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Full Length Research Paper

Assessment of the antifungal activities of polyhexamethylene-guanidine hydrochloride (PHMGH)based disinfectant against fungi isolated from papaya (*Carica papaya* L.) fruit

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The antifungal activity of the Polyhexamethylene-guanidine hydrochloride (PHMGH)-based disinfectant against fungi isolated from papaya fruit was evaluated. The aim of this work was to show that the PHMGH can be used as a disinfectant for papaya preservation. Thirty one strains of fungi were isolated as *Mucor* sp., *Botrytis* sp., *Penicillium* sp., *Geotrichum* sp., *Aspergillus* sp. and *Colletotrichum* sp. *Mucor* sp. was the most isolated with a frequency of 52.77% followed by *Botrytis* (47.22%), *Aspergillus* and *Penicillium* (8.33%), *Colletotrichum* (5.56%) then, *Geotrichum* (2.78%). The antifungal activity of the PHMGH was tested through the determination of the minimal inhibitory concentrations (MICs) and the minimal fungicidal concentrations (MFCs). All the strains tested were sensitive to the disinfectant. However, the activity of PHMGH varies according to the strain tested. The values of the MIC and MFC of 1.9 mg/ mL whereas the most sensitive to PHMGH were the genera *Botrytis* and *Colletotrichum* with a MIC <0.01 mg/ mL. No matter the strains of fungi studied, the MIC and MFC values were equal. The PHMGH could therefore be considered as a fungicidal agent that could serve for the preservation of papaya fruit after harvesting.

Key words: Antifungal activity, minimum inhibitory concentrations (MICs), minimum fungicidal concentrations (MFCs), papaya preservation, polyhexamethylene-guanidine hydrochloride.

INTRODUCTION

The economy of most developing countries, based primarily on their agricultural resources, is strongly dependent on the often rigorous and rigid quality standards set by developed countries (Dembele et al., 2009). Côte d'Ivoire, which is the second exporter of papaya (*Carica papaya* L) after Ghana, sends about 1163 tons of papaya every year toward the European Union

market (N'da et al., 2008). Unfortunately, the marketing of this crop is threatened by a multitude of insects, bacterial, viral and fungal diseases that may lead to a loss of profit (Luciana et al. 2008; Hadizadeh et al., 2009). In Côte d'Ivoire, the main diseases of papaya are of viral and fungal origin (Diallo et al., 2007). Filamentous fungi are found to be responsible for many postharvesting losses and are therefore, a real problem for the preservation of the quality of papaya, especially after harvesting.

Fungal activity can lead to a contamination with mycotoxins, and could pose a health risk to the consumers. That can be a major condition to foreign

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exchange, may cause some losses of market and a notable decrease of the income of producers (Dembele et al., 2009). Therefore, the reduction or elimination of fungal contamination of the Ivorian papaya would undoubtedly prevent the risk of mycotoxin production, increase the shelf life of the fruit and its commercial quality and keep the leading edge of the production. To improve the preservation of fruit, physical or chemical treatments are used to fight post-harvest diseases. The chemical treatment remains the primary means to reduce the incidence of post-harvest disease of various fruits and vegetables. However, the misuse of antimicrobial chemicals (antibiotics, food preservatives ...) in recent years has increased the resistance of microorganisms (Harbottle et al., 2006; Ouahiba et al., 2010) so that the significant economic losses in foods and feeds and the significant increase of microbial infections are increasing over time. To reduce food poisoning with the resulting epidemic diseases, the development and use of new bioactive molecules that can act more effectively on new resistant microorganism generations is essential (Coates et al., 2002; Ouahiba et al., 2010).

To promote new chemicals that can help improve preservation, the chemical-based papaya polyhexamethylene quanidine hydrochloride (PHMGH) disinfectant, a member of the polymeric guanidine family, which has proved a powerful bactericide (Krebs et al., 2005; Müller and Kramer, 2005; Oulé et al., 2008) has been tested. The PHMGH disinfectant known to be a biocide, odourless, noncorrosive and nontoxic to humans and animals, fast at low concentrations and with a broad spectrum of action (Krebs et al., 2005; Müller and Kramer, 2005; Oulé et al., 2008) is gaining increasing interest because of its relatively safe status, its wide acceptance and its exploitation for potential multi-purpose functional use.

