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ORIGINAL ARTICLE

Microencapsulation of *Eucalyptus globulus* essential oil anti-fungal sachet against blue mold on peaches

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Abstract

The present study was specifically designed to develop bio-fungicides that may help mitigate reliance on hazardous synthetic chemicals which give rise to environmental safety concerns. A survey (2021–2022) of local fruit markets in Islamabad, Pakistan included morpho-molecular disease identification. It revealed *Penicillium chrysogenum* as a major phytopathogenic fungi causing fruit rot. The fungicidal action of plant essential oils might be boosted by the technique of application therefore, nanoencapsulation of essential oil and chitosan was performed. To attain the objective of antimicrobial packaging, essential oil and chitosan tablets were encapsulated in spun bond sachets against *P. chrysogenum* using a sachet volatile phase technique. *In vitro* screening of nano encapsulated eucalyptus essential oil showed significant inhibition of radial growth of *P. chrysogenum* colonies at 1.6 mg · ml^{-1} followed by 1.4, 1.2 and 1.0 mg · ml^{-1}. Results of Gas Chromatography and Mass Spectrometry revealed the presence of eucalyptol in eucalyptus EO as a major antifungal component. An *in vivo* experiment analyzing the efficacy of essential oil tablets against pre-inoculated peach fruit with *P. chrysogenum* at ambient temperatures (7–37°C) showed significant reduction in lesion diameter, disease severity and prolonged shelf-life of peaches of more than 2 weeks. The natural ripening process of peach was not affected by the presence of antifungal sachets as no significant alteration in weight loss of fruit was recorded. The suppressiveness of fungal mycelial growth of *P. chrysogenum* was directly proportional to increases in *E. globulus* oil concentration. This research may have a significant impact on prolonging the shelf-life of peach fruit.

Keywords: nano-encapsulation, *Penicillium chrysogenum*, postharvest fungal rot

Introduction

Peach is the most substantial deciduous fruit of the Rosaceae family due to its tender quality, pleasant taste, and nutrient content (Hameed et al. 2022). The nutritional attributes of peach fruit, as an important indicator of fruit quality, provide other health effects. Phytopathogenic postharvest fungal rots are important issues limiting the storage period and selling life of peach fruit, and ultimately result in severe economic losses around the globe. Different postharvest fungal diseases of peach fruit include gray mold, black mold, green mold, blue mold, brown rot, softening and rotting, caused by: *Botrytis cinerea, Aspergillus niger, Aspergillus flavus, Penicillium* spp, *Monilinia* spp., *Rhizopus stolonifera, Fusarium* spp, *Alternaria* spp., *Collototrichum* spp., and *Cladosporium* spp. all over the world. All of these reduce the nutritional, medicinal and economic value of peaches and affect the storage period (Sugar 2002; Pscheidt and Ocamb 2019). Accordingly, postharvest deteriorations primarily develop from damages that occur before and, most prominently, during or after harvest. Once spores of rot fungi are inoculated into these wounds, rapid fruit

deterioration starts. Germinating conidia of fungi can also enter the intact fruit cuticle and establish themselves internally in the host (Adaskaveg *et al*. 2000). During storage *Penicillum* spp. is the major cause of postharvest rot, which affects peach quality, shortens its shelf life, and causes the production of mycotoxins (Baggio *et al*. 2016). *Penicillium* rot is a major fungal pathogen initiating blue mold on perishable fruits. It is considered to be one of the most significant and widely reported postharvest pathogens worldwide.

The marketability of peach fruit is significantly reduced by both qualitative and quantitative losses as peaches are prone to injuries especially mechanical wounds and bruises during the logistics of harvest (Santoro *et al*. 2018). Tissue rupturing of fruit occurs after physical injuries and become prone to microbe contamination. Consequently, post-injury active-control management, to increase resistance to injury-accelerated contagious decomposition, is needed. Fungicide use is very common at postharvest stages but as far as fruits are concerned, the use of fungicide after harvest is strictly prohibited by FAO (Sharma et al. 2019). The application of synthetic fungicides has rapidly increased recently. About 400,000 tons of fungicides are globally applied, which represent 17.5% of universal pesticide applications (Sharma *et al*. 2019).

Eco-friendly use of plant essential oils (PEOs) against contagious pathogens present in many horticultural products has resulted in positive outcomes that can lead to efficient options concerning fruit protection. For instance, essential oils from various natural herbs and plants have shown strong antimicrobial activity against different fungal pathogens, *in vitro*. As a result, they may be used as a natural and eco-friendly strategy against the decay of perishable fruits. However, the efficacy of plant essential oils has significant importance in restraining the mode of pathogen dispersal, by minimizing the spore load on fruit surfaces under storage conditions (Singh *et al*. 2017). Usually, plant essential oils are harmless both for the ecosystem and anthropological well-being, hence attention to their use as antifungal agents of postharvest fungal rots is increasing rapidly.

Nanoencapsulation is an emerging technique that plays a crucial role in protecting essential oils against various abiotic factors *viz,* moisture, temperature, humidity, and evaporation under various storage conditions with respect to their antifungal potential at specified controlled rates. Moreover, chitosan and tripolyphosphate nanoparticles are being researched as an impending medium of biocompatibility. These nanoparticles can also encapsulate and protect essential oils from evaporation and denaturation hence, completely protecting their antifungal nature (Fan *et al*. 2012).

Antifungal sachets enclosing volatile constituents are excellent examples of profitable antimicrobic packaging and are used widely. Bioactivity of plant EO's vapor phase is known as a distinctive practice making it effective against postharvest fungal rots in storage commodities.

Materials and Methods

A random survey of commercial fruit markets in Islamabad, Pakistan was conducted to detect postharvest fungal rot on peach fruits. Isolation, characterization, and preservation techniques were followed. For comprehensive microscopic studies, the microscopic features of postharvest fungal isolates Normarski interference contrast microscopy (USA) at 100x magnification was conducted (Aktaruzzaman *et al*. 2017). A pathogenicity test was conducted, and highly pathogenic isolates were confirmed following the percentage disease index (PDI) (Ushall *et al*. 2015).

