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Antifungal Activity of Essential Oils of *Melaleuca alternifolia*, *Psidium guajava* and *Zingiber officinale* in the Management of Grey Mould of Chilli

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A B S T R A C T

Essential oils of *Melaleuca alternifolia*, *Psidium guajava* and *Zingiber officinale* were tested against the test pathogen *Botrytis cinerea* the causal organism of grey mold of chilli. The pathogenicity of the test fungus was confirmed by inoculating the pathogen in the chilli fruits. The MIC of essential oils of *M. alternifolia*, *P. guazava* and *Z.officinale* were found to be 200µl/l, 300µl/l and 500µl/l respectively. The essential oils of *M. alternifolia* and *Z. officinale* were found to be 100 percent inhibitory to spore germination at 0.02% and 0.05% respective concentrations of the oils, while the essential oil of *P. guajava* was found to be 90.5 percent active at the 0.03% concentration of oil. The essential oils of *M. alternifolia*, *P. guajava* and *Z. officinale*, were found to enhance the shelf life of chilli fruits when applied as fumigant and enhanced the shelf life for 5 days, 4 days and 6 days respectively. The fruits were given dip treatments with the essential oils of *M. alternifolia*, *P. guajava* and *Z. officinale* and it was found that the oils by dip treatment enhanced the shelf life for 7 days, 4 days and 8 days respectively.

***Key words*:** *Capscicum annuam, Melaleuca alternifolia, Psidium guajava, Zingiber officinale,* Antifungal activity

Chilli (*Capscicum annuam* L.) is most widely used and universal spice of India. India is the world leader in chilli production followed by China, and Thailand. Indian chilli is important as it has commercial qualities of color and pungency levels. Indian chilli is exported to Asian countries like China, Sri Lanka, Malaysia, Bangladesh, Singapore, Thailand, UAE, etc. In India, major chilli producing states are Andhra Pradesh, Telangana, Tamil Nadu, Karnataka and Madhya Pradesh. Green chillies are rich source of Vitamin A and Vitamin E. It is widely used in the curry powder, curry paste, all kinds of pickles and preparing sauce, soups, etc. In spite of high production, in a sub-tropical country like India, it is difficult to maintain the quality and storability of chilli after harvest. It is estimated that about 6.7–17.1% loss of chilli occurs during marketing [1]. Fresh produce like chilli and other fruits and vegetables need low temperature and high relative humidity (RH) during storage and transportation. Therefore, reducing the temperature and increasing the RH are primary means of maintaining product quality during storage and transportation [2]. Traditionally, cultural techniques such as good sanitation practices (farm cleanliness) proper disposal of rotten fruits, using clean equipment and proper harvesting techniques have been employed to control post-harvest losses.

Maximum loss to the chillies is caused during storage by fungal diseases. *Botrytis cinerea* is one of the most important fungal pathogens responsible for the post-harvest loss caused during storage. Synthetic fungicides such as propiconazole, difenoconazole, carbendazim, benomyl, maneb and captan [3-4] have been used in the pre-harvest control of chilli fungal diseases as a pre-requisite for the post-harvest control. It is imperative to note that benomyl and its associated fungicides carbendazim and thiophanate methyl (both of which registered) has raised major health concerns such as causing eye defects, and other birth related effects by disrupting the process of cell division making their use unacceptable and dangerous [5].

The emergence of resistant strains of fungi in chilli fruit against benomyl, which were cross-resistant to thiophanate methyl and carbendazim was reported in Malaysia [6]. Recently, resistance of *C. truncatum* to benomyl has also been reported in Trinidad [7]. In addition, long term usage of synthetic chemicals is known to have a negative impact on the environment especially soil and water resources [8-9]. The increasing health concerns expressed by consumers and the intention of governments to regulate pesticide use and their residues in fresh produce have necessitated the development of non-toxic alternative management techniques for management of fungal losses of fruits during storage.

A number of antimicrobial or metabolic compounds are synthesized by plants and considered to be the alternative for controlling diseases of tropical fruits and vegetables. These

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antimicrobials of plant origin can be obtained from plant in the form of extracts or essential oils. In chilli, several *in vitro* studies have shown the efficacy of certain medicinal herbs or plant extracts against phytopathogenic fungi of chilli [10-14]. Essential oils are concentrated hydrophobic mixture of volatile aroma compounds resulting from plant secondary metabolism and have long been known to provide effective control over phytopathogens. They are usually obtained by steam or hydro-distillation and are known for their antiseptic (bactericidal, viricidal and fungicidal) properties and the rich fragrance they produce [15]. The purpose of the present study was to evaluate the *in vitro* effects of *Melaleuca alternifolia*, *Psidium guazava* and *Zingiber officinale* essential oils on radial colony growth and spore germination of *Botrytis cinerea* in addition to this to assess *in vivo* efficacy for enhancement of shelf life of chilli fruits.

