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Research article

Combined Effect of Acid Salts with Clove and Cinnamon Oils on Controlling of Postharvest Decay in Carrot

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Abstract

Fungal decay in carrots is a serious problem during postharvest storage. The present work aimed to study the antifungal effect of ammonium carbonate (AC) or potassium metabisulfite (PM) in combination with cinnamon or clove oils on delaying carrot decay caused by black rot mold during chill storage. *In vitro* trials were conducted to determine the antifungal activity of those acid salts, essential oils and their combinations against selected fungi isolated from carrots. *Alternaria* sp. C7D7 isolated from carrots was inhibited by AC and PM at 0.5-1.0% w/v minimum inhibitory concentration (MIC), while cinnamon and clove oils possessed strong anti-*Alternaria* effect with 0.025% w/v MIC. The combination of AC or PM at 0.25-0.5% w/v with cinnamon or clove oils at 0.04-0.25% w/v in potato dextrose agar produced a 100% antifungal index. Synergy testing by the checkerboard method showed that the combination of AC and cinnamon oil had partial synergistic effects against the mold. The optimum concentrations of combined AC or PM and cinnamon or clove oil were selected to formulate dipping solutions for carrot treatments. Their effect on controlling black rot decay in carrots during chill storage was investigated. The treatment with the dipping solutions consisting of combined 0.5% AC with 0.25% cinnamon oil or 1% AC alone caused a lower percentage of black rot decay (13.9-19.4% decay) in carrots compared to the others during storage at 5°C for 10 weeks. These findings show the usefulness of cinnamon oil and AC or their combination on delaying black rot decay in carrots.

Keywords: *Alternaria*; antifungal effect; antifungal effect; ammonium carbonate; potassium metabisulfite

1. Introduction

Carrot (*Daucus carota* L.), one of the most commonly consumed root vegetables, belongs to the Apiaceae family, commonly known as the Umbelliferea family. Fresh carrots are sensitive to decay during storage due to fungal growth. This problem can occur throughout storage period resulting in postharvest loss. Carrot decay can be due to the action of soil-borne fungi including *Alternaria* spp. (black rot) and *Botrytis cinerea* (gray mold rot)

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(Papoutsis & Edelenbos, 2021). Moreover, some molds including *Alternaria dauci* (Alternaria leaf blight), *Aspergillus niger*, *Geotrichum candidum*, *Rhizopus oryzae* and yeasts were reported to cause spoilage of carrots (Akhtari et al., 2016; Perrin et al., 2017; Ahamad et al., 2023). Various chemical fungicides such as strobilurins and chlorothalonil are normally applied to carrots during preharvest and postharvest. These fungicides can control a wide range of diseases. However, the continuous use and improper handling of fungicides can lead to their accumulation in the environment and have a negative impact on biodiversity (Deresa & Diriba, 2023).

To control the postharvest fungal pathogens in carrots, one would prefer non-toxic methods to the environment and human. Thus, alternative techniques have been developed and applied, e.g., GRAS salt treatment (Papoutsis & Edelenbos, 2021), active coating with pectin (Ranjitha et al., 2017), and plant extract and essential oil treatments (Khetabi et al., 2022). Essential oils, which are potential natural products obtained from different spices and herbs, have been reported to possess antifungal properties (Matrose et al., 2021). Cinnamon and clove oils are rich sources of natural compounds. These plant oils have been shown to be the potent oils against several fungi (Hu et al., 2019; Gadhi et al., 2020). Studies have suggested that essential oils are probably not effective enough as a single inhibitor (Jackson-Davis et al., 2023) and should be used in combination with other hurdles for postharvest fungal control. In recent years, synergy testing has been applied to evaluate the antimicrobial interaction for some reasons such as the need to decrease the dosage of inhibitors, the development of microbial resistance, and the need to broaden the spectrum of antimicrobial properties. When two or more control methods are applied together, a good outcome such as synergistic effect can be expected. However, indifferent, additive, or antagonistic effects can also occur (Laishram et al., 2017).

Many organic and inorganic salts are classified as food-grade additives. Some salts such as carbonates, acetates, metabisulfites, sorbates, and others are approved as generally recognized as safe (GRAS) (US FDA, 2024), and have been suggested as alternative substances for controlling fungal pathogens in fresh produce (Palou et al., 2016). The use of salts as fungicides has some advantages including their availability at inexpensive cost, solubility in water, low toxicity to organisms and environment. These allow them to be suitable choices for hurdle technology used in postharvest treatment. Several GRAS salts have been reported to control black rot caused by *Alternaria alternata* in yellow pitahaya (*Selenicereus megalanthus*) (Vilaplana et al., 2018) and sour rot caused by *Geotrichum citri-aurantii* in citrus fruits (Soto-Muñoz et al., 2020). Previous studies reported the synergistic effects of essential oil combination against mold species (Pekmezovic et al., 2015; Ribes et al., 2018; Ji et al., 2019). Thus, one approach is to use a combination of cinnamon or clove oils with these GRAS salts in the formulation of carrot dipping solutions for their synergistic effects against black rot during storage.

To extend the shelf life of carrots, some researchers have relied on physical methods such as modified atmosphere packaging (Larsen & Wold, 2016) and chill storage (Condurso et al., 2020). Fresh carrots are perishable and prone to moisture loss. Chill storage of less than 10°C is essential to delay their quality deterioration. While psychrotrophic microorganisms can still grow under these conditions, their growth rate is significantly slower. Although chill storage alone does not serve as a fungal control treatment, it can alter microbiological changes, helping to prevent moisture loss and allowing plant tissues to maintain resistance against fungal invasion (Usall et al., 2016; Zhang et al., 2019). However, each fungal control treatment has limitations when used alone, and some may induce tissue damage or lead to the accumulation of unwanted chemicals (Romanazzi et al., 2016). Therefore, combining multiple control methods has been suggested to enhance effectiveness. The success of these combined treatments

depends on the compatibility of the selected methods. The aim of this research was to evaluate the antifungal activity of combining cinnamon or clove oils with specific acid salts against fungi isolated from rotting carrots. Additionally, this study assessed the synergistic effects of these combinations against black rot mold and formulated dipping solutions with optimized concentrations of cinnamon and clove oils and acid salts to delay black rot decay in carrots during chill storage.

2. Materials and Methods

2.1 Plant materials

Dried cinnamon (*Cinnamomum verum*) stem bark and clove (*Syzygium aromaticum*) buds were obtained at the local market in Bangkok, Thailand. Fresh carrots were purchased from the central market in Pathum Thani, Thailand in January 2021.