Molecular characterization

Target gene region (ITS 1-forward primer and ITS 4-reverse primer) sequence 5'-3' CTTGGTCATTTAGAGGAAGTAA and 3'-5' TCCTCCGCTTCTTGATATGC with 600 aligned fragment length (bp) were used for genomic DNA sequencing and amplification was performed in a (Bio-Rad T100) programmable thermocycler. PCR temperature was adjusted, viz. denaturation at 94°C for 3 min, followed by 35 cycles at 95°C for 30 sec, annealing at 59°C for 1 min, and a final extension step for 10 min at 72°C, respectively. Gel electrophoresis was conducted for visualizing the PCR product using (Nanopac-300), with 1.0% agarose gel (w/v) (Act gene USA). An ExoSAP-IT DNA purification kit was used for refining and purifying the PCR product following standard protocol. A 96-well DNA sequencing plate was used loaded with primers (forward and reverse) along with 2 µl aliquots of purified DNA in a specific standard order. DNA sequencing was carried out at the Iowa State University DNA sequencing facility center (USA). MEGAX software was used for alignment of DNA sequences and were further confirmed in the National Center for Biotechnology Information (NCBI) on the GenBank database website followed by the Basic Local Alignment Search Tool (BLAST) program. Aligned sequences were submitted to NCBI for accession numbers allocation. Geneious Prime sequence analysis software was used for alignment of DNA sequences by removing all sequence gaps. For construction of a phylogenetic tree, the latest version of Molecular Evolutionary Genetic Analysis (MEGA) software was used (Tamura *et al*.

2021). Furthermore, all sequences obtained from the NCBI website along with our sequences were aligned by MUSCLE alignment in MEGAX (Kumar *et al.* 2016). A phylogenetic tree was constructed using the neighbor joining method with 1000 bootstrap values and standard cut off value.

Essential oil extraction

The drying and crushing of selected plant material for oil extraction was performed in the sun and by using a grinder machine. Extraction of essential oil was carried out with a Soxhlet apparatus following the extraction procedure (Lu and He 2010). Plant material ground into powder form was used in sufficient quantities in the Soxhlet apparatus. After evaporation in a rotary evaporator the obtained essential oil was transferred to sterilized glass containers and kept at 4°C.

Screening of eucalyptus essential oil against *Penicillium Chrysogenum*

Different eucalyptus essential oil (EEO) concentrations (1.0, 1.2, 1.4, 1.6 mg · ml^{-1}) were prepared in Tween 20 and sterilized distilled water. For *in vitro* screening of plant EO efficacy against postharvest fungal peach fruit rots, the poisoned food technique (PFT) as a reliable method was applied (Singh *et al.* 2017). The percentage of mycelial growth inhibition (MGI%) after 3 days incubation was measured and calculated according to the following formula (Sharma and Tripathi 2008):

$$\text{Mycelial growth inhibition (\%)} = \frac{dc - dt}{dc} \times 100,$$

where: *dc* mean colony diameter of control sets and *dt* – mean colony diameter of treatment sets.

GC-MS analysis

Eucalyptus (*Eucalyptus globulus)* essential oil was evaluated in a thermo scientific instrument with software version (2.0.7.1). Evaluation was conducted using a silica tube (CP-Sil) and column 5. Nitrogen was the carrier gas at a movement rate of 0.49 ml · min^{-1}. The temperature was adjusted to 250 and 300°C, at injector and detector points, respectively. Whereas the oven temperature was adjusted to 40°C with a gradual rise to 300°C isothermally for 25 minutes. Major constituents were detected followed by peak area % and retention time. Volatile constituents present in plant EO's were detected by the assessment of retention directories and mass spectra of major existing compounds by smearing a multi-dimensional process. Constituents' comparative concentrations were attained by peak area retention time. No major response factors were considered.

Nanoencapsulation of essential oil

Nanoencapsulation of the eucalyptus essential oil (EEO) was performed following the ionic gelation method according to the protocol of Sawtarie *et al.* (2017). Initially, chitosan and 1% (w/v) acetic acid were dissolved to a 1 mg · ml^{-1} concentration. The solution was sonicated and 10 ml tripolyphosphate (TPP) 1 mg · ml^{-1}) was added dropwise to a 25 ml chitosan solution (pH = 5), followed by constant stirring at room temperature, producing chitosan-TPP nanoparticles. Moreover, chitosan-TPP nanoparticles were loaded with eucalyptus EO, 20% (w/v) to the chitosan solution prior to the addition of tripolyphosphate TPP solution.

Essential oil tablets encapsulated in spun bond sachets

Tablets containing nano-encapsulated eucalyptus essential oil (EEO) were prepared by using hard paraffin wax purchased from International Petrochemicals (Pvt) Ltd. Pakistan. The wax melted at its melting point of 58–60°C. Eucalyptus essential oil concentrations (1.0, 1.2, 1.4 and1.6 mg · ml^{-1}) were added to melted paraffin wax separately. In less than 10 seconds the solution was resolidified to its original form. Small, round to oval tablets (12–16 mm) were prepared and placed in crystal vessels hermetically airtight for 24 hours at 25 ± 1°C. The nonwoven spun bond polypropylene fabric that weighed 35.4 gram was purchased from a local fabric store in Pakistan. Small sachets (4 × 4 cm) were prepared with polypropylene spun bond fabric and nano-encapsulated paraffin wax essential oil tablets were placed in sachets (2 each). Sachets were then sewed and heat-sealed at equal sides (Fig. 1).

Sachet volatile phase method

In vitro screening of the suppressive effect of essential oil tablet sachets against *P. chrysogenum* was performed by the sachet volatile phase method. For each EO concentration a single EO sachet was taped inside the lid of a PDA plate that had been pre-inoculated with a mycelial plug of the isolated pathogen. There was no direct contact between the sachet and mycelial plug. The Petri dishes were sealed and incubated at 26 ± 1°C for 7 days, after which the colony diameter was measured.