Several studies have been done in Côte d'Ivoire on Papaya regarding viral diseases (Diallo et al., 2007) and maturation of the fruit (Yao et al., 2011). To our knowledge, little has been done in Côte d'Ivoire to prevent postharvest spoilage of papaya due to fungi. This work was designed to promote new products that can contribute to improve the preservation of fruit and vegetables such as papaya. The aim of this study was to determine the fungal profiles of the papaya produced in Côte d'Ivoire and assess the antifungal properties of the PHMGH disinfectant against fungi that spoiled papaya to the field in order to improve its use in areas such as agriculture and food industry. Thus, the antifungal activity tests were performed on the in vitro growth of fungi through the determination of the minimum inhibitory (MIC) concentrations and minimal fungicidal concentrations (MFC).

MATERIALS AND METHODS

The study was carried out on infected *Carica papaya* L. var. Solo 8' (the most cultivated variety in Côte d'Ivoire). The fruit were

harvested from six different papaya farms, which are among the three main papaya producing zones located in the southern area of Côte d'Ivoire: Azaguié (site 1), Abengourou (site 2) and N'Zianoua (site 3). The polyhexamethylene guanidine hydrochloride disinfectant from the Department of Biological Sciences, University of Manitoba (Canada) was used.

From each zone, two (2) farms or collecting sites were chosen. The sampling was therefore realized from the six farms chosen randomly from the 3 papaya producing zones. During the sampling, papayas were checked for obvious symptoms of rots. Fungal mycelium or fruiting bodies on the fruits indicated infection. The papayas with rot symptoms were collected for fungal isolation. A sample consists of an infected papaya of 400 to 500 g taken directly from a papaya tree. For each sampling day, 12 samples were collected from each farm, making it 144 samples per zones and a total of 432 samples from the 6 collecting sites over a period of 9 months. Each sample was placed inside a sterile plastic bag, labelled and stored in an ice box and transported to the laboratory for analysis.

Isolation and identification of the fungi

From samples with symptoms of rots, parts of the diseased tissue area were either directly cultivated on Sabouraud agar (Biomérieux, France), or twenty five grams (25 g) of the infected area of each sample were placed aseptically in 225 ml of buffered peptone water (Merck, Germany) contained in a sterile plastic bag and mixed in a stomacher (laboratory blender Model 80 Seward Medical, London) for 30 seconds at normal speed into a homogeneous mixture. 1 ml of the homogenate was serially diluted in an aseptic condition and used for the isolation of the fungi. 0.1 mL of each dilution was spread-plated onto Sabouraud agar. The plates were incubated at 28 ± 2°C for 3 to 5 days until the sporulation of the fungus. During this incubation, Petri dishes were often checked and the fungi purified. Fungal isolates were identified by culture appearance and microscopical observation using taxonomic keys (André, 1997; Gams et al., 1998; Boerema et al., 2004), and recorded to determine the predominant type of fungi from the collected samples. These fungi were then used to test the antifungal activity of the PHMGH disinfectant.

Determination of the antifungal activity of the PHMGH

Inoculum preparation

The inoculum was obtained according to the method described by Moroh et al. (2008). The strains of moulds were plated on Sabouraud agar with chloramphenicol (Biomérieux, France). The plates incubated at 30° C for 48 h. Part of a 48 h colony from each strain was added to 10 mL of Sabouraud broth (Biomérieux, France) to achieve a final concentration of 10^6 cfu/ mL (equivalent to MC- Farland test tube number 3 (0.5 Marc-Farland)). These inocula served for the determination of the MIC and MFC.

Preparation of the different concentrations of PHMGH

The different concentrations of PHMGH were performed on Sabouraud broth. A series of solutions of different concentrations were made using the method described by Oulé et al. (2008). Serial dilutions from 0.01 to 1.9 mg/ mL of the Polyhexamethylene-guanidine hydrochloride PHMGH were made in the growth medium, inside 15 x 150 mm test tubes labelled from T₁ to T₁₀. The following concentrations were prepared to determine the MIC across the 10 /11 dilutions: 0.01- 0.04 - 0.07- 0.1- 0.4- 0.7- 1- 1.3- 1.6 and 1.9 mg/ mL. Two tubes, T₀ and Tc containing 10 mL of Sabouraud broth only

were considered as mould growth controls (Tc) or no growth control (T_0) .