2.2 Extraction of essential oils

Dried cinnamon and cloves were ground and extracted by hydrodistillation process. Then, oil extraction was performed using a Clevenger's apparatus. Briefly, 150 g of each plant powder was placed in the distillation flask. Then, water was added and heated until the amount of oil in the receiver was steady. The distillation process was done for approximately 3 h. To remove the excess water, sodium sulfate anhydrous was added into the oil, stirred and filtered using Whatman no. 1 filter paper (Nanasombat & Wimuttigosol, 2011).

2.3 Study of contaminated fungi in carrot

Ten samples of carrot were determined for total yeast and mold count using the methods as described by Tournas et al. (2001). Each different fungal colony was transferred to Czapek yeast extract agar (CYA) and malt extract agar (MEA). After a 7-day incubation at 30°C for molds and 3 days for yeasts, the morphological characteristics of each isolate were observed. They were re-isolated to get pure culture and maintained on potato dextrose agar (PDA) and yeast malt agar (YMA) for the identification process. The mold isolates were identified morphologically using the methods as described by Samson et al. (2004), while yeast isolates were identified using an API 20 C AUX biochemical test kit (bioMérieux, France).

2.4 Molecular identification of the fungal isolate

The selected mold isolate was identified by molecular technique. Briefly, DNA was extracted by the FavorPrep™ Fungi/Yeast genomic DNA extraction mini kit (Flavorgen, Taiwan) and subjected to internal transcribed spacer (ITS)-based PCR amplification. PCR was performed using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGA TATGC-3') (Bellemain et al., 2010). The 50- µL reaction mixture was composed of 1x KAPA *Taq* ready mix (Sigma, USA) containing each 0.25 µM primer and 50 ng of genomic DNA. The reaction involved initial denaturation at 95°C for 10 min, followed by 30 cycles in series of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 90 s, with a final step of one cycle at 72°C for 10 min to final extension. The PCR product was subsequently subjected to sequencing (U2Bio company, Thailand). The nucleotide sequences were compared with other ITS1-ITS4 sequences

deposited in Genbank using the Nucleotide Basic local alignment search tool (BLASTn) program (Altschul et al., 1990). The phylogenetic analysis of ITS1-ITS4 sequences was performed by the maximum likelihood (ML) algorithm using Molecular Evolutionary Genetics Analysis (MEGA11) software with 1,000 bootstraps (Tamura et al., 2021).

2.5 Minimum inhibitory concentration (MIC) determination of acids and salts

Firstly, the MIC determination of ascorbic and lactic acids, AC and PM was conducted using the agar dilution technique (Collins et al., 2001) against the selected fungal isolates from rot carrots as well as a reference strain, *A. alternata* TISTR 3282 obtained from Bangkok MIRCEN, the Microbiological Resources Centre for Southeast Asian Region, Thailand. Briefly, stock solution of each tested sample in 10% dimethyl sulfoxide (DMSO) was diluted and mixed with molten PDA for molds and YMA for yeasts to obtain a final concentration in the range of 0.05-8% for ascorbic acid and lactic acid and 0.01-10 % for AC and PM. The spore suspension (10^6 spore/ml, 5 µL in 0.1% v/v tween 80) was then inoculated onto the medium surface and incubated at 30°C for 7 days. The presence or absence of fungal growth was examined visually. The lowest concentration inhibiting mold growth was recorded as the MIC value. Chlorothalonil and 10% DMSO were used as a positive control and a negative control, respectively. These tests were conducted in triplicate.

2.6 MIC determination of cinnamon and clove oils

The MIC test of cinnamon and clove oils as well as some positive controls (cinnamaldehyde, eugenol and chlorothalonil) against the two mold strains was performed using the same procedure as described above. However, the final concentration of all tested samples after mixing with molten PDA were different, and as follows: 0.006-0.8% of cinnamon and clove oils, 0.008-30.27 mM of cinnamaldehyde, 0.006-24.36 mM of eugenol and 50-5,000 ppm of chlorothalonil.

2.7 Synergy assay of combination of acid salt and essential oil

Agar dilution checkerboard procedure was used to conduct synergy testing of combined GRAS salts (AC and PM) with clove or cinnamon oil against the two mold strains using the protocol as described by Rosato et al., 2007. Briefly, each sample of the combination was prepared at 1/2, 1/4, 1/8 and 1/16 of their MIC. Then, each combination of essential oil and acid salt (0.125 mL each, a total of 0.25 mL) and 4.75 mL molten PDA were mixed together and left to solidify. Then, 10^6 spore/ml (5 µL) were inoculated and incubated at 30°C for 7 days. The fractional inhibitory concentration index (FICI) was calculated and interpreted (Pekmezovic et al., 2015).

2.8 Antifungal assay of acid salts in combination with clove or cinnamon oil

The antifungal activity of selected combinations of AC or PM and clove or cinnamon oil on growth of the two mold strains was tested according to Xie et al. (2017). The PDA medium was incorporated with AC or PM at 0.25-0.5% w/v and cinnamon or clove oil at 0.04-0.25% w/v. These PDA plates were inoculated with each tested mold at the center of the plate. After 7-day incubation at 30°C, mycelial growth was observed, and the diameter of radial growth was measured. The antifungal index (AI) was calculated as follows: AI (%) = (1-

$D_e/D_n) \times 100$, where D_e and D_n are the diameter of the mycelial growth on the surface of the experimental plate and the negative control plate, respectively.

2.9 *In vivo* antifungal activity of clove or cinnamon oil in combination with acid salts against *Alternaria* in carrots stored at abusive temperature

Fresh mature carrots were washed and sanitized by soaking in 0.5% v/v sodium hypochlorite and rinsing with sterile water. After surface drying, a sterile 4-mm diameter corkborer was used to puncture into the surface of each carrot to make three wounds (3 mm deep each) according to Kolaei et al. (2012) with slightly modification. All punctured carrots were divided into six groups (9 carrots each) for dipping treatments in solutions: T1, control (sterile water); T2, 7,500 ppm chlorothalonil; T3, 0.25% AC mixed with 0.125% cinnamon oil; T4, 0.5% AC mixed with 0.25% cinnamon oil; T5, 0.25% PM mixed with 0.04% clove oil; and T6, 1% AC. The T3-T6 dipping solutions were incorporated with tween 80 (0.2% v/v) and glycerol (2.5% v/v) before stirring vigorously for 1 min using hand mixer (Sokany 800W, China). Then, all carrots were left to dry, and 10^6/mL spore suspension (50 µL) of each tested mold was inoculated into each wound. Each inoculated carrot was placed on a sterile moistened container, and stored at 30°C in 95% relative humidity condition for 10 days. All carrots were examined at day 0, 6 and 10 of storage for determination of black rot control efficiency (BRCE), weight loss and pH value using Testo 207 pH meter, Testo AG, Germany. Each carrot showing visible black rot decay was sorted and measured for lesion sizes. Then, the diameter and number of lesions were assessed. The BRCE was calculated as follows: BRCE (%) = $[(N-T) \times 100]/ N$, where N is lesion diameter in a negative control sample and T is lesion diameter in a treated sample.

