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In Vitro Antifungal Activity of Several Antimicrobial Compounds against *Penicillium expansum*

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ABSTRACT

Fungicides used in the prevention and control of mold rots in stored apples are subjected to legal, social, and biological limitations. The aim of this study was to find an alternative to postharvest fungicides currently used in the prevention and control of blue mold rot caused by *Penicillium expansum* in apples. For this purpose, the antimicrobial activity and MIC of several substances against *P. expansum* were evaluated in vitro using different end point methods: agar diffusion assay, volatility method, and agar dilution and broth dilution MIC assays. Most of the substances tested are common food ingredients and have a recognized antimicrobial activity. Essential oils, such as thymol, eugenol, citral and cineole, vanillin, sodium hypochlorite, acetic acid, potassium sorbate, and hydrogen peroxide, were the substances evaluated. Thymol and citral were the essential oil components that showed the greatest inhibitory effects. The effectiveness of 5 and 10% hydrogen peroxide in growth inhibition of *P. expansum* in the agar diffusion assay was total, and its MIC as determined by the agar and broth dilution assays was less than 0.025%. These results indicate that the application of small quantities of hydrogen peroxide to the apple skin might be an alternative to fungicides in the elimination of *P. expansum*.

Apple (*Malus domestica*) is a fruit with a large economic relevance in Spain, especially the Golden Delicious variety, which is produced in the northeast area. Usually, after harvest, apples are stored at 0°C in a controlled atmosphere for at least 9 months. During this period, a large number of mold genera are able to invade the fruit and cause important economic losses. Blue mold rot by *Penicillium expansum* is the most common pathologic condition associated with stored apples. In our country, the blue mold rot is responsible for 80 to 90% of mold spoilage of stored fruit. To prevent this deterioration, the apples are washed with a fungicide mixture between harvest and storage. However, the use of synthetic fungicides has a large number of limitations and disadvantages. These include progressively stricter laws, social rejection due to toxicologic problems affecting humans and environment, and biological limitations due to fungicide resistance. For instance, the genus *Penicillium* has developed resistance to thiabendazole *(4)* and *Botritys* to the fungicide benomyl *(31)*. Moreover, these compounds have a bad reputation with consumers, who demand more natural and safe foods. A large number of health organizations and researchers noted that, although synthetic fungicides prevent microbial spoilage, their use in food products is no longer recommended because of their toxicity and oncogenic risk *(19, 34)*. These problems will cause the drastic reduction or elimination of the use of these chemicals in the near future. However, this reduction depends on the development of new antimicrobial agents similar in effectiveness at low concentrations but more natural and less harmful to the consumers and environment and also economically affordable.

Essential oils are generally volatile substances produced by several plant species as a chemical defense mechanism against phytopathogenic microorganisms *(17)*. Therefore, several investigators have used them in the control of postharvest microbial spoilage of vegetable, fruit, and flower commodities *(9, 30, 33)*. There are numerous in vitro and in vivo assays for the antifungal activity of essential oils and their major components *(11, 32)*.

Vanillin, a major constituent of vanilla beans (*Vanilia planifolia, Vanilia pompona*, or *Vanilia tahitensis*), has been reported as a possible antimicrobial agent *(1, 3, 13)*. It has been used successfully in inhibiting yeast development and mold growth in laboratory and semisynthetic culture media *(5, 15)*.

Chlorine and a number of hypochlorites have been used for many years to treat drinking water and wastewater and to sanitize food processing equipment and surfaces in processing environments. They are also used as disinfectants in wash, spray, and flume waters in the raw fruit and vegetable industries *(35)*. To disinfect whole and cut fruits and vegetables, chlorine is commonly used at concentrations of 50 to 200 ppm, with a contact time of 1 to 2 min. Treatment of whole and shredded lettuce leaves in water containing 200 to 250 ppm of chlorine reduced populations of yeasts and molds by 50 to 90%. Therefore, it would seem that chlorine could be effective in the elimination of *P. expansum* from whole apples, alone or in combination with other sanitizing agents.

The use of acetic acid and its vapors as sanitizing agents in horticultural commodities appears promising. The

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antifungal effectiveness of acetic acid in several fruits (apples, peaches, nectarines, and apricots) has been verified recently *(26–28)*.