Determination of the PHMGH's MIC and MFC

The MIC (mg/ mL) was taken as the lowest concentration of PHMGH in broth tube which did not show any turbidity. The MIC was determined using the NCCLS two-fold serial dilution broth method (National Committee for Clinical Laboratory Standards, documents M-38A for filamentous fungi, 2000). One (1) mL of each inoculum of the mould isolated was added to each of the tube from T₁ to T₁₀ and also T₀ containing 10 mL of the disinfectant. Before incubation, a first reading of the optical density (di= initial optical density) was measured with a spectrophotometer (Milton Roy Spectronic 601) at 560 nm. The tubes were incubated at 30°C for 48 h, then a second reading of the optical density induced by the growth of the fungi studied was performed giving a final optical density (df: final optical density). Fungi growth was indicated by the turbidity and the absence of growth meant an inhibitory activity. The variation in turbidity induced by the growth of the fungi according to the concentration of the PHMGH was determined. The MIC was determined by measuring the optical density induced by the growth of the microorganism studied. Thus, the MIC (mg/ mL), was the lowest concentration of PHMGH at which the initial optical density (di) is equal to the final optical density (df) (Moroh et al., 2008).

The MFC was the lowest concentration of antimicrobial agent (PHMGH) at which the tested organism was killed. That means the concentration of PHMGH that did not show any growth on a new set of agar plates. To determine the MFC, a sample (0.1 mL) taken from each test tube used to determine the MIC which did not show any turbidity was streaked on Sabouraud agar plates without the PHMGH and incubated at 30°C for 48 to 72 h. The growth plates was compared to those of the control consisted of a plate of Sabouraud with chloramphénicol not inoculated. Each test was performed in duplicate and repeated three times. The determinations of the MICs were carried out in duplicate, repeated three times and the mean values were used. The lowest concentrations of the PHMGH that did not permit any visible growth on the plates after 48 to 72 h of incubation at 30°C were recorded as the MFCs.

Determination of the percentage of survivors

The percentage of survivors was done according to the method described by Moroh et al. (2008) and Zrihi et al., (2007) and allowed the determination of the PHMGH median inhibition concentration (IC₅₀). The IC₅₀ was the lowest concentration of the PHMGH that exhibits 50% of survivors. The percentage of survivors was determined from the tubes used for the MIC determination. Thus, the variation in the optical density value (OD) obtained for the tube Tc (growth control) represents 100% survivors. The values obtained for the ten other experimental tubes were then expressed as a percentage of survivors using the following formula:

$$S(\%) = \frac{(df-di)}{(Df-Di)} \times 100$$

Where S= percentage of survivors, di = initial OD, df = final OD (after incubation), Df = OD value of the control tube before incubation, Di = OD value of the control tube after incubation.

Determination of the efficacy of the PHMGH

A ratio was established between the MIC and the MFC values to

demonstrate the PHMGH fungicidal activity for the fungi studied (Moroh et al., 2008). According to Marmonier (1990), when the ration of the MFC: MIC of an antimicrobial agent is below 4 (\leq 4), this agent is qualified as microbicidal. However, if this ratio is above 4 (> 4), the agent is microbistatic. Thus, if the PHMGH MFC: MIC ration is \leq 4, the PHMGH will be qualified as fungicidal ou fungistatic if this ratio is above 4. Each test was performed in duplicate and repeated three times.

Statistical analysis

The data were submitted to an analysis of variance, followed by Duncan multiple comparison test. Mean values of the frequency of isolation of the strains of fungi and percentage of survivors were compared. In order to determine which means were significantly different from others, differences between means were assessed by Duncan's multiple range test at $\alpha = 0.05$.