2.10 Application of dipping solutions in non-inoculated carrots

All mature carrots were tested using a procedure similar to that described above, but no inoculation process was performed. After surface-sterilization, carrots were treated with each dipping solution. Then, they were left to dry and stored at 5°C for 10 weeks. Black rot decay was assessed visually every 2-week time interval. Then, the percentage of black rot decay was calculated (Eshel et al., 2009).

2.11 Effect of dipping solution on membrane integrity

The mycelia of the studied mold treated with the selected potential dipping solutions were evaluated for membrane integrity using Evans blue staining technique as described by Peralta-Ruiz et al. (2020) with slightly modification. To prepare the slide culture, 30 µL spore suspension was inoculated into 100 µL potato dextrose broth (PDB) previously added on a sterile coverslip. After 7-day incubation at 30°C, the hyphae attached on the coverslip were soaked with each dipping solution (500 µL) for 6 h before rinsing with phosphate buffer saline (PBS). Then, Evans blue (1% (w/v), ACROS ORGANICS, USA) in PBS was used to stain the hyphae for 5 min before washing with PBS. The hyphae were then examined under a bright-field microscope.

2.12 Statistical analysis

Data obtained from the testing of combined acid salt and essential oil were subjected to analysis of variance (ANOVA) and Duncan's multiple range test using version 26.0 SPSS

statistical package (MBI, USA) to determine if significant difference existed between each treatment mean ($P \leq 0.05$).

3. Results and Discussion

3.1 Fungal contamination in fresh carrots

Fresh carrots were highly contaminated with fungi in the range of 1.2×10^6 - 3.1×10^7 and 2.6×10^5 - 3.2×10^7 CFU/g on DRBC and acidified PDA, respectively. The main mold isolates were *Geotrichum* spp. (26.7%) and *Absidia* spp. (20%). Other molds and yeasts found with 6.7% each were *Aspergillus* spp., *Penicillium* spp., *Geotrichum* spp., *Candida famata, Candida lustaniae*, *Trichosporon asahii*, *Rhodotula minuta* and *Trichosporon asahii*. The morphological characteristics of the selected mold isolate C7D7 were similar to those of *Alternaria*, which is a common mold isolated from rot carrots (Samson et al., 2004; Ahamad et al., 2023). It was selected for molecular identification and further study.

3.2 Morphological and molecular identification

The results of morphological and molecular identification of the mold isolate C7D7 from rot carrots (Figure 1A) are illustrated. This mold colony appeared pale brown in color with the diameter of 7.4 cm on PDA after 7-day incubation at 25°C (Figure 1B). Its conidiophore was also pale brown with smooth wall. The conidia were ellipsoidal in shape, with transverse and longitudinal septa (Figure 1C). They occurred in chains with up to 8 conidia consecutively. Each conidial chain arose from a short conidiophore. Secondary branch chains occasionally occurred.

By molecular identification of the selected fungal isolate C7D7, the PCR product of the ITS1-ITS4 fragment with an approximate size of 500 bp was amplified and sequenced. The obtained ITS1-ITS4 sequence of isolate C7D7 was deposited in GenBank with the accession number PP455647. The result of BLAST sequence analysis showed that the ITS1-ITS4 of the isolate C7D7 was highly homologous (99.81-100 %) with those of many *Alternaria* strains. Thus, the fungal isolate C7D7 was classified by molecular analysis in the genus *Alternaria* and named as *Alternaria* sp. C7D7. By phylogenetic tree analysis based on ITS1-ITS4 sequences using the maximum likelihood (ML) method, *Alternaria* could be divided into two clades (Figure 1D). Clade I was divided into 2 subgroups, IA and IB. *Alternaria* sp. C7D7 was clustered in the clade IA which included many strains such as *Alternaria alternata*, *A. arborescens*, and *A. burnsii*, whereas Clade IB contained *A. blumeae, A. solani* and *A. porri* (Figure 1D). The second clade was composed of *A. papavericola*, *A. chlamydospore, A. infectoria, A. armoraciae,* and *A. breviramosa* (Figure 1D).

In the current study, the mold grew very well on DRBC agar medium. This medium was highly recommended by Pitt & Hocking (1985), who noted that rose Bengal and dichloran (2, 6-dichloro-4-nitroaniline) had been added to the medium to decrease the spreading growth of mold colonies, enabling the accurate counting of the mold colonies. *Geotrichum* spp. was the most common mold isolated from rotted carrots. Akhtari et al. (2016) reported similar results. They found many strains of fungi contaminating carrots cultivated in the State of Odisha, India. *Geotrichum candidum* was found the most (49.1%). *Candida* spp. was also the most common yeast in carrots. Similarly, Horita & Hatta (2016) isolated molds from rotted carrots in Hokkaido, Japan. They found *G. candidum* at up to 83%.