MATERIALS AND METHODS

Phytopathogenic fungi

The fungul culture used throughout the present study was of *Botrytis cinerea* the causal agent of grey mould of chilli. *B. cinerea* was isolated from the disease infested chilli fruits collected from local market of Kanpur during winter season. The fruits were surface sterilized using 70% ethyl alcohol. The small pieces of infected parts of chilli were inoculated in the petri plate containing PDA media and incubated at 27 ± 1°C for six days. Seventh day the mycelial growth of fungus was observed. The morphology of the fungus was observed and identified using the microscope. The morphological characteristics of *B. cinerea* were found to be similar to the descriptions of [16-17]. The fungus culture was stored at 4°C for experimentation.

Pathogenicity test

To confirm the pathogenicity of *B. cinerea*, healthy chilli fruits were washed with distilled water and surface sterilized with 70% ethyl alcohol for one minute, and ultimately, washed with sterilized water. Some fruits were wounded with the help of a sterilized cork borer. The fungus inoculums were inoculated in the wounded part and wrapped with clean polythene bag, and were kept at 27±1°C for a week. After a week, characteristics symptoms were produced which were found to be similar as that of the previous one. The fungal pathogen was isolated, studied under microscope and compared with previously isolated fungus.

Essential oils

The essential oils from leaves of *M. alternifolia* and *P. guajava* and rhizomes of *Z. officinale* were isolated separately by hydro distillation through Clevenger's apparatus. The isolated fractions of plant parts exhibited two distinct layers an upper oily layer and the lower aqueous layer. Both the layers were separated and the essential oils were stored in clean glass vials after removing water traces with the help of capillary tubes and anhydrous sodium sulphate.

In vitro evaluation of antifungal activity of M. alternifolia, P. guajava and Z. officinale essential oil and determination of MIC of oils

The antifungal activity of each essential oil was evaluated at the concentrations (viz. 100 µl/l, 200 µl/l, 300 µl/l, 400 µl/l, 500 µl/l, 1000 µl/l and 2000µl/l). The requisite amount of oils were added to emulsifier Tween 80 (0.05% v/v) for the homogenization of the essential oils. The PDA medium was cooled to 40°C and poured to the Petri plates. The requisite amount of each oil was mixed separately with Tween 80 (0.05% v/v) (used for the homogenization of the oil in PDA) and was separately added to the Petri plates. The Petri plates were allowed to revolve with the help of finger for mixing oils to the PDA medium. A control containing only the emulsifier and the PDA medium, was also employed. After solidification of the PDA medium, a disk (5 mm in diameter) of *B. cinerea* from a culture plate was transferred to the center of the respective essential oil treated plate. Petri plates were sealed with parafilm to prevent the leak of test oils. Plates were incubated at 27 ± 1°C for six days and observations were taken on seventh day. Measurement of radial colony growth was taken in two perpendicular directions (diameter in cm). Percentage of fungal mycelial growth inhibition (PI) of essential oils were measured by following equation:

$$\text{Percentage of mycelial inhibition} = \frac{Dc - dt}{dc} \times 100$$

Where;
dc = mean colony diameter of control sets
dt = mean colony diameter of treatment sets

The minimum inhibitory concentration of oils was determined by observing the lowest concentration at which the oil inhibited the complete growth of the test fungus.

Spore germination assay

The spore germination tests were carried out using assay developed by Pimentel *et al*. [18], with some modifications. An aliquot of 100 µL of *B. cinerea* conidia suspension containing 2×10^5 conidia per mL were incubated with 100 µL of essential oils of *M. alternifolia*, *P. guazava* and *Z. officinale* in the respective concentrations of 0.02%,0.03% and 0.05% separately in sterile glass depression slides. Tween 80 (0.05% v/v) was used as the negative control. The depression slides were incubated at 27 ± 1°C for 24 h in a Petri dish containing wet filter paper. Inhibition of germination was observed under microscope. Approximately 200 spores were counted, and the percentage of germinating of conidia was done with the help of following equation:

$$\text{Germinating conidia (\%)} = \frac{\text{Number conidia germinated}}{\text{Total number of conidia}} \times 100$$

In vivo testing of M. alternifolia, P. guajava and Z. officinale essential oil on gray mould of chilli fruits
By dipping method

Sterilized water containing essential oils of *M. alternifolia*, *P. guajava* and *Z. officinale* at different concentrations i.e., 0.02%, 0.03% and 0.05% (v/v) were tested to study their effect against gray moulds incidence on chilli fruits. Fresh chilli fruits apparently free from physical damage and diseases were used for *in vivo* test. All the fruits were disinfected [19] with sodium hypochlorite solution (2.5%) for 2 min. After repeated washing with distilled water (three times) the fruits were placed in sterile cartons.