RESULTS

Thirty one strains of fungi were isolated as Mucor sp., Botrytis sp., Penicillium sp., Geotrichum sp., Aspergillus sp. and *Colletotrichum* sp. The prevalence of the strains isolated per collecting site was presented in Figure 1. Among them, Mucor and Botrytis were predominant with a frequency of isolation of 66.67 and 33.33% for the site 1 samples, 41.67 and 75% for those of site 2, and 33.33 and 33.33% for site 3 samples. Geotrichum was identified only on the collecting site 3 with a prevalence of 16.67%. Colletotrichum and Penicillium were isolated only on site 1 samples with a frequency of 11.11 and 16.67% respectively. Except Geotrichum, the strains of fungi were found on site 1 samples, followed by site 2 and 3 with only 3 strains isolated per site. Mucor sp., Botrytis sp., and Aspergillus sp. for site 2 samples, Mucor sp., Botrytis sp. and Geotrichum sp. for site 3 samples. Mucor and Botrytis were detected with a high prevalence, 52.77 and 47.22% respectively from all the papaya sampled analysed (Figure 2). The frequency of isolation was not significantly different for these two strains (P>0.05). The less isolated strains of fungi were Penicillium sp (8.33%), Aspergillus sp. (8.33%), Colletotrichum sp. (5.56%) and Geotrichum sp. (2.78%). There was no significant difference in the frequency of isolation for these 4 strains (P >0.05).

Effect of the PHMGH on the growth of the fungi

Table 1 shows the growth of the fungi at different concentrations of the PHMGH disinfectant. The susceptibility test revealed the presence of an antifungal activity of the disinfectant at various concentrations. Since the lowest concentration that inhibited *Botrytis* sp. and *Colletotrichum* sp. was 0.01 mg/ mL, this concentration can be defined as the MICs for the PHMGH against these microorganisms tested. Thus, *Botrytis* sp. and *Colletotrichum* sp. were the most



Figure 1. Frequency of isolation of the fungi according to papaya collecting sites.



Genera of molds isolated

Figure 2. Prevalence of the fungi isolated in all the papaya samples studied curves with the same letters are not significantly different (α >0.05).

Table 1. Effect of different conce	entrations of the PHMGH on the	growth of the fungi strains	tested with the broth dilution technique
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	Concentrations of PHMGH (mg / mL)										
Strains of fungi tested	Tc (0.00)	T1 (0.01)	T2 (0.04)	T3 (0.07)	T4 (0.1)	T5 (0.4)	Т6 (0.7)	T7 (1)	T8 (1.3)	T9 (1.6)	T10 (1.9)
Mucor sp.	+	+	+	+	+	+	-	-	-	-	-
<i>Botrytis</i> sp.	+	-	-	-	-	-	-	-	-	-	-
Penicillium sp.	+	+	+	+	+	-	-	-	-	-	-
Geotrichum sp.	+	+	+	+	+	+	-	-	-	-	-
Aspergillus sp.	+	+	+	+	+	+	+	+	+	+	-
Colletotrich sp.	+	-	-	-	-	-	-	-	-	-	-

The plus sign + indicates a microbial growth in broth after exposure to PHMGH and the minus sign - indicates no growth; T = test tube numbers; Tc = mould growth controls. Collectorich = Collectorichum.

Strains of fungi tested	MICs (mg/mL)	MFCs (mg/mL)			
<i>Mucor</i> sp.	0.7	0.7			
<i>Botrytis</i> sp.	<0.01	<0.01			
Penicillium sp	0.4	0.4			
Geotrichum sp.	0.7	0.7			
Aspergillus sp.	1.9	1.9			
Colletotrichum sp.	<0.01	<0.01			

 Table 2. The minimal inhibitory concentrations (MICs) and minimal fungicidal concentrations (MFCs) for the PHMGH against the molds tested.



PHMGH disinfectant concentration (mg/m])

Figure 3. Percentage of survivors of the molds isolated according to the PHMGH disinfectant concentration.

sensitive to the effect of PHMGH. The highest concentration was recorded with *Aspergillus* sp. for which a concentration of 1.9 mg/ mL was needed to inhibit its growth. Therefore, *Aspergillus* sp. with a MIC value of 1.9 mg/ mL was the most resistant strain. The concentration that inhibited the other strains (*Mucor, Geotrichum* and *Penicillium*) with an intermediate susceptibility was 0.7 mg/ mL, 0.7 mg/ mL and 0.4 mg/ mL respectively. The results obtained in this work indicated that the effect of PHMGH on the growth varies according to the strain of mould.