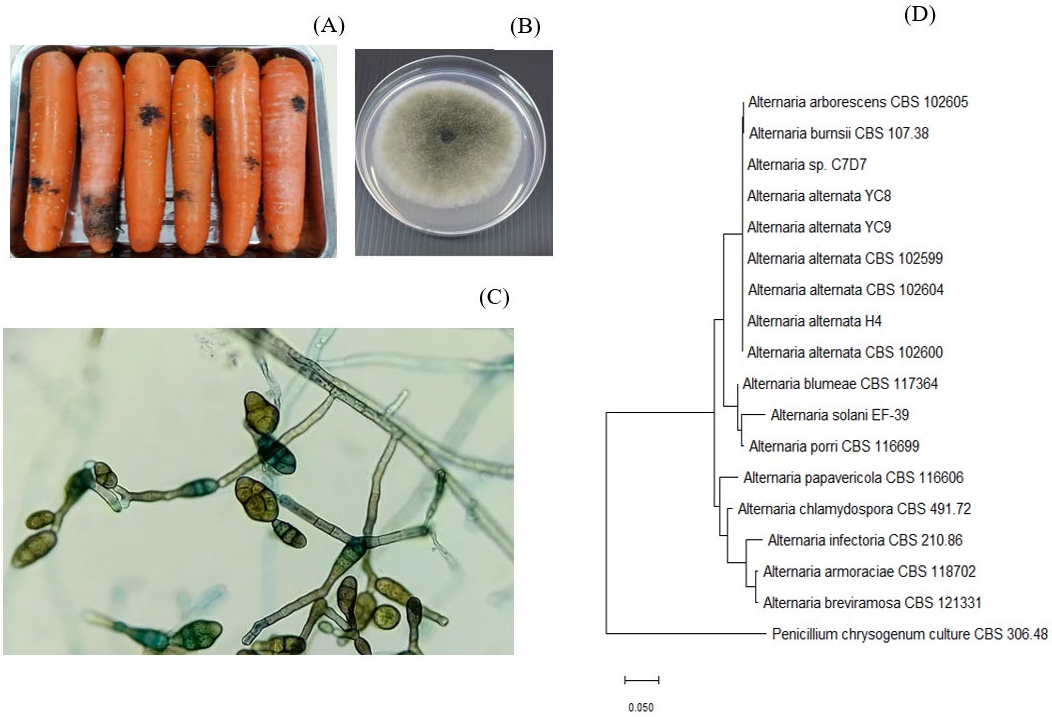

Figure 1. Morphological and molecular identification of the mold isolate C7D7 isolated from rotted carrots: (A) black rot in carrots; (B) colony morphology on PDA plate at 25°C for 7 days; (C) microscopic observation (400X); (D) phylogenetic tree analysis of ITS1-ITS4 using maximum likelihood method with 1,000 bootstraps

As previously known, the analysis of conserved ITS sequences was widely used for molecular identification of several fungi. In this study, the mold isolate C7D7 was identified as *Alternaria* by both molecular and morphological results. Interestingly, *Alternaria* sp. C7D7 was in the same cluster and showed very high similarity to many strains of *Alternaria alternata* (Figure 1D). Previous study showed that by phylogenetic tree analysis based on the ITS region, the small-spored *Alternaria* was distinct with the large-spored *Alternaria* (Landschoot et al., 2017). *Alternaria* sp. C7D7 contained spores of small size as same as *A. alternata*. Among the small-spored *Alternaria*, *A. alternata* is the most widespread strain in this group. Therefore, *Alternaria* sp. C7D7 formed a monophyletic group together with *A. alternata* species. Since there are many species classified in genus *Alternaria*, phylogenetic analysis of multilocus genes can help to distinguish among *Alternaria* species. Besides the ITS region, phylogenetic tree analyses of other genes including glyceraldehyde-3-phosphate dehydrogenase (*gpd*), endopolygalacturonase (*endoPG*), Alternaria major allergen (*Alt a1*), RNA polymerase II (RPB2), plasma membrane ATPase (ATPase), Calmodulin , translation elongation factor 1-alpha (TEF 1-α) have been reported in the study of the genetic diversity of *Alternaria* species (Andrew et al., 2009; Deng et al., 2013; Lawrence et al., 2016; Wang et al., 2021; Woudenberg et al., 2013; Zhu & Xiao, 2015).

3.3 Antifungal activity of acids and salts

The MIC values implied that AC and PM could inhibit the growth of all fungal strains more than lactic and tartaric acids. Mostly, AC possessed more potent antifungal activity compared to PM. The growth of two *Alternaria* strains was restricted by AC and PM at 0.5% and 1%, respectively. However, the yeast isolate, *Candida famata* C1A3 was more sensitive to AC with the MIC of 0.25% compared to other yeast strains (Table 1).

Table 1. Antifungal activity of acids and salts

Fungal strains	Minimum Inhibitory Concentration (% w/v)			
	Salts		Acids	
	Ammonium Carbonate	Potassium Metabisulfite	Ascorbic Acid	Lactic Acid
Molds				
Alternaria sp. C7D7	0.5	1.0	8.0	2.0
Alternaria alternata TISTR 3282	0.5	1.0	8.0	1.0
Yeasts				
Candida lusitaniae C1D3	0.5	0.5	>8.0	4.0
Candida famata C1A3	0.25	0.5	>8.0	4.0
Rhodotorula minuta C1A4	0.5	1.0	>8.0	>8.0
Trichosporon asahii C9D7	0.1	1.0	>8.0	>8.0

The current study revealed that AC effectively inhibited mold growth. This was in agreement with those reported by Türkkan (2013). Similarly, some researchers previously confirmed the strong inhibitory effect of carbonates and succeeded in using these salts to control the growth of *Monilinia fructicola* on plums (Karaca et al., 2014). Carbonate anions are responsible for the growth inhibition of fungal pathogens. Their effect on suppression of mold growth was likely related to their alkaline pH value, which was found to be 8.00-8.39 for 0.25-1.0% AC solution used in the current study. Most microorganisms are vulnerable to this pH range. A number of researchers previously confirmed the strong inhibitory effect of chemical sanitizers such as organic acids and their salts. In case of PM, which is a sulfur-containing salt, was quite effective at 0.5-1%. Its efficacy was revealed as an effective fungicide to control the growth of pathogenic fungi (Arslan, 2015). Metabisulfites at 10-100 mM in PDA were reported to completely inhibit mycelial growth of citrus blue mold, green mold and sour rot mold (Martínez-Blay et al., 2020). PM is a sulfur dioxide salt approved for use in food, especially in fruit and vegetable products for controlling spoilage and fermentative fungi. It has been suggested that metabisulfites act through sulfur dioxide liberation. The interaction of liberated sulfur dioxide with cellular components produces antifungal activity (Papoutsis & Edelenbos, 2021).

3.4 Antifungal activity of essential oils

Cinnamon oil displayed stronger antifungal activity against *Alternaria* sp. C7D7 and *A. alternata* TISTR 3282 (0.0125-0.025% MIC) than clove oil. *Alternaria alternata* TISTR 3282 was more susceptible to cinnamon oil compared to the other strains. The positive controls, cinnamaldehyde and eugenol had antifungal activity against these two *Alternaria* strains. *Alternaria* sp. C7D7 was more resistant to eugenol than *A. alternata* TISTR 3282 (Table 2).