Fig. 1. Eucalyptus essential oil tablets in sachet

In vivo screening of *Eucalyptus globulus* essential oil sachet against pre-inoculated peach fruit with *Penicillium chrysogenum* at room temperature and in cold storage

Ripened, asymptomatic peaches were collected from selected commercial fruit markets in Pakistan and an *in vivo* analysis was carried out for management of *P. chrysogenum*. Selected peach fruits were dipped in 70% (v/v) ethanol for 1 minute, then washed with running tap water. A (3 mm diameter) mycelial plug of *P. chrysogenum* was placed midway between the calyx and stem ends in contact with the fruit (Chen *et al.* 2016). Four inoculated peaches were placed in surface-sterilized equal size (8"L × 8"W × 4"H) cardboard boxes (four fruits per box). A total of nine fruit boxes (four fruits per box) with treated fruits and three fruit boxes (four fruits per box) as a control were used in the experiment. *Eucalyptus globulus* essential oil tablet sachets (two sachets in each fruit box with two tablets in each sachet) of 1.4 mg · ml^{-1} and 1.6 mg · ml^{-1} were placed in each fruit box with peach fruits. The control boxes were identical to treated boxes except that the paraffin tablets in sachets contained no clove essential oil. Fruit boxes were incubated at room temperature for 9 days and in cold storage for 15 days.

A vernier caliper was used to measure lesion diameter (LD) on all DPI (days post inoculation). Disease severity was also calculated by analyzing the disease progress on peach fruits. The rating scale was: (Scale 0) 0 mm LD – no rot symptoms, (Scale 1) 1 mm ≤ LD ≤ 20 mm, (Scale 2) 20 mm < LD ≤ 40 mm, (Scale 3) 40 mm < LD ≤ 50 mm, (Scale 4) lesion diameter > 50 mm. For disease severity m = calculation the following formula was applied:

$$\text{Disease severity (\%)} = \frac{\sum \text{disease scale} \times \text{number of fruits in each scale}}{\text{highest disease scale} \times \text{number of total fruits}} \times 100,$$

Average weight loss measurement

Evaluation of weight loss was conducted consecutively every other day during storage for 9 days, using the following formula (Sharma *et al.* 2002; Asghari *et al.* 2013):

$$\text{Average weight loss (AWL) \%} = (A–B)/A \times 100,$$

where: A – weight of fresh peaches prior to storage, B – weight of treated peach fruits after end of experiment. All treatments were repeated thrice in the weight loss assessment experiment.

$$\text{Average weight loss} = \frac{\text{initial weight of fruit} - \text{total weight of fruit}}{\text{initial weight of fruit}} \times 100.$$

Statistical analysis

Statistical analysis was performed by using Statistix 8.1 and Tukey's HSD test at $p \leq 0.05$ was used for means separation followed by ANOVA. ANOVA analysis was performed for significant and non-significant interactions.

Results

Symptom based identification of our results depicted *Penicillium* rot as a major postharvest pathogen from peach fruit with bluish green sporulation on the peach surface (Fig. 2). Florida King 8-A was the most common peach cultivar abundantly found in all surveyed markets showing significant infections by blue mold. A sum of 28 isolates were morphologically identified as *Penicillium* spp. (Table 1; Fig. 3).

Pathogenicity assay

Pathogenicity assay revealed that symptoms developed by blue mold on peaches after artificial inoculation were observed to be a bluish powdery mass with light blue sporulation on the fruit's surface resembling previously cultured *P. chrysogenum* on PDA plates from peach samples. Four isolates, PEN11S, PEN43K, PEN21R, and PEN13SM, were the most infectious, initiating about 75% decaying of peach fruits as compared to the rest of the isolates where 16 isolates were observed moderately virulent, and eight isolates were seen as having low virulence (Table 1).

Molecular characterization

Molecular identification of four representative and highly virulent isolates of *Penicillium* spp. from all

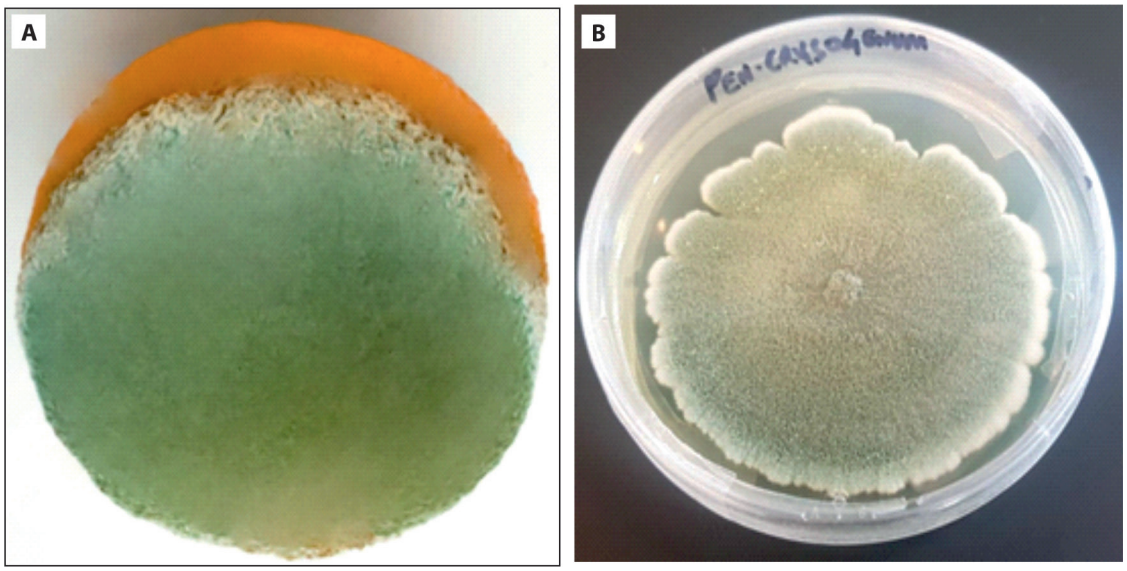

Fig. 2. A – *Penicillium chrysogenum* on peach; B – *Penicillium chrysogenum* colony growth