Table 2 shows the MICs and MFCs values of the PHMGH against the tested organisms. When aliquots from the tubes where no growth was observed were inoculated in Sabouraud agar without PHMGH for 72 h at 30°C, no growth was observed. The values of MFC were equal to those of the MIC. Figure 3 showed the percentage of survivors of the fungi exposed to different concentrations of the PHMGH. The inactivation of the

fungi presented a decreasing pattern to an undetectable level at 0.01 mg/ mL for *Colletotrichum*, followed by *Penicillium* at 0.4 mg/ mL, *Mucor* and *Geotrichum* at 0.7 mg/ mL, *Aspergillus* at 1.9 mg/ mL. *Aspergillus* and *Geotrichum* presented the same median inhibition concentration (IC₅₀) which is about 0.05 mg/ mL. However, the median inhibition concentration for *Mucor*, *Penicillium*, *Botrytis* and *Colletotrichum* was <0.01. No matter what type of fungi was tested, the MFC has the same value as the MIC. The ratio of MFC: MIC gave the value of 1 for the strains studied. This value is less than 4, thus the PHMGH was fungicidal for the mould isolated from the mouldy papaya fruit studied.

DISCUSSION

The strains of fungi isolated in this study were similar to those reported in the literature regarding papaya spoilage, except *Penicillium* sp. and *Geotrichum* sp. Species of *Colletotrichum*, *Phoma*, *Phomopsis*,

Phytophthora, Alternaria, Mucor, Botrytis as well as Aspergillus have been reported as common postharvest fungi frequently responsible for papaya spoilage (Luciana et al., 2008; Hadizadeh et al., 2009; Naureen et al.,

2009). Phoma, Phomopsis, Phytophthora and Alternaria were not detected in the papaya samples examined in this study. The absence of these 4 strains could be explained by the location of the sampling sites and the climatic conditions. Côte d'Ivoire is an equatorial country; the climate is warmer and wetter than in temperate zones. It should be noted that some strains of mold such as *Alternaria* sp grow preferentially on plants at low temperatures (10 to 12°C). This could explain that despite its great involvement in the degradation of papaya, it was not found on any of the samples analyzed.

According to Joseli et al. (2002), the anthracnose, a mildew caused by Colletotrichum gloeosporioides contributes significantly in the postharvest losses of the papaya. The presence of *Colletotrichum* on the papaya samples studied could be explained by the fact that this strain of mold can infect the intact immature green fruits, not injured in the field as indicated by Noel et al. (2002). The presence of the other fungi isolated could be attributed to their persistence as spores in the environment, the soil and decaying organic matter. Mucor and Botrytis were the major contaminant isolated. Botrytis is known to be responsible for the gray rot of many foods while *Mucor* is involved in the post-harvest rots of many fruits and other foods. Moreover, species of the genera Penicillium, Aspergillus, Botrytis and Geotrichum are reported as common fungi to the post-harvest problems (Jorjandi et al., 2009; Naureen et al., 2009).

Sampling site 1 (Azaguié) samples seemed to be infected by most of the strains isolated except Geotrichum. In fact, the soils in the site 1 zone were wet. Therefore, the ambient temperature $(30 \pm 2^{\circ}C)$ and the high relative humidity are factors that could promote microbial activity, particularly fungi and may explain the greatest number of fungi in this area. In all the countries with a warm and wet climate, the moisture and the poor agronomic conditions are favourable to fungi growth and product contamination which could consequently affect the food quality. It is also under these conditions that produce toxic pathogenic fungi may secondarv metabolites, namely mycotoxins. The presence of Aspergillus and Penicillium implies a risk of mycotoxins such as aflatoxin and ochratoxin production and could represent a health risk for the consumers (Tahani et al., 2008; Reddy et al., 2010).

The PHMGH showed strong activity on the *in vitro* growth of the six (6) strains of fungi isolated from the papaya fruit. Indeed, the growth of the filamentous fungi decreases progressively as the concentration of the PHMGH increases. However, the MIC and MFC vary according to the strain of fungi. The PHMGH was active

to varying degrees and exhibits an antifungal activity by inhibiting the *in vitro* growth of the mould.

Colletotrichum and Botrytis were the most susceptible fungi to the PHMGH and Aspergillus, the most resistant. The difference of sensitivity between the genera of mould investigated could be explained by the difference of the cell wall and cytoplasmic membrane composition. According to McDonnell and Russell (1999) and Oulé et al. (2008), the main target of the PHMGH is the cell envelope. The product penetrates the cell envelope, and attacks the membrane. Indeed, the PHMGH provokes the degradation of the cell envelope, the destruction of the cytoplasmic membrane and a release of the cellular contents (Guan et al., 2008; McDonnell and Russell, 1999), thereby causing death of the cell. Microbial cells that are less sensitive to PHMGH have therefore a rigid envelope compared to those who are very sensitive. Aspergillus could therefore be thought to have a rigid envelope compared to that of the other strains.