Table 2. Antifungal activity of essential oils and active compounds against *Alternaria*

Essential Oils and Compounds	Minimum Inhitbitory Concentration	
	***Alternaria* sp. C7D7**	***Alternaria alternata* TISTR 3282**
Cinnamon oil (%w/v)	0.025	0.0125
Clove oil (%w/v)	0.025	0.05
Cinnamaldehyde (mM)	7.60	0.05
Eugenol (mM)	>24.40	6.10
Chlorothalonil (ppm)	2,000	2,000

The MIC test of the cinnamon and clove oils revealed the strong antifungal effect of these essential oils against all tested fungi. Cinnamon oil possessed stronger antifungal activity than clove oil. These results were in agreement with those reported by Yooussef et al. (2016). They reported that cinnamon oil at ≥ 1,000 ppm completely inhibited the mycelial growth of the tested fungi, while clove oil needed higher concentrations (≥ 1,500 ppm). This was probably due to the action of their active components. Cinnamaldehyde and eugenol are the two main antifungal compounds in these two oils (Xie et al. 2015; Ribeiro-Santos et al., 2017). Cinnamon oil from cinnamon stem bark was reported to contain 60.58 g cinnamaldehyde/100 g oil (Bakr et al., 2024), while clove oil was found to contain eugenol (80.15%) as the main constituent (Sethunga et al., 2023). Thus, it can be assumed that the activity of these two oils was due to the presence of cinnamaldehyde and eugenol.

3.5 Synergy characteristic of combined acid salt and essential oil

Combined ammonium carbonate and cinnamon oil could inhibit the growth of *Alternaria* sp. C7D7 with partial synergistic effect as the FIC index was between 0.5 and 0.75. However, the combination of potassium metabisulfite and clove oil showed synergistic effect against *A. alternata* TISTR 3282 with the FIC index of less than 0.5 (Table 3).

Table 3. Synergy effect of combined acid salt and essential oil against *Alternaria*

Combinations	MIC		MIC_C		FIC		FICI	Interpretation of FICI
	MIC_1	MIC_2	MIC_{C1}	MIC_{C2}	FIC_1	FIC_2		
Alternaria sp. C7D7								
Combination of ammonium carbonate (1) and cinnamon oil (2)								
	0.5	0.025	0.03125	0.0125	0.0625	0.5	0.5625	Partial synergism
Combination of potassium metabisulfite (1) and clove oil(2)								
	1.0	0.025	0.5	0.0125	0.5	0.5	1.0	Indifference
Alternaria alternata TISTR 3282								
Combination of ammonium carbonate (1) and cinnamon oil (2)								
	0.5	0.0125	0.25	0.00625	0.5	0.5	1.0	Indifference
Combination of potassium metabisulfite (1) and clove oil(2)								
	1.0	0.05	0.125	0.00625	0.125	0.125	0.25	Synergism

MIC_1 is MIC of salt (1) alone, and MIC_2 is MIC of oil (2) alone. MIC_{C1} is MIC of salt (1) combined with oil (2), and MIC_{C2} is MIC of oil (2) combined with salt (1). FIC_1 was calculated using MIC_{C1} divided by MIC_1, and FIC_2 was calculated using MIC_{C2} divided by MIC_2. The sum of FIC_1 and FIC_2 is FIC index (FICI); FICI ≤ 0.5 indicates synergistic effect; 0.5<FICI≤0.75 indicates partial synergistic effect; 0.75< FICI≤ 2 indicates indifference; FICI>2 indicates antagonistic effect.

3.6 Combined effect of acid salts and cinnamon or clove oils on growth inhibition of *Alternaria*

Our preliminary study revealed the effect of single acid salt (AC and PM) and essential oil (cinnamon and clove oils) on mycelial growth inhibition of *Alternaria* sp. C7D7 and *A. alternata* TISTR 3282 on PDA. AC at 0.25-1% could inhibit the growth of *Alternaria* sp. C7D7 and *A. alternata* TISTR 3282 with 21.9-95.3% and 27.9-100% AI, while PM at 0.25-1% showed 67.2-100% and 56.4-100% AI, respectively. For the inhibitory effect of single essential oils, almost all concentrations of cinnamon and clove oils at 0.04-0.25% on PDA could completely inhibit the mycelial growth of the two *Alternaria* strains with 100% AI. However, cinnamon oil at 0.04% was found to inhibit *Alternaria* sp. C7D7 and *A. alternata* TISTR 3282 with only 72.5% and 52.7 AI, respectively. Similarly, the combinations of PM (0.25-0.5%) and clove oil (0.04-0.06%) were also strong with 100% AI against these two mold strains (Table 4). However, 64.29-68.93% AI of *Alternaria* sp. C7D7 was found when this mold strain was grown on PDA added with combined 0.04-0.06% cinnamon oil and 0.25-0.5% AC, but 100% AI was observed at higher concentrations. Similarly, all combined cinnamon oil (0.04-0.25%) and ammonium carbonate (0.25-0.5%) in PDA completely inhibited the growth of *A. alternata* TISTR 3282. Based on the synergistic interaction, some of these combinations were selected for use in the dip treatment of carrots.

Table 4. Antifungal activity of combined acid salt with essential oil on potato dextrose agar against *Alternaria*

Treatments	Antifungal index[x] (%) ± SD[y]	
	***Alternaria* sp. C7D7**	***Alternaria alternata* TISTR 3282**
AC (0.25%) + Cinnamon oil (0.04%)	64.29±5.05[a]	100±0[a]
AC (0.5%) + Cinnamon oil (0.06%)	68.93±4.39[a]	100±0[a]
AC (0.25%) + Cinnamon oil (0.12%)	100±0[a]	100±0[a]
AC (0.5%) + Cinnamon oil (0.25%)	100±0[a]	100±0[a]
PM (0.25%) + Clove oil (0.04%)	100±0[a]	100±0[a]
PM (0.5%) + Clove oil (0.06%)	100±0[a]	100±0[a]

[x]Data are means of two replications. [y]Different letters in different rows of the same column indicate significant difference ($P<0.05$).

Increased concentrations of AC in combination with cinnamon oil produced an increase antifungal potential. Although the synergistic inhibitory effect of combined acid salt and essential oil against fungi has rarely been reported, only the synergistic effects of cinnamon oil and other food ingredients against foodborne mold were studied (Clemente et al., 2019). The advantage of using the oil and salt in combination as different hurdles is to lower treatment intensities. These can minimize the sensory adverse effect of the essential oil in food. Moreover, the potency of different functional groups in the essential oils with different biochemical properties may enhance their antifungal potential (Khan et al., 2017; Gurtler & Garner, 2022).