Potassium sorbate is the most readily soluble of the sorbates. Sorbic acid and its derivatives are commonly used to inhibit mold growth and extend shelf life of several foods because of physiological harmlessness and organoleptic neutrality *(16)*.

Diluted hydrogen peroxide (H_2O_2) and its vapors have been used successfully in the disinfection of fresh mushrooms *(23)*, minimally processed fruits and vegetables *(25)*, and prunes *(29)*. Other experimental antimicrobial applications of H_2O_2 include preservation of fresh fruits and vegetables *(12)*, control of postharvest decay in table grapes *(10, 21)*, and decontamination of fresh-cut melons *(24)*.

In vitro evaluation of the antimicrobial effectiveness of a sanitizer is the first step to finding the most suitable sanitizing treatment for the prevention of growth and development of a microorganism in a food system. Therefore, this study was undertaken to investigate the effectiveness of the following antimicrobials on the survival and growth of *P. expansum*: thymol, the main component of thyme (*Thymus vulgaris*) essential oil; eugenol obtained from clove (*Caryophilli flos*) essential oil; citral, obtained from lemon (*Citrus limum*) peel; cineole or eucalyptol, the major component of the oil obtained from eucalyptus (*Eucalyptus globulus*) leaves; vanillin; sodium hypochlorite; acetic acid; potassium sorbate; and H_2O_2. This allows us to find an alternative to postharvest fungicides currently used in the prevention and control of blue mold rot caused by *P. expansum.*

MATERIALS AND METHODS

Preparation of inoculum. A strain of *P. expansum* (MUCL 29381) isolated from apples was supplied by the Spanish Microorganisms Collection Type Cultures. Stock culture of the mold was recultivated monthly on potato dextrose agar (PDA; Merck, Darmstadt, Germany) slants and maintained at 5°C.

Cultures of the mold were grown on PDA slants for 5 to 7 days at 23 to 25°C, and inoculum was prepared by adding 10 ml of sterile 0.01% Tween 80 (Merck) in distilled water. Spores were loosened by gentle brushing of the conidiophores with a sterile inoculating loop. The spore suspension was filtered through four layers of sterile cheese cloth to remove mycelial debris and then diluted with a sterile 0.01 M phosphate-buffered solution (pH 7.0) to obtain the number of spores per ml required for the different assays. The number of spores per ml was determined by direct microscopic count.

Inhibitor solutions. Purity and source of antimicrobial compounds used in this study are listed in Table 1. Thymol, citral, eugenol, and cineole were prepared just before use in appropriate concentrations. Because of their poor solubility, they were first dissolved in a small volume of 95% ethyl alcohol (3% [vol/vol] final concentration of ethanol in the solution) and Tween 80 (0.05% [vol/vol] final concentration in the solution). To measure the effects of Tween 80 and ethyl alcohol, a solution containing the same amount of Tween 80 and ethyl alcohol but without inhibitor was also evaluated. Vanillin, sodium hypochlorite, acetic acid, potassium sorbate, and H_2O_2 solutions were prepared in distilled water. Fungicides (imazalil sulfate and thiabendazole) currently used for control of postharvest mold rots in apples were also included in this study. Imazalil sulfate and thiabendazole were also solubilized in sterile distilled water. A new solution of each inhibitor was prepared just before each replication of every experiment.

TABLE 1. *Purity and source of the substances tested against* P. expansum

Substance	Purity (%)	Source
Thymol	98	Panreac, Barcelona, Spain
Eugenol	99	Panreac
Citral	95	Panreac
Cineole	99	Panreac
Vanillin	99	Panreac
Sodium hypochlorite	5[a]	Henkel, Barcelona, Spain
Acetic acid	99	Panreac
Potassium sorbate	99	Panreac
Hydrogen peroxide	30	Merck, Darmstadt, Germany
Imazalil sulfate	7.5	ELF Atochem Agri, Paterna, Spain
Thiabendazole	60	ELF Atochem Agri

[a] Expressed as percentage of free chlorine.