The results obtained in this study on the resistance of *Aspergillus* agreed with those of Ouattara et al. (2009). These authors indicated that some species of *Aspergillus* such as *Aspergillus fumigatus* were known to be particularly resistant to therapeutic agents. In addition, *Aspergillus niger* is one of the most frequently resistant microorganisms to antifungal agents. It was reported that resistance to disinfectants and antiseptics can be acquired by mutation or acquisition of plasmids or transposons (McDonnell and Russell, 1999; Poole, 2002). Generally, the resistance genes are multifunctional.

That means that one gene can provide resistance or sensitivity to many chemical antibacterial agents (Sasatsu et al., 1995). Allen et al. (2006) indicated that the contact between the PHMGH and nucleic acids could prevent the expression of genes. The same authors reported that the PHMGH provokes deterioration of many genes of *E. coli* involved in the transcription and changes the expression of several genes.

In this study, the lowest MIC value obtained was 0.01 mg/ mL. In a similar study conducted by Oulé et al. (2008) on *Staphylococcus aureus* resistant to methicillin and *Escherichia coli* to the PHMGH, MIC values obtained were 0.04 and 0.005%, respectively. The resistance of the fungi cells could be due to their cell wall which is made of chitin, glucane and mannose. The mould cell wall is a rigid structure preventing phagocytosis and protecting the cell from mechanical, physical and chemical aggressions. Indeed, the chitin and glucane present in the wall protect them and allow them to resist to antimicrobial effects (Oulé et al., 2008).

The PHMGH disinfectant can be characterized as essentially a fungicide for all the strains isolated from the infected papaya. Indeed, the results obtained in this work showed that regardless of the strains of fungi tested, the MIC and MFC were equal and the MFC / MIC ratio was 1. These results were in accordance with those obtained by Oulé et al. (2008). These authors found the same values for the MIC and the MBC respectively for *E. coli* and *S. aureus* resistant to methicillin.

of The absence survivors for Botrytis and Colletotrichum could be explained by their high sensitivity to the disinfectant. Mucor and Geotrichum have the same MIC, but different IC₅₀. The genus Geotrichum has an IC₅₀ of about 0.05 mg / mL and Mucor an IC₅₀ <0.01 mg / mL. Mucor has therefore, a higher sensitivity to the disinfectant than Geotrichum. But, Geotrichum has the same IC₅₀ as Aspergillus, the most resistant strain isolated, followed by Geotrichum, Mucor, Penicillium and finally Botrytis and Colletotrichum.

Guan et al. (2008) stated that chemical products such as PHMGH act by destroying the plasma membrane of microorganisms. In addition, these types of products have an irreversible action and microorganisms cannot be used to; when the concentration used is microbicidal, this product cannot cause any accustomed phenomenon (FAO, 1993). The PHMGH is thus revealed as an up coming disinfectant to fight against microorganisms accustomed to chemical products used for the preservation of papaya fruit after harvesting. With its many innovative properties (odorless, colorless, non corrosive, nonvolatile, nontoxic to humans and animals, pH neutral), in addition to its significant antimicrobial activity, this disinfectant may be recommended for the disinfection and conservation of food, especially to preserve papaya fruit from mycotoxin producing fungi such as Aspergillus and Penicillium.

Conclusion

Mucor, Botrytis, Penicillium, Geotrichum, Aspergillus and Colletotrichum are the main fungi involved in the spoilage of papaya fruit in Côte d'Ivoire with Botrytis and Mucor as the major contaminants. The PHMGH exerts an antifungal activity on all the strains at low concentrations with different susceptibility. Thus the PHMGH could be part of the up coming products used in food preservation to limit the number of post-harvest losses and also contribute to improve the storage quality of food products. The use of the PHMGH for postharvest disease control of fresh produce, as a novel emerging chemical to hazardous anti-fungal treatments will allow а safer and environmentally more acceptable management of postharvest diseases.

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