3.7 *In vivo* antifungal effect of combined acid salt and essential oil

3.7.1 Black rot control efficiency

Inoculated carrots treated with each dipping solution were stored at 30°C for rapid observation of decay patterns to delay carrot decay. The results showed that carrots treated with T2-T6 dipping solutions and inoculated with *Alternaria* sp. C7D7 or *A. alternata* TISTR 3282 had significantly higher BRCE after 6-day storage as compared to the negative control ($P<0.05$, Figure 2). After 6-day storage, the T2, T4 and T6-dipped carrots with inoculation of *A. alternata* TISTR 3282 showed higher %BRCE of 88.89, 79.17 and 71.88, respectively, compared to the others (Figure 2B). Interestingly, the T3 dipping solutions gave the best protection with 100% BRCE in carrots after 6-day storage. However, higher %BRCE were observed in T5 and T6 carrots inoculated with *Alternaria* sp. C7D7 after 10-day storage (Figure 2A).

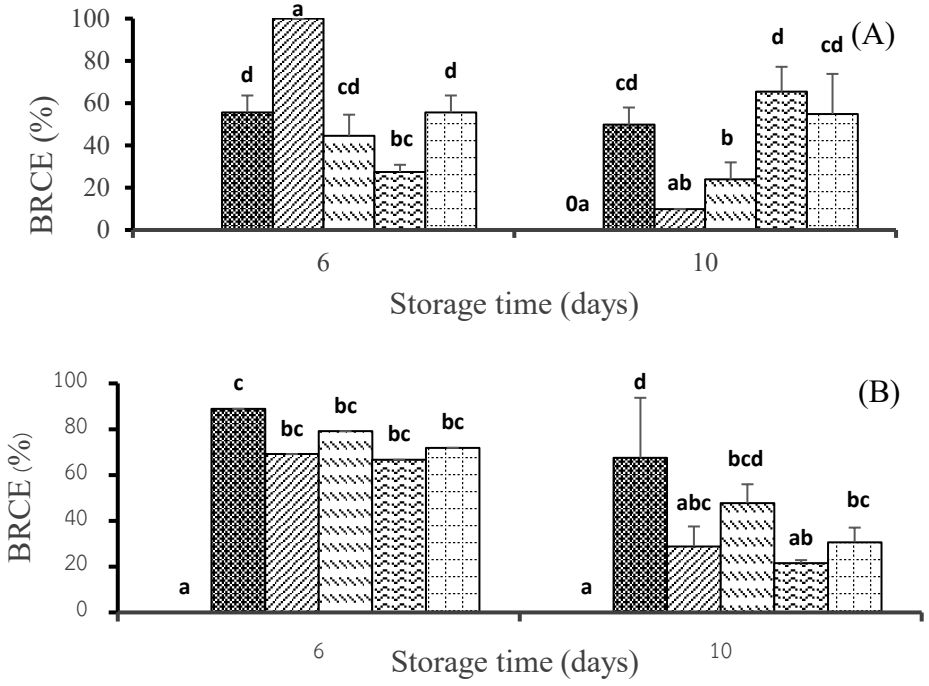

Figure 2. Effect of combined acid salt and essential oil on black rot control efficiency (BRCE) in carrots inoculated with *Alternaria* sp. C7D7 (A) and *Alternaria alternata* TISTR 3282 (B) during storage at 30°C for 10 days: T1, ■ Control (Sterile water); T2, ▩ 7,500 ppm Chlorothalonil; T3, ▨ 0.25% ammonium carbonate + 0.125% cinnamon oil; T4, ▨ 0.5% ammonium carbonate + 0.25% cinnamon oil; T5, ▩ 0.25% potassium metabisulfite + 0.04% clove oil; T6, □ 1% ammonium carbonate, Different letters in different bar chart indicate significant different ($P<0.05$).

3.7.2 Weight loss

After 6-day storage, carrots inoculated with *Alternaria* sp. C7D7 and *A. alternata* TISTR 3282 had 1.99-10.49% and 1.69-4.18% weight loss, respectively (Figure 3). After 10-day storage, weight loss increased to 5.22-8.39% and 5.42-14.02%, respectively. However, most of carrots treated with the dipping solutions had less weight loss after 6-day storage compared to the negative control samples.

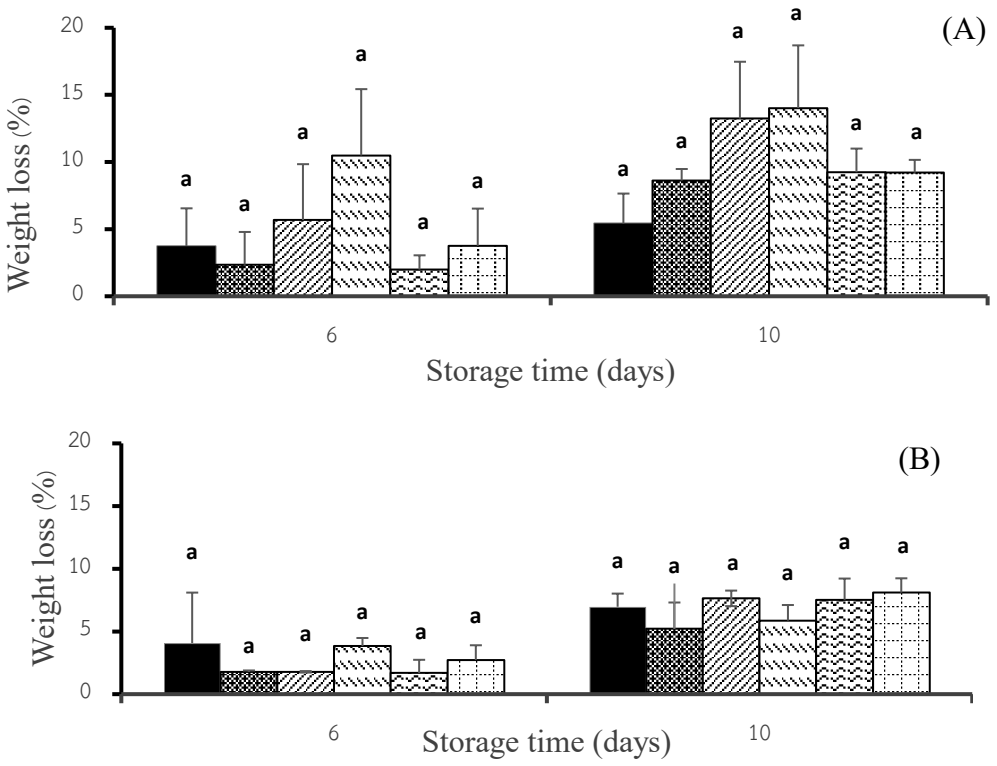

Figure 3. Effect of combined acid salt and essential oil on weight loss in carrots inoculated with *Alternaria* sp. C7D7 (A) and *Alternaria alternata* TISTR 3282 (B) during storage at 30°C for 10 days: T1, ■ Control (sterile water); T2, ▩ 7,500 ppm Chlorothalonil ;T3, ▨ 0.25% ammonium carbonate + 0.125% cinnamon oil; T4, ▨ 0.5% ammonium carbonate + 0.25% cinnamon oil; T5, ▩ 0.25% potassium metabisulfite + 0.04% clove oil ; T6, ▯ 1% ammonium carbonate: Different letters in different bar chart indicate significant difference ($P<0.05$).