Techniques for measuring inhibition. Antimicrobial activity was previously tested by the agar diffusion and volatility methods, and quantitative data (MIC and minimum lethal concentration) were determined by the agar dilution and broth dilution methods. A semisynthetic medium consisting of a mixture of a commercially available medium and apple juice was used to evaluate the effects of inhibitors on the growth of *P. expansum (22)*.

In the agar diffusion assays, PDA was modified by adding apple juice; 39 g of PDA was dipped into 500 ml of water and autoclaved for 15 min at 121°C. Apple juice (500 ml) obtained from Golden Delicious apples was filtered, autoclaved as described above, and added to the PDA just before the agar began to solidify. The apple juice PDA mixture (AJPDA) was poured into petri plates and allowed to solidify. Each plate was inoculated with 0.1 ml of spore suspension (10^4 spores per ml^{-1}), which was spread on the agar surface and allowed to dry for approximately 2 h. Sterile, 12.7-mm-diameter, absorbant Sensi-discs (Albet, Barcelona, Spain) were dipped in the appropriate solution, blotted, and then placed on the surfaces of inoculated plates. To measure the effects of Tween 80 and ethyl alcohol, a solution containing the same amounts of Tween 80 and ethyl alcohol was also evaluated. Plates were incubated at 25°C for 7 days, after which the inhibition zones were measured and recorded in millimeters. Concentrations of inhibitory solutions tested are listed in Table 2. Each inhibitory solution was assayed in triplicate plates on at least three separate occasions.

Volatile effects were assayed on essential oil components, acetic acid, H_2O_2, and commercial fungicides, at the concentrations listed in Table 3. Medium and inoculum were prepared as described above, but in this case, only a single paper disc (12.7-mm diameter) was placed on the lid of the petri plate. The plates were incubated upside down at 25°C for 7 days. Each inhibitor solution was assayed in three replicates on three separate occasions and the zone of inhibition was measured daily.

In the agar dilution MIC assay test, chemicals were incorporated into the molten agar medium (AJPDA), which was then dispensed in 20-ml quantities into sterile petri plates. These plates were inoculated with a loop of a microbial suspension containing

TABLE 2. *Effects of inhibitors on* P. expansum *growth by the agar diffusion assay*[a]

Treatment	Concentrations tested (%)	Inhibition zone diameter (mm)
Thymol	0.02, 0.1, 0.3	NI
	0.5	4.3 ± 1.4
	1	11 ± 2.0
Eugenol	0.02, 0.1, 0.3, 0.5	NI
	1	2.0 ± 1.6
Citral	0.02, 0.1	NI
	0.3	2.0 ± 0.9
	0.5	4.0 ± 2.0
	1	8.0 ± 2.3
Cineole	0.02, 0.1, 0.3, 0.5, 1	NI
Vanillin	0.05, 0.1, 0.15, 0.2, 0.3, 0.5, 1	NI
Chlorine	0.01, 0.015, 0.02, 0.025, 0.03, 0.05, 0.1	NI
Acetic acid	0.1, 0.5, 1	NI
	3	1.0 ± 0.5
	5	6.0 ± 1.7
Potassium sorbate	0.1	0.8 ± 1.3
	0.3	5.3 ± 2.0
	0.5	8.3 ± 0.5
	1	12.0 ± 1.4
Hydrogen peroxide	1	2.0 ± 2.2
	3	17.5 ± 0.6
	5	24.3 ± 3.0
	10	27.3 ± 2.6
Imazalil sulfate	0.5	25.0 ± 2.3
Thiabendazole	0.2	NI
I + TBZ	0.5 + 0.2	26.0 ± 2.8

[a] Values for zone of growth inhibition measured as the diameter (mm) of the clear zone around the paper disc are presented as average mean ± SE of three replicates. The diameter of the paper disc (12.7 mm) is not included. NI, no inhibition; I + TBZ, imazalil sulfate plus thiabendazole.