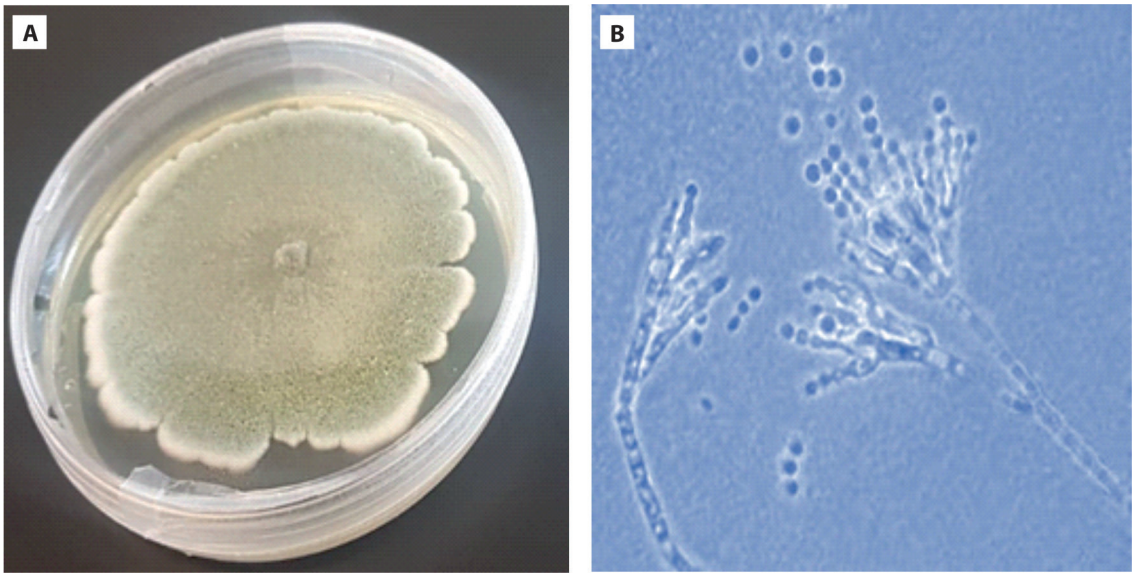

Fig. 3. A – *Penicillium chrysogenum* colony growth; B – tetra-verticillate fruiting body

Table 1. Morphological features of *Penicillium* spp. isolates ID, viz. colony color, spores and conidia

No.	Isolate ID	Colony [C]	Conidiophore [s]	Conidia [s]	L [µm]	B [µm]	Pathogenicity test
1.	PEN11S	bluish-green sporulation	mono-verticillate	ellipsoidal	3.16 ± 0.14	1.3 ± 0.07	1
2.	PEN12S	bluish-green sporulation	tetra-verticillate	spherical	3.17 ± 0.2	2.64 ± 0.16	2
3.	PEN13S	bluish-green sporulation	mono-verticillate	ellipsoidal	3.34 ± 1.15	2.42 ± 0.17	5
4.	PEN14S	bluish-green sporulation	mono-verticillate	sub-globose	3.4 ± 0.8	2.34 ± 0.41	3
5.	PEN41K	bluish-green sporulation	tetra-verticillate	ellipsoidal	3.1 ± 0.08	3 ± 0	1
6.	PEN23R	bluish-green sporulation	mono-verticillate	ellipsoidal	3.16 ± 0.14	1.3 ± 0.07	1
7.	PEN24R	bluish-green sporulation	tetra-verticillate	spherical	3.17 ± 0.2	2.64 ± 0.16	2
8.	PEN42K	bluish-green sporulation	mono-verticillate	ellipsoidal	3.16 ± 0.14	1.3 ± 0.07	1
9.	PEN43K	bluish-green sporulation	tetra-verticillate	spherical	3.17 ± 0.2	2.64 ± 0.16	2
10.	PEN44K	bluish-green sporulation	mono-verticillate	ellipsoidal	3.34 ± 1.15	2.42 ± 0.17	5
11.	PEN21R	bluish-green sporulation	mono-verticillate	sub-globose	3.4 ± 0.8	2.34 ± 0.41	3

Table 1. Morphological features of *Penicillium* spp. isolates ID, viz. colony color, spores and conidia – continued

No.	Isolate ID	Colony [C]	Conidiophore [s]	Conidia [s]	L [µm]	B [µm]	Pathogenicity test
12.	PEN22R	bluish-green sporulation	tetra-verticillate	ellipsoidal	3.1 ± 0.08	3 ± 0	1
13.	PEN25R	bluish-green sporulation	mono-verticillate	ellipsoidal	3.34 ± 1.15	2.42 ± 0.17	5
14.	PEN1KP	bluish-green sporulation	mono-verticillate	sub-globose	3.4 ± 0.8	2.34 ± 0.41	3
15.	PEN3KP	bluish-green sporulation	mono-verticillate	ellipsoidal	3.16 ± 0.14	1.3 ± 0.07	1
16.	PEN9SM	bluish-green sporulation	tetra-verticillate	spherical	3.17 ± 0.2	2.64 ± 0.16	2
17.	PEN10SM	bluish-green sporulation	mono-verticillate	ellipsoidal	3.34 ± 1.15	2.42 ± 0.17	5
18.	PEN11SM	bluish-green sporulation	mono-verticillate	sub-globose	3.4 ± 0.8	2.34 ± 0.41	3
19.	PEN12SM	bluish-green sporulation	tetra-verticillate	ellipsoidal	3.1 ± 0.08	3 ± 0	1
20.	PEN13SM	bluish-green sporulation	mono-verticillate	ellipsoidal	3.16 ± 0.14	1.3 ± 0.07	1
21.	PEN14SM	bluish-green sporulation	tetra-verticillate	spherical	3.17 ± 0.2	2.64 ± 0.16	2
22.	PEN15SM	bluish-green sporulation	mono-verticillate	ellipsoidal	3.34 ± 1.15	2.42 ± 0.17	5
23.	PEN1RT	bluish-green sporulation	mono-verticillate	sub-globose	3.4 ± 0.8	2.34 ± 0.41	3
24.	PEN2RT	bluish-green sporulation	tetra-verticillate	ellipsoidal	3.1 ± 0.08	3 ± 0	1
25.	PEN1HA	bluish-green sporulation	mono-verticillate	ellipsoidal	3.16 ± 0.14	1.3 ± 0.07	1
26.	PEN2HA	bluish-green sporulation	tetra-verticillate	spherical	3.17 ± 0.2	2.64 ± 0.16	2
27.	PEN1AR	bluish-green sporulation	mono-verticillate	ellipsoidal	3.34 ± 1.15	2.42 ± 0.17	5
28.	PEN2KP	bluish-green sporulation	tetra-verticillate	ellipsoidal	3.1 ± 0.08	3 ± 0	1

C – color, S – shape, L – length (µm), B – breadth

surveyed districts was conducted. Genomic DNA amplification was done using ITS gene region (ITS 1 and 4). All sequences were submitted to GenBank, and accession numbers assigned to each sequence *viz.* ON208825.1, ON208826.1, ON208827.1 and ON208828.1 (Table 2).