3.7.3 Change of pH value

The initial pH value of carrots inoculated with *Alternaria* sp. C7D7 and those with *A. alternata* TISTR 3282 was 6.09-6.35 and 6.00-6.62, respectively. Carrot pH value decreased as storage time increased. After 10-day storage, the pH value dropped to 5.61-6.04 and 4.99-5.97, respectively (Table 5). These storage temperatures at 30°C accelerated growth and fermentation of microorganisms in carrots.

Table 5. Effect of combined acid salt and essential oil on pH value of inoculated carrots

Storage Time (Days)	Treatments	pH[x] ± SD[y]	
		***Alternaria* sp. C7D7**	***Alternaria alternata* TISTR 3282**
0	T1: Control: sterile water	6.26±0.03[ab]	6.34±0.09[ab]
	T2: 7500 ppm CTN	6.35±0.12[b]	6.62±0.04[b]
	T3: 0.25% AC + 0.125% Cin	6.22±0.00[ab]	6.23±0.04[a]
	T4: 0.5% AC + 0.25% Cin	6.19±0.08[ab]	6.34±0.25[ab]
	T5: 0.25% PM + 0.04% Clo	6.20±0.00[ab]	6.13±0.08[a]
	T6: 1% AC	6.09±0.11[a]	6.00±0.02[a]
6	T1: Control: sterile water	5.88±0.023[a]	6.15±0.05[a]
	T2: 7500 ppm CTN	5.86±0.11[a]	5.16±1.12[a]
	T3: 0.25% AC + 0.125% Cin	5.97±0.01[a]	5.57±0.22[a]
	T4: 0.5% AC + 0.25% Cin	5.92±0.11[a]	5.93±0.14[a]
	T5: 0.25% PM + 0.04% Clo	5.76±0.04[a]	4.86±0.95[a]
	T6: 1% AC	5.97±0.18[a]	5.74±0.57[a]
10	T1: Control: sterile water	5.63±0.26[a]	4.99±0.89[a]
	T2: 7500 ppm CTN	5.71±0.19[a]	5.86±0.51[ab]
	T3: 0.25% AC + 0.125% Cin	5.76±0.09[a]	5.68±0.02[ab]
	T4: 0.5% AC + 0.25% Cin	5.61±0.55[a]	5.97±0.15[ab]
	T5: 0.25% PM + 0.04% Clo	5.91±0.13[a]	5.47±0.36[ab]
	T6: 1% AC	6.04±0.04[a]	5.74±0.33[ab]

[x]Data are mean of two replications. [y]Different letters in different row of the same column indicate significant difference ($P<0.05$).
CTN, chlorothalonil; Cin, cinnamon oil; Clo, clove oil; AC, ammonium carbonate; PM, potassium metabisulfite

3.8 Effect of dip treatments in combination with chill storage on controlling of carrot decay

After 10-week storage at 5°C, control carrot samples had the highest percentage of decay (83.3%), followed by the carrot samples dipped with T2, T3 and T5 solutions (38.9%, 33.3 and 27.8% decay, respectively). Interestingly, combined chilling storage condition and dip treatments of 0.5% AC mixed with 0.25% cinnamon oil (T4), and the treatment with 1%AC alone (T6) affected lower black rot decay in carrots (13.9-19.4% decay) as compared to other treatments (Figure 4).

In the current study, the combination of AC or PM with cinnamon or clove oils affected the postharvest quality of carrots. Carrots treated with AC in combination with cinnamon oil showed delayed black rot decay during chill storage. This was probably due to the action of the active compounds combined with chilling condition. Larsen & Wold (2016) studied the effects of different storage conditions on the quality of carrots. They revealed that after 15-day storage, the percentage of rotted carrots stored at chilling conditions was lower compared to those stored at retail conditions. Regarding the weight loss of carrots, Vilaplana et al. (2018) reported that non-treated fruits had higher weight loss compared to the fruits treated with chemicals or fungicidal substances. This may be due to their thin peel which was prone to water loss. Thus, carrots should be stored at chilling temperature with high relative humidity conditions of more than 95% (Seljåsen et al., 2013).