TABLE 3. *Effects of inhibitors on* P. expansum *growth by the volatility assay*[a]

Treatment	Concentrations tested (%)	Inhibition zone diameter (mm)
Thymol	0.02, 0.1, 0.3, 0.5, 1	NI
Eugenol	0.02, 0.1, 0.3, 0.5, 1	NI
Citral	0.02, 0.1, 0.3, 0.5, 1	NI
Cineole	0.02, 0.1, 0.3, 0.5, 1	NI
Chlorine	0.01, 0.015, 0.02, 0.025, 0.03, 0.05, 0.1	NI
Acetic acid	0.1, 0.5	NI
	1	7.1 ± 1.9
	3	25.3 ± 7.0
	5	40.0 ± 2.2
Hydrogen peroxide	1	NI
	3	NI
	5	25.2 ± 5.6
	10	TI
Imazalil sulfate	0.5	TI
Thiabendazole	0.2	NI
I + TBZ	0.5 ± 0.2	TI

[a] For assay of volatile effects, the paper disc (12.7-mm diameter) impregnated with the inhibitor solution was placed on the lid of the petri plate. The plate inoculated with the microorganism was incubated upside down at 25°C for 7 days. Values for zone of growth inhibition measured as the diameter (mm) of the clear zone in the surface of the agar are presented as average mean ± SE of three replicates. NI, no inhibition; TI, total inhibition (no growth on the petri plate); I + TBZ, imazalil sulfate plus thiabendazole.

TABLE 4. *MICs of inhibitors for* P. expansum *by the agar dilution assay*

Treatment	MIC (%)
Thymol	0.02
Eugenol	0.03
Citral	0.03
Chlorine	0.1
Vanillin	0.1
Acetic acid	0.25
Potassium sorbate	0.5
Hydrogen peroxide	0.05

10^6 spores per ml^{-1} and incubated at 25°C for 7 days. Results of the assays were determined after 7 days of incubation. A single colony, a haze, or no growth was considered negative *(8)*. Each inhibitory solution was assayed in three replicates. The experiment was repeated three times. The MICs were the lowest concentrations inhibiting the visible growth of *P. expansum* on the agar plate.

In the broth dilution assay, substances were evaluated for antifungal activity based on their capacity to inhibit the increase in mycelia dry weight of the fungi in liquid cultures. The activities of all of these inhibitors were evaluated at the corresponding MICs as determined in the agar dilution assay (Table 4) and at concentrations below the MIC to find the lowest concentration that caused irreversible inhibition in liquid media (Table 5). The basal semisynthetic medium used consisted of Czapek-Dox medium (Merck) and apple juice supplemented with yeast extract (Merck) and casein hydrolysate (Merck), each 0.1% (wt/vol); 50 ml of liquid medium was dispensed into a 250-ml Erlenmeyer flask and autoclaved at 121°C for 15 min. Before the inoculation with the test fungi, the inhibitory solutions at the concentrations listed in Table 5 were placed into the Erlenmeyer flasks containing the liquid medium, then inoculated with 1 ml of spore suspension containing 5×10^7 spores ml^{-1} and incubated at 25°C without agitation for 9 days. When essential oil components were used, the final concentration of ethanol in the medium never exceeded 0.5% (vol/vol). Three flasks of every treatment were removed after 9 days of incubation, and the mycelia from individual flasks were collected by filtration on Whatman no. 1 filter paper (Whatman Ltd., Maidstone, England). Mycelia were dried at 80°C for 48 h and then weighed. The percentage of inhibition (I%) was calculated using the formula $I = (C - T)C^{-1} \times 100$, where C is the weight of the mycelia in the untreated flasks and T is the weight of the mycelia in the treated flasks. The absence of turbidity was deemed to be total (100%) inhibition of growth.

Before filtration, an aliquot of medium (0.1 ml) from any Erlenmeyer flask that demonstrated no growth was transferred to a fresh medium (AJPDA) that contained no antimicrobial. If after 7 days of incubation no growth occurs in the medium, the concentration of inhibitory substance tested is lethal for the fungi.

RESULTS AND DISCUSSION

Results obtained by the agar diffusion and volatility methods are qualitative, and the microorganism is generally termed susceptible, intermediate, or resistant, depending on the diameter of the inhibition zone *(8)*. These tests provided preliminary information to determine potential utility of the test compound and allowed us to remove cineole from the subsequent assays. Cineole did not show any inhibitory activity at concentrations of more than 1% (data not shown).