Table 2. *Penicillium chrysogenum* isolates along with assigned accession numbers

Serial. No	Isolate ID on NCBI	Accession No. GenBank [ITS]
1	PEN11S	ON208825.1
2	PEN13SM	ON208826.1
3	PEN21R	ON208827.1
4	PEN43K	ON208828.1

The morphological identification, pathogenicity confirmation, ITS gene region amplification and nucleotide sequence analysis provided strong evidence toward the confirmation of *P. chrysogenum* as a pathogen causing blue mold on peaches in Pakistan. A neighbor-joining phylogeny tree of the ITS region of rDNA nucleotide sequences of *Penicillium* spp. causing blue mold disease of peach fruit was constructed (Fig. 4).

In vitro screening of eucalyptus EO's against *Penicillium chrysogenum*

The colony diameters of *P. chrysogenum* in the poisoned food technique after 7 days of incubation was 12.3 mm at a conc. of 1.6 mg · ml^{-1} eucalyptus essential oil followed by 16.1, 19.2 and 23.0 mm at 1.4, 1.2 and 1.0 mg · ml^{-1} conc., respectively. With the control treatment there was no reduction in mycelial growth of blue mold (Table 3).

Major constituents detected from eucalyptus essential oil in GC-MS

Eucalyptus essential oil contains certain major constituents. Eucalyptol, having a molecular weight of 164, is the key constituent with excellent antifungal characteristics. Other constituents detected from clove EO include: phenol, benzaldehyde, benzyl alcohol, 1,2-ethanediol, 1,2-diphenyl, acetic acid phenylmethyl ester, eugenol acetate, beta-caryophyllene, 1,2-butanediol, 1-phenyl, alpha-humulene and caryophyllene. Eugenol was detected as having the highest peak followed by 5.49 retention time (RT) (Table 4).

Sachet volatile phase method

In vitro application of an eucalyptus EO tablet sachet (1.6 mg · ml^{-1}) against *P. chrysogenum* cultured on Petri plates was evaluated by measuring radial growth after 2, 5 and 7 days of incubation (Fig. 5). Mycelial growth recorded on the 3rd, 5th and 7th days of incubation (DI) was 2.3, 6.1 and 9.13 mm, respectively, compared to the clove EO tablet sachet (1.4 mg · ml^{-1}) where mycelial growth was observed to be 4.17, 9.3 and 13.1 mm, respectively. The control treatment (no

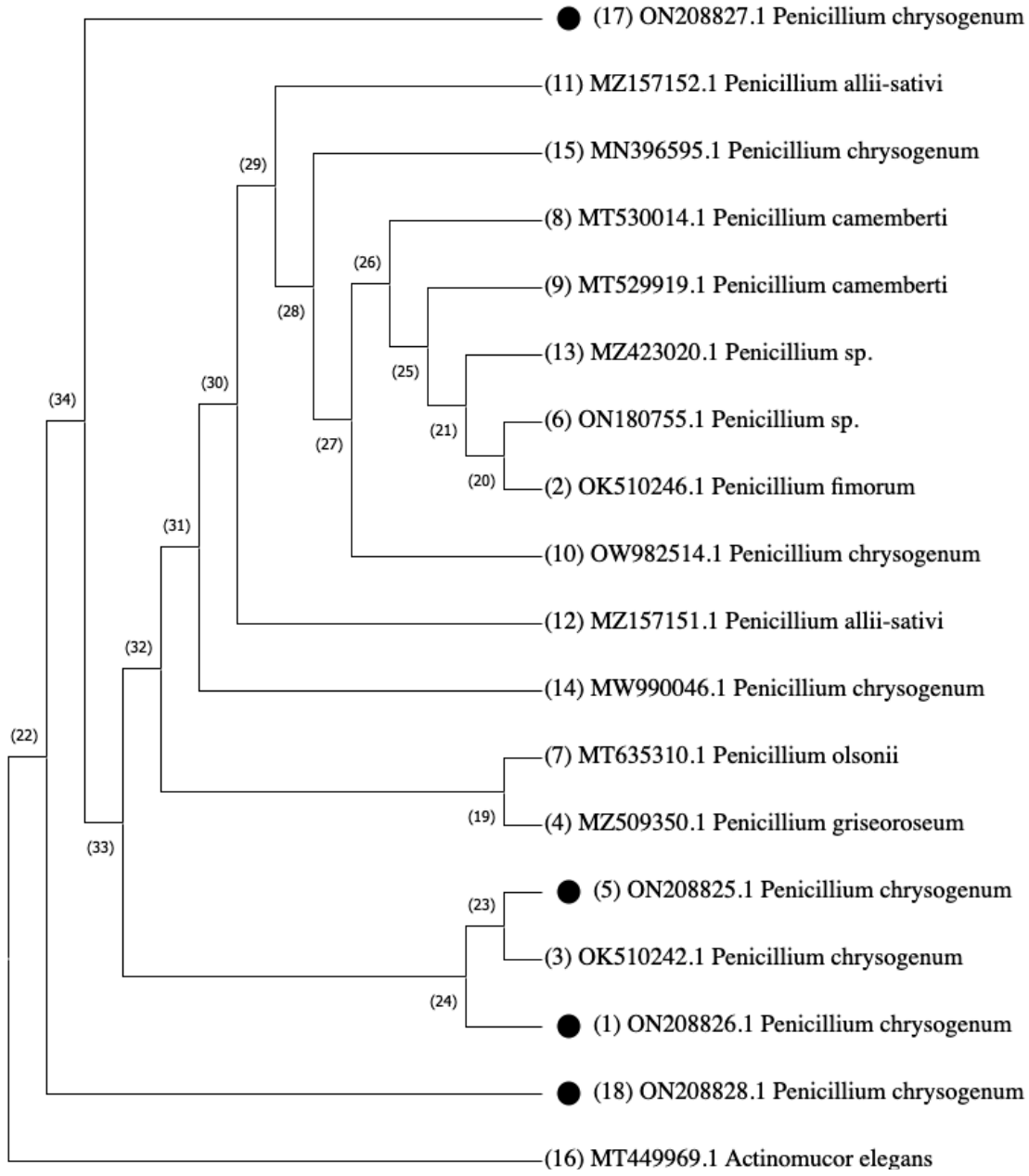

Fig. 4. A phylogenetic tree constructed using MEGAX software and MUSCLE alignment. Isolates inferred with ITS gene regions of rDNA nucleotide sequences of *Penicillium* spp. isolates causing blue mold on peach fruit. The neighbor joining tree construction method was applied along with 1000 bootstrap replicates. All missing data and gaps were removed while aligning in MEGAX software. *Actinomucor elegans* isolate (MT449969.1) was used to root the tree

EO sachet) showed no growth suppression of *P. chrysogenum* (Table 5).