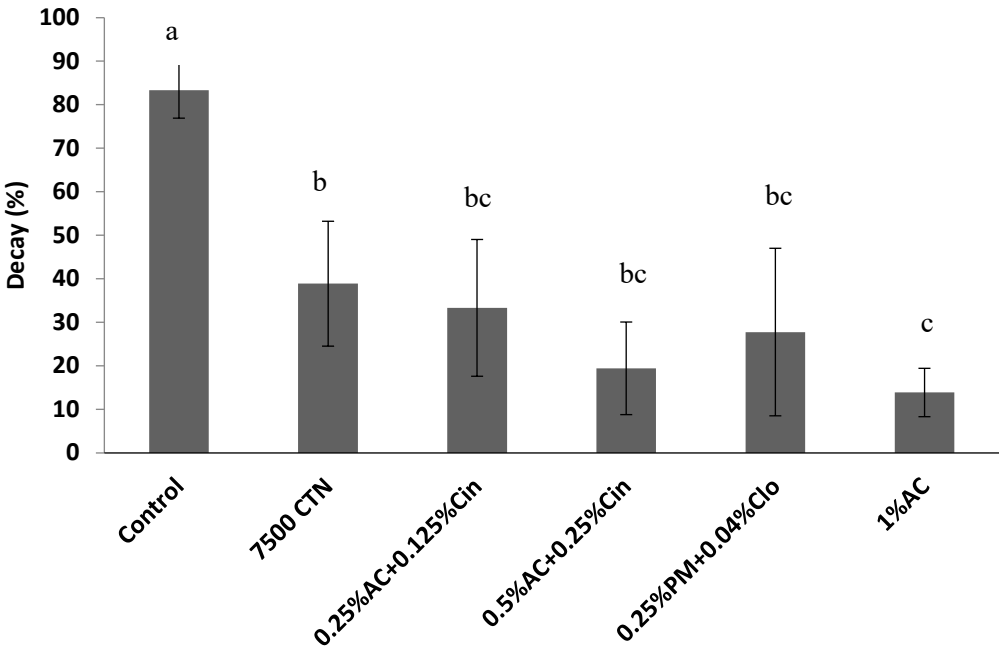

Figure 4. Effect of combined dip treatment and cold storage on controlling of black rot decay in non-inoculated carrot during storage at 5°C for 10 weeks: T1, Control (sterile water); T2, 7,500 ppm chlorothalonil (CTN); T3, 0.25% ammonium carbonate (AC) + 0.125% cinnamon oil (Cin); T4, 0.5% AC + 0.25% Cin; T5, 0.25% potassium metabisulfite (PM) + 0.04% clove oil (Clo) ; T6, 1% AC
Different letters in different bar charts indicate significant difference ($P<0.05$).

3.9 Response of dipping solution on membrane integrity

Microscopic observation of *Alternaria* sp. C7D7 mycelia treated with dipping solutions revealed some morphological alteration (Figure 5B-5F). The T4 (0.5% ammonium carbonate plus 0.25% cinnamon oil) and T6 (1% ammonium carbonate) dipping solutions, which produced a lower percentage of carrot decay, were selected for this test. Chlorothalonil (T2) solution clearly produced deformations of fungal morphology with lots of debris of conidium and mycelium fragments (Figure 5B). When the fungal hyphae were soaked with these dipping solutions, they were stained in blue which indicated dead cells. Previous researchers reported the change of cytoplasmic membrane integrity in fungal cells after treatment with chlorothalonil (2,4,5,6-tetrachloro-1,3-benzene-benzenedicarbonitrile), a broad-spectrum fungicide. The number of damage cells depended upon the fungicide concentration (Scariot et al., 2022). Moreover, morphological changes were more pronounced in hyphae treated with cinnamon oil and cinnamaldehyde which definitely implied severe damage of fungal cytoplasmic membrane and cell death (Figures 5E and 5F).

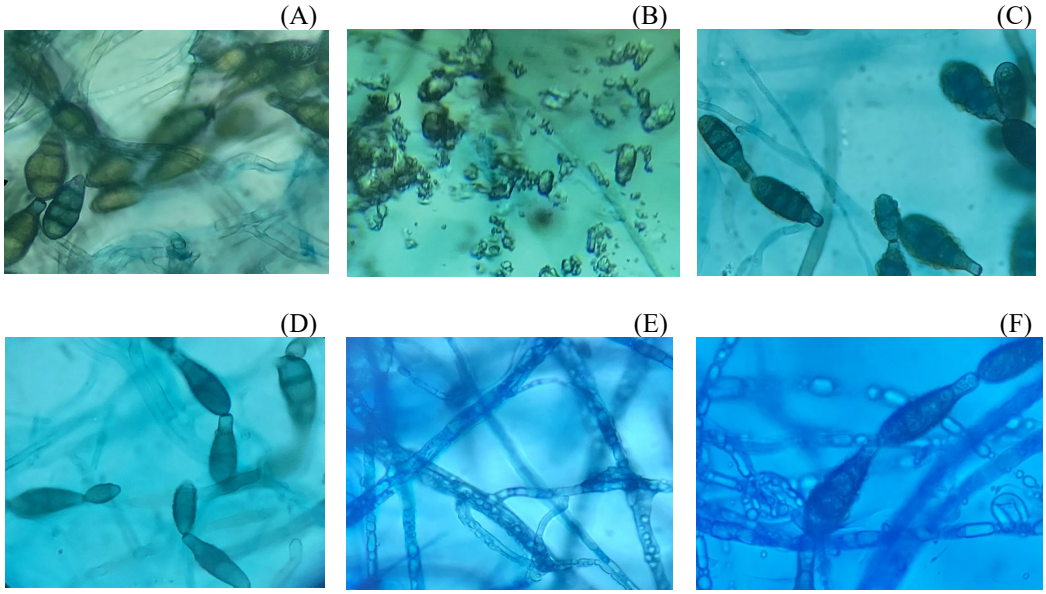

Figure 5. Microscopic observation (1000 × total magnification) of *Alternaria* sp. C7D7 after treatment with dipping solutions: (A) the negative control, sterile water (T1); (B) 7,500 ppm chlorothalonil (T2); (C) 0.5% ammonium carbonate + 0.25% cinnamon oil (T4); (D) 1% ammonium carbonate (T6); (E) the positive control, cinnamon oil; (F) the positive control, cinnamaldehyde

The antifungal activity of cinnamaldehyde and its mode of action against *A. alternata* were previously reported (Xu et al., 2018). Their study described morphological alterations in *A. alternata* hyphae and the destruction of its cytoplasmic membrane after treatment with cinnamaldehyde. As shown in the current study, Evans blue dye was used to treat the mycelia of *Alternaria* sp. C7D7 that had been previously soaked with cinnamon oil or cinnamaldehyde. Evans blue ($C_{34}H_{24}N_6Na_4O_{14}S_4$) is synthetic dye commonly used for staining to characterize cell death. Additionally, it exhibits toxicity that can inhibit the activity of various microorganisms (Kameche et al., 2022). After treatment with cinnamon oil and cinnamaldehyde, *Alternaria* sp. C7D7 appeared blue upon staining, indicating fungal cell death (Figures 5E and 5F). This suggests that the tested compounds penetrate the fungal cells, leading to their destruction. These findings support the potential use of cinnamon oil as an effective treatment for eradicating fungal infections in carrots.

4. Conclusions

This research confirmed that *Alternaria* sp. C7D7, isolated from carrots, was effectively inhibited by AC and PM. The synergistic effect was observed when these acid salts were used in combination with cinnamon and clove oils. Notably, the synergism between AC and cinnamon oil was more pronounced when used together. The application of a dipping treatment with either 0.5% AC and 0.25% cinnamon oil in combination or 1% AC alone effectively delayed black rot decay in carrots during chill storage. Therefore, this combination of acid salts and essential oils could be a valuable approach for carrot dip

treatments, helping to extend the shelf life of fresh-cut carrots during long-term chill storage.

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6. Conflicts of Interest

There are no conflicts of interest of this research project.

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