TABLE 5. *Mycelial growth inhibition and lethal concentrations of inhibitors for* P. expansum *by the broth dilution assay*

Treatment	Concentration (%)	Dry weight (mg)	Reduction in dry weight (%)[a]
Control	—	370.2	—
Thymol	0.01	48.1	89.9 ± 2.5
	0.02	0.0	100.0 ± 0.0[b]
Eugenol	0.015	110.9	70.0 ± 2.4
	0.03	85.7	76.8 ± 3.5
Citral	0.015	118.5	68.0 ± 1.5
	0.03	42.9	88.4 ± 1.7[b]
Vanillin	0.07	140.8	61.9 ± 6.1
	0.1	26.2	92.9 ± 7.3
Chlorine	0.025	72.1	80.5 ± 1.3
	0.05	33.4	91.0 ± 0.6[b]
	0.1	5.6	98.5 ± 1.5
Acetic acid	0.1	369.9	0.1 ± 0.1
	0.2	0	100.0 ± 0.0[b]
	0.25	3.9	98.9 ± 1.1
Potassium sorbate	0.25	323.0	12.8 ± 11.6
	0.5	239.5	35.3 ± 9.0
Hydrogen peroxide	0.02	73.3	80.2 ± 9.9
	0.025	0	100.0 ± 0.0[b]
	0.03	1.1	99.7 ± 0.3
	0.05	7.1	98.1 ± 1.9

[a] Values for reduction in dry weight are presented as average mean ± SE of three replicates.

[b] Minimum lethal concentration. Lethality was determined by transferred an aliquot of medium (0.1 ml) from any Erlenmeyer flask that demonstrated no growth to a fresh medium (AJPDA) that contained no antimicrobial. If after 7 days of incubation no growth occurs in the medium, the concentration of inhibitory substance tested is lethal for the fungi.

However, we cannot establish the range of diameters of the clear zone round the paper disc at which the microorganism is classified as susceptible, intermediate, or resistant, because it depends on several factors, such as rate of diffusion, microorganism tested, nutrient medium, and other variables. This is the reason why food microbiologists have never adopted a standardized measure for the inhibition zone.

The agar and broth dilution assays describe the inhibition of the microorganism at a specific end point in time and provide quantitative information. In this study, MICs determined by the agar dilution method (Table 4) proved to be lethal in the broth dilution assay, except for vanillin, eugenol, and potassium sorbate. It is worth noting that 0.03% of citral yielded some growth but nevertheless appeared to be lethal by the end of the assay (Table 5). In the cases of chlorine, acetic acid, and H_2O_2, concentrations above the MICs obtained in solid media showed a lethal effect on *P. expansum* growth in liquid media (Table 5).

Among essential oil components, citral and thymol showed the greater inhibitory effects, but in both cases, MICs were higher than those reported by Moleyar and Narasimham *(18)* as inhibitory on *Aspergillus niger* and *Penicillium digitatum* growth on petri plates (less than 0.01%).

Vapors of essential oil components did not show any inhibitory activity. This was highly surprising in the case of thymol vapors, which have been successfully tested for controlling gray mold rot of cherries *(7)*. In liquid culture, MICs of thymol and citral totally inhibited the germination of *P. expansum* spores. When concentrations were reduced by half, mold growth was inhibited by 89.9% for thymol and 68.0% for citral, but no lethal effect was observed.

It should be noted that none of the vanillin concentrations tested by the agar diffusion assay (Table 2) inhibited *P. expansum* growth even though its MIC was 0.1% (1,000 ppm) (Table 4). This result is similar to that reported on its inhibitory action on *Aspergillus,* which was in the range of 1,000 to 2,000 ppm, depending on the species and agar composition *(15)*. Cerruti and Alzamora *(5)* found that the addition of 2,000 ppm of vanillin had an important inhibitory effect on the growth of the yeasts *Saccharomyces cerevisiae, Zygosaccharomyces bailii, Zygosaccharomyces rouxii*, and *Debaromyces hansenii* in laboratory media and apple puree.

In the agar diffusion and volatility assays (Tables 2 and 3), inhibitory effects of sodium hypochlorite were not detected. MIC for this sanitizer in *P. expansum* growth inhibition was 1,000 ppm, a concentration too high for food decontamination.