In vivo Evaluation of Antifungal Sachets of Eucalyptus EO against *P. chrysogenum* at Room Temperature Incubation

Eucalyptus EO tablet sachets at concentrations of 1.4 and 1.6 mg · ml^{-1} significantly reduced the lesion diameter and disease severity of peach fruits inoculated with *P. chrysogenum*. Lesion diameters were 5.1, 10.3 and 20.2 mm at a concentration of 1.6 mg/mL followed by 9.3, 13.7 and 22.12 mm, respectively, at 1.4 mg · ml^{-1}. Disease severity rates observed on the 3rd, 6th and 9th days were 5.6, 17.8 and 24.1 mm, respectively, at 1.6 mg · ml^{-1} followed by 9.1, 22.4 and 35.9 mm, respectively, at 1.4 mg · ml^{-1} concentration (Table 6). Disease severity recorded in the control treatment was 31.2, 46.9 and 77.2% on the 3rd, 6th and 9th days, respectively, followed by lesion diameter on the 3rd, 6th and 9th days post inoculation 29.18, 44.11 and 74.2 mm, respectively. The disease severity and lesion diameter significantly differed ($p < 0.05$) from the control treatment.

Table 3. Evaluation of eucalyptus essential oil at four concentrations (1.0, 1.2, 1.4, and 1.6 mg · ml^{-1}) against *Penicillium chrysogenum*

Index	Treatments	1.6 [mg · ml^{-1}]	1.4 [mg · ml^{-1}]	1.2 [mg · ml^{-1}]	1.0 [mg · ml^{-1}]
Colony diameter Measured	eucalyptus EO	12.3 ± 1.50 e	16.1 ± 1.11 e	19.2 ± 1.61 e	23.0 ± 1.22 e
	control	100 ± 0.00 a	100 ± 0.00 a	100 ± 0.00 a	100 ± 0.00 a

Mean values are separated using Tukey's test at $p \leq 0.05$

Table 4. Major compounds detected from eucalyptus essential oil in GC-MS

Retention time	Peak area	Compound detected	Peak height
3.61	3620274.35	acetic acid, phenylmethyl ester	209054.63
4.53	3286946125.56	eucalyptol	71226675.20
7.07	753019711.98	eucamalol	29588.21
8.97	117735.32	citronellol	43442161.37
9.23	93827.68	limonene	19515.09
10.46	294134.28	Y-terpinene	9195.44
11.01	785664.71	alpha-pinene	30441.67
12.57	2331622.43	benzyl Alcohol	137966.79
13.43	339155.13	1,1′:4′,1″:4″,1‴:4‴,1⁗-quinquephenyl	19371.67
21.58	628192.33	1,2,3,4-tetrahydroisoquinolin,2-acetyl-6,7-dimethoxy-1-phenmethylene	17754.43

Table 5. *In vitro* evaluation of eucalyptus EO volatile tablet sachet with 1.4 and 1.6 mg · ml^{-1} concentrations against growth of *Penicillium chrysogenum* on the 2nd, 5th and 7th DPI

Index	Eucalyptus EO concentrations [mg · ml^{-1}]	Days after inoculation		
		2	5	7
Colony Diameter (mm)	1.6	2.3 ± 1.11 d	6.1 ± 1.50 c	9.13 ± 2.20 b
	1.4	4.17 ± 1.14 c	9.3 ± 1.32 b	13.1 ± 1.56 b
	Control*	100 ± 0.00 a	100 ± 0.00 a	100 ± 0.00 a

Tukey's test at $p \leq 0.05$ applied for mean values separation. Similar letters are significantly not altered. Control* (without application of eucalyptus EO volatile tablet sachet)

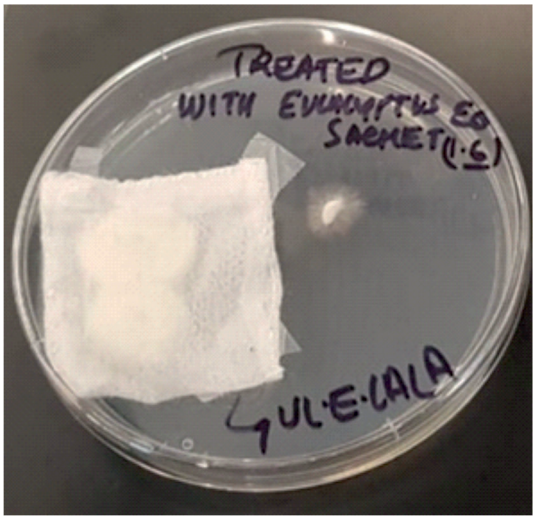

Fig 5. *In vitro* application of eucalyptus EO tablet sachet (1.6 mg · ml^{-1}) against *P. chrysogenum* cultured on Petri plates after 7 days of incubation

In vivo evaluation of antifungal sachets of eucalyptus EO against *Penicillium chrysogenum* in cold storage incubation

Peach fruits inoculated with *P. chrysogenum* were treated with antifungal sachets of eucalyptus EO at 1.4 and 1.6 mg · ml^{-1} concentrations in cold storage for 15 days, where disease severity and lesion diameter significantly differed from the control treatment ($p < 0.05$). The lesion diameter on peach fruits measured on the 5th, 10th and 15th days was 3.23, 9.15 and 14.1 mm, respectively, at a concentration of 1.6 mg · ml^{-1} of eucalyptus EO sachet followed by 5.7, 9.2 and 17.4 at a concentration of 1.4 mg · ml^{-1}. Disease severity rates observed on the 5th, 10th and 15th days were 4.3, 10.4 and 15.2 mm, respectively, at 1.6 mg · ml^{-1} followed by 7.1, 13.5 and 21.2 mm at 1.4 mg/ml conc., respectively (Table 7). In the control treatment lesion diameters were 20.1, 29.12 and 40.91 mm on the 5th,

