*, pp.1-47.
55. Silva, E. K., *et al*. "Process Engineering Applying Supercritical Technology for Obtaining Functional and Therapeutic Products." *Advances in Biotechnology for Food Industry*, 2018, pp. 327-358.
56. Campaniello D., *et al*. "Antifungal Activity of Eugenol against Penicillium, Aspergillus, and Fusarium Species." *J Food Prot*, 2010, vol. 73, no. 6, pp. 1124-1128.
57. de Oliveira Pereira, *et al*. "Investigation on mechanism of antifungal activity of eugenol against Trichophyton rubrum." *Medical mycology*, 2013, vol. 51, no. 5, pp. 507-513.
58. Abbasszadeh S., *et al*. "Antifungal efficacy of thymol, carvacrol, eugenol and menthol as alternative agents to control the growth of foo-relevant fungi." *Journal de mycologie medicale*, 2014, vol. 24, no. 2, pp. e51-e56.
59. Šimović, Mirela, *et al*. "Antifungal effect of eugenol and carvacrol against foodborne pathogens Aspergillus carbonarius and Penicillium roqueforti in improving safety of fresh-cut watermelon." *Journal of intercultural ethnopharmacology*, 2014, vol. 3, no. 3, pp. 91.
60. Vazquez B. I., *et al*. "Inhibitory effects of eugenol and thymol on Penicillium citrinum strains in culture media and cheese." *International Journal of Food Microbiology*, 2001, vol. 67, no. 1-2, pp. 157-163.
61. Guy St. Germain and Summerbell R., "Identifying fungi: A clinical laboratory handbook" 2010. ISBN: 9780898633115
62. Pitt J. I. and Hocking A.D., "Fungi and Food Spoilage" 2012, *Springer*, Third Edition.
63. Raja H. A., *et al*. "Fungal identification using molecular tools: a primer for the natural products research community." *Journal of Natural Products*, 2017, vol. 80, no. 3, pp. 756-770.
64. White, T. J., *et al*. "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics." *PCR protocols: a guide to methods and applications*, 1990, vol. 18, no. 1, pp. 315-322.