Acetic acid showed only a slight inhibitory effect on the growth of *P. expansum* in the agar diffusion assay and even that required extremely high concentrations (3 and 5%) (Table 2). Sholberg and Gaunce *(27)* showed that low concentrations of acetic acid in air (2.7 to 5.4 mg liter$^{-1)}$ reduced germination of *Botrytis cinerea* and *P. expansum* conidia to zero after they had been dried on 0.5-cm square pieces of dialysis tubing. However, in our study the inhibitory volatile effects of acetic acid appeared at concentrations of 1%. This shows that the action of acetic acid is closely related to its vapors, but at concentrations higher than those used in apple fumigation application. As a liquid, acetic acid was lethal for *P. expansum* spores at a concentration of 0.25%, whereas when it was reduced to 0.1%, no antimicrobial effect was detected (Table 5).

Potassium sorbate showed a light inhibitory effect at concentrations of 0.1 and 0.3%, substantially increasing at concentrations above 0.5 %, with a clear zone around the paper disc of 8.3 and 12.0 mm (0.5 and 1%, respectively) (Table 2). In the agar dilution assay, mold growth was inhibited by 0.5% potassium sorbate (Table 4). This level is higher than those reported as MIC (0.02 to 0.1%) on *Penicillium* spp. growth by Lück and Jager *(16)*. Similar to our results and those reported by others *(2, 3, 22)*, the MIC of potassium sorbate was above 0.5%, whereas its vapors did not show any antifungal activity. Lennox and McElroy *(14)* found that concentrations of 0.3% potassium sorbate (the maximum accepted for use in bakery products) would result only in a 57% *P. expansum* growth reduction in liquid culture. In our study, 0.25% of potassium sorbate resulted in only a 12.8% reduction in growth (Table 5).

H_2O_2 was the most effective substance in inhibiting *P. expansum* growth in both agar diffusion and volatility assays (Tables 2 and 3). This activity was detected with concentrations of 1% with a clear zone around the paper disc of 2 mm, and a notable inhibition of growth was clearly visible at a concentration of 3% (Table 2). However, the effectiveness of its vapors was only detected at higher concentrations (5%), showing a total inhibition at 10% (Table 3). H_2O_2 MIC against *P. expansum* in solid medium was as low as 0.05% (Table 4), and the inhibitory effect was maintained for up to 10 days (data not show). In liquid medium, concentrations of 0.03 and 0.025% had a lethal effect in *P. expansum* development, and 0.02% H_2O_2 led to a 80.2% reduction in mycelia dry weight (Table 5).

It has been verified that among the fungicides currently used for the control of mold development only imazalil sulfate inhibited *P. expansum* growth in both agar diffusion and volatility assays (Tables 2 and 3). Thiabendazole did not inhibit *P. expansum* growth in any assay. Although this compound is a well-known fungicide, it was not effective because the strain tested in this study was one of the most common strains of *P. expansum* resistant to benzimidazole *(4, 20)*.

These results confirm that substances known as antimicrobials have a different inhibitory effect and MIC, depending on the genus or species in study and the method used to evaluate the antifungal activity.

H_2O_2 was the best alternative to synthetic fungicides against blue mold rot. H_2O_2 showed a lethal effect on *P. expansum* spores even at concentrations as low as 0.025 to 0.05%. Furthermore, H_2O_2 is a natural metabolite of many microorganisms and totally miscible in water. In addition, using low concentrations, neither oxidizing effects nor peroxide residues would be detected in H_2O_2. The *Code of Federal Regulations* (21 CFR 184. 1366) *(6)* specifies that H_2O_2 is permitted for use as an antimicrobial agent at maximum levels of 0.05 to 0.15%, depending on application and provided that "residual hydrogen peroxide is removed by appropriate physical and chemical means during processing."

Our results point to the possibility of successfully using H_2O_2 to avoid *Penicillium* rots. In a commercial application, the dose needed to eliminate the risk of rots could fulfill the maximum levels accepted in the *Code of Federal Regulations*. Additional research is needed to optimize H_2O_2 postharvest treatment of Golden Delicious apples and to establish its legal and economical feasibility.

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