Table 6. Peach fruits treated with 1.4 and 1.6 mg · ml^{-1} concentrations of eucalyptus essential oil tablets encapsulated in polypropylene sachets at room temperature for 9 days along with the control* having no material

Index	Clove EO concentrations [mg · ml^{-1}]	DPI		
		3rd Day	6th Day	9th Day
Lesion Diameter	1.6	5.1 ± 1.11 d	10.3 ± 1.29 d	20.2 ± 1.16 c
	1.4	9.3 ± 1.14 d	13.7 ± 1.21 c	22.12 ± 1.27 b
	control*	29.18 ± 1.39 a	44.11 ± 1.72 a	74.2 ± 1.96 a
Disease Severity	1.6	5.6 ± 1.21 d	17.8 ± 2.37 c	24.1 ± 2.25 b
	1.4	9.1 ± 2.33 d	22.4 ± 2.18 b	35.9 ± 3.11 b
	control*	31.2 ± 3.91 a	46.9 ± 5.17 a	77.2 ± 0.00 a

Each value is the mean of three replicates ± standard error (SE). Means within a row with different letters (a, b, c and d) for each time point (3rd, 6th and 9th day) indicate significant differences ($p < 0.05$) between different concentrations of eucalyptus EO by the Tukey test

Table 7. Peach fruits treated with 1.4 and 1.6 mg · ml^{-1} concentrations of eucalyptus essential oil tablets encapsulated in polypropylene sachets placed in cold storage for 15 days along with the control* having no material

Index	Clove EO concentrations [mg · ml^{-1}]	DPI		
		5th Day	10th Day	15th Day
Lesion Diameter	1.6	3.23 ± 1.20 d	9.15 ± 1.62 d	14.1 ± 2.51 c
	1.4	5.7 ± 2.14 d	9.2 ± 1.21 c	17.4 ± 1.77 b
	control*	20.1 ± 1.29 a	29.12 ± 2.72 a	40.91 ± 1.96 b
Disease Severity	1.6	4.3 ± 2.21 d	10.4 ± 2.37 d	15.2 ± 2.15 c
	1.4	7.1 ± 1.73 d	13.5 ± 3.10 c	21.2 ± 3.21 b
	control*	26.2 ± 4.91 a	47.4 ± 6.14 a	56.1 ± 0.00 b

Each value is the mean of three replicates ± standard error (SE). Means within a row with different letters (a, b, c and d) for each time point (5th, 10th and 15th day interval) indicate significant differences ($p < 0.05$) between different concentrations of eucalyptus EO by the Tukey test

10th and 15th DPI, respectively, followed by disease severity of 26.2, 47.4 and 56.1% on the 5th, 10th and 15th DPI, respectively. Disease severity and lesion diameter were significantly different ($p < 0.05$) from the control treatment in cold storage temperature.

Average weight loss

AWL of peach fruits measured at room temperature with 1.6 mg · ml^{-1} eucalyptus EO tablet sachet on the 3rd, 6th and 9th days was 5.9, 10.1 and 13.42%, respectively, followed by 7.8, 13.6 and 19.2% with 1.4 mg · ml^{-1} conc., respectively, of eucalyptus essential oil tablet sachet. AWL of the control treatment was 34.11, 46.32 and 53.13% on the 3rd, 6th and 9th days, respectively, of room incubation (Table 8). Thereafter, weight loss measured before and after 1.6 mg/ml concentration of eucalyptus EO sachet against *P. chrysogenum* on peaches in cold storage on the 5th, 10th and 15th days was 4.7, 7.3 and 12.1%., respectively,

Table 8. Effect of eucalyptus EO tablet sachets on average weight loss of peaches during room and cold storage incubation at different temperatures along with the control*

Index	Clove EO concentrations [mg · ml^{-1}]	Days after inoculation		
		3	6	9
Weight loss at room temperature	1.6	5.9 ± 1.23 d	10.1 ± 1.18 d	13.42 ± 2.1 c
	1.4	7.8 ± 1.11 d	13.6 ± 1.15 c	19.2 ± 2.11 b
	control*	34.11 ± 2.42 a	46.32 ± 2.53 a	53.13 ± 2.89 a
		Days after inoculation		
		5	10	15
Weight loss in cold storage	1.6	4.7 ± 2.21 d	7.3 ± 2.27 c	12.1 ± 3.15 b
	1.4	6.7 ± 1.33 d	9.42 ± 3.10 b	16.1 ± 2.11 b
	control*	13.9 ± 3.71 a	26.7 ± 5.17 a	35.91 ± 2.94 a

All values are the mean of three replicates ± standard error (SE). Means within a row with different letters (a, b, c and d) for each time point indicate significant differences ($p < 0.05$) between different concentrations of eucalyptus EO by the Tukey test

followed by 6.7, 9.42 and 16.1% with 1.4 mg · ml^{-1} conc., respectively. Weight loss in the control treatment of cold storage was 13.9, 26.7 and 35.91% on the 5th, 10th and 15th days post inoculation, respectively.

Discussion

To date, in Pakistan, no broad surveys for evaluation of postharvest phytopathogenic fungal peach fruit rots from commercial fruit markets had been conducted. The purpose of our survey was to detect the incidence of postharvest fungal rots responsible for latent infections on fruits which develop after maturity under various storage conditions. Mantyka (2010) reported that postharvest rots are responsible for producing quiescent infection, that can limit not only the storage period but also the market value by causing considerable losses for retailers and consumers. Postharvest deterioration primarily develops from damages that occur before and, most prominently, during or after harvest. Once spores of fungi are inoculated into these wounds, rapid fruit deterioration starts. Germinating conidia of fungi can also enter the intact fruit cuticle and then become established internally in the host (Adaskaveg *et al.* 2000). In recent years, quiescent infections were considered to be only a minor cause of fungal postharvest fruit decay. Perishable fruits are usually sensitive to softening and rotting during storage. Cultural and microscopic studies of *Penicillium* spp. found light blue to green colonies (3.7–5.5 mm) on CYA media plates on the 3rd day of incubation at 25°C. Smooth-walled conidiophores were observed (13 × 3 µm) whereas the size of phialides was 10 × 2 µm and conidia were ellipsoidal, globose, smooth and subglobose with 3.5 × 2.5 µm, respectively. *Penicillium* is a large and pervasive genus which presently contains 354 recognized species (Visagie *et al.* 2014). Colonies of *Penicillium* are typically fast growing bluish-green, with intense conidiophores. Microscopically, basipetal shaped chains of single-celled conidia in *Penicillium* spp. were observed from phialide.

Plant essential oils (PEOs) are widely used aromatic, volatile liquids extracted by various techniques from plants and herbs. They contain many natural bioactive constituents with anti-fungal characteristics (Xing *et al.* 2016; Sawtarie et al. 2017).

Eucalyptus EO at a conc. of 1.6% was extremely efficacious in retarding the growth of blue mold after a week of incubation and revealed the strongest suppression of radial growth of *P. chrysogenum*. Eucalyptus EO treatment has been seen to have an efficacious impact against blue mold in citrus by *P. italicum*. The effect of various volatile oils including clove, eucalyptus, thyme, mint and fenugreek was screened against postharvest fungal rots of perishable stone fruits. Among all these oils eucalyptus EO resulted in a very significantly reduced lesion diameter and disease severity leading to the collapse, squashing and deformation of fungal cell walls. It is pertinent to mention that during quantification of major components in *Eucalyptus* spp. EO following were recorded viz; eucalyptol, α-pinene Ocymen, β-pinene, limonene, α-phellandrene, globulol, and 9-octadecenamide (5.414%). On the other hand, significantly reported components in *E. globulus* EO were eucalyptol, 1, 8-cineole (54.7%), aromadendrene (14.9%), α-phellandrene (6.3%), and globulol (3.054%), respectively (Taghavi *et al.* 2018).

Moreover, eucalyptus (*Eucalyptus globulus*) essential oil possesses broad biotic activity, including anti-fungal, anti-microbial, acaricidal and nematicide features. The central components present in eucalyptus EO, including eucalyptol, γ-terpinene, limonene, *p*-cymene, 1,8-cineole, α-pinene, ocimene α-terpineol, camphene and linalool, play a major and fundamental role in disrupting the cell walls of phytopathogenic rot fungi (Hameed *et al.* 2022).

The sachet volatile phase method (SVPM) is a reliable technique applied against postharvest fungal rot inhibition of perishable papaya fruits using plant essential oils where maximum growth inhibition was recorded in agar plates. Results differed significantly with the control where no treatment was applied.

Postharvest fruit losses which lower consumption quality and the market value of produce primarily arise laterally in the entire food supply chain from handling to consumption (Parfitt *et al.* 2010). Usually, immature peaches show no visible rot symptoms but as they mature symptoms are clearly visible. These latent infections usually become active when the fruit ripen. Similarly, blue mold results in peach rotting with soft bluish, light to medium white mycelia and bluish-green colonies with abundant sporulation on the fruit surface (Natasa *et al.* 2020). Spores of post-harvest fungal rots are inoculated into wounds and result in rapid fruit deterioration as germinating conidia of fungi enter the intact fruit cuticle and develop internally in the host (Adaskaveg *et al.* 2000). *Penicillium* is a large and pervasive genus which presently contains 354 recognized species (Visagie *et al.* 2014). Morphological features of *P. chrysogenum* were observed showing bluish green colonies with sporulation having white margins. Similarly, blue mold results in peach rotting with soft bluish, light to medium white mycelia and bluish-green colonies with abundant sporulation on the fruit surface (Natasa *et al.* 2020). Plant essential oils play a significant role against these phytopathogenic fungal rots. Clove essential oil is well known for its excellent antifungal activity due to the presence of volatile constituents. Eucalyptus essential oil has excellent antifungal and antimicrobial properties.

A major antifungal component present in eucalyptus essential oil is eucalyptol. The possibility of eucalyptol was observed interacting with cell wall enzymes (chitin synthase and α- and β-glucanases) resulting in the suppression of fungal post-harvest rots. Moreover, in another study it was found that the anti-fungal effect of volatile oils resulted in collapse, squashing and deformation of post-harvest fungal rots (Hameed et al., 2022).

Micro-encapsulation is an efficient and reliable alternative technique to protect the volatility of essential oil along with their antifungal components. Encapsulation generally helps to protect the oil matrix from diffusing into the tissues of the fruits followed by augmentation of their bioactivity. Various eco-friendly materials, *viz.* edible coatings, paraffin wax and Arabic gum are being used as excellent packaging agents for encapsulating essential oils. *In vitro* application of eucalyptus EO against *Penicillium* rot was evaluated with the poisoned food technique as the most authentic and reliable method of application against post-harvest fungal rots (Balouiri *et al.* 2016). The eucalyptus essential oil sachet volatile phase method showed more than 96% inhibition in mycelial growth of *P. chrysogenum*. Paula *et al.* (2012) reported that the sachet volatile phase method applied against postharvest fungal rot inhibition of perishable papaya fruits using plant essential oils resulted in collapse of the post-harvest fungal phytopathogenic rots. Essential oils have a significant impact on the growth of blue mold in citrus fruits caused by *P. italicum* (Chen *et al.* 2019).

Eucalyptus EO is an excellent biopesticide against postharvest fungal rots resulting in disruption of fungal growth and conidial malformation in a dose dependent manner. These findings suggest that sachets containing eucalyptus essential oil tablets are economical and non-hazardous against post-harvest rot fungi which in further *in vivo* studies may enhance the shelf-life of peaches.

Conclusions

This research is the first reported evidence that eucalyptus essential oil can suppress the growth of fungal postharvest peach fruit rot and presents the beneficial inhibitory effects of eucalyptus EO on post-harvest fungal peach fruit rot. Essential oil-infused edible nanocoating is an excellent innovation for maintaining the stability of essential oils and boosting their effectiveness. It is suggested that eucalyptus essential oil (EEO) treatments using the sachet volatile phase method have a significant impact on the growth of *Penicillium* fruit rot caused by *P. chrysogenum*. The possible mechanism of eucalyptus EO treatment in controlling *Penicillium* fruit rot may alter in a dose dependent manner. Nanoencapsulation of EO with chitosan is an efficacious, applicable, and economical technique to protect antifungal components of plant essential oils. It may be the best antifungal packaging technique against post-harvest rots of perishable fruits for prolonging shelf-life. Therefore, plant essential oil nanoencapsulation is suggested as a desirable antimicrobial packaging alternative to chemical additives.

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