30 fruits were randomly distributed into three replicates and were artificially wounded using sterilized scalpel. Fruits were arranged by groups in carton having three layers of moisten blotters at the bottom. Fruits were dipped in sterilized water containing essential oils of *M. alternifolia, P. guazawa* and *Z. officinale* at concentrations 0.02%, 0.03% and 0.05% (v/v) respectively containing 0.01% Tween 80 for 3 min, then air dried. For control fruits were dipped in sterilized water containing 0.01% Tween 80.

Conidia of *B. cinerea* were recovered from one week old culture by adding 10 ml of sterile water to each plate. The

conidia suspension was filtered through three layers of sterile cheese cloth. The concentration of the conidial suspension was adjusted to 10^5 spores/ml of *B. cinerea*. All treated or un-treated fruits were placed into carton boxes at the rate of 10 fruits/box. Each particular concentration as well as control treatment was represented by three carton box. All boxes were stored at 27±1°C for 20 days. Initiation of rotting was observed after 6 days of inoculation.

By fumigation method

For this experiment, essential oil emulsions of *Melaleuca alternifolia*, *Psidium guazava* and *Zingiber officinale* oil were prepared at 0.02%, 0.03% and 0.05% (v/v) respectively in distilled water using Tween 80 (0.01% v/v) as surfactant. Emulsions were prepared by mixing the essential oil phase and the aqueous phase in a high-speed mixer for 5 min at 5.000 rpm. All the fruits were disinfected with sodium hypochlorite solution (2.5%) for 2 min. Fruits were punctured by sterilized scalpel at different sides for inoculation and were inoculated with 30 µL of a spore suspension (10^5 spores mL^{-1}) of *B. cinerea*. Ten fruits were placed in transparent plastic containers. The treatments were performed with the addition of 1 mL of each essential oil emulsion in cotton attached to the edge of the plastic containers. The plastic containers were sealed with Parafilm® to prevent vapor leakage. The fruits were stored at 27 ± 1°C. The antifungal activity of the treatments was evaluated by the incidence of the disease in the fruit. Disease incidence was calculated from the number of symptomatic fruits in relation to the total number of fruits in each treatment, evaluated after seven days of storage.

RESULTS AND DISCUSSION

In vitro evaluation of antifungal activity of M. alternifolia, P. guajava and Z. officinale essential oil

Impact of essential oils on mycelial growth of *Botrytis cinerea* is depicted in (Fig 1). The essential oil of *Melaleuca alternifolia* showed 80 percent inhibition at 100µl/l while at higher concentrations of 200µl/l to 2000 µl/l oil has shown 100 percent inhibition. The oil of *Psidium guazava* showed 75 percent at100 µl/l and 90 percent inhibition at 200µl/l while at 300µl/l to 2000 µl/l it was found to inhibit the complete growth of the fungus. On the other hand, the essential oil of *Zingiber officinale* showed 40percent inhibition at 100µl/l, 65 percent inhibition at 200 µl/l, 70 percent inhibition at 300 µl/l and 80 percent inhibition at, 400 µl/l while the oil was totally potent to inhibit the 100 percent growth at 500 µl/l, 1000 µl/l and 2000 µl/l. Therefore 200µl/l, 300µl/l and 500µl/l were respective lowest concentrations for the essential oils of *Melaleuca alternifolia, Psidium guazava and Zingiber officinale at* which the oils have been found to inhibit complete growth of the fungus. Therefore, the MIC of *Melaleuca alternifolia* oil was found to be 200 µl/l, for *P. guajava* it was 300 µl/l and for *Zingiber officinale* the MIC was determined to be 500µl/l (Table 1).

Table 1 Minimum inhibitory concentration of essential oils

Essential oils	MIC (µl/l)
Melaleuca alternifolia	200
Psidium guazava	300
Zingiber officinale	500

In vitro inhibitory effects of M. alternifolia, P. guajava and Z. officinale essential oils on spore germination of B. cinerea

The germination of *Botrytis cinerea's* spores was notably inhibited by all the three essential oils at their respective MIC (Table 1). The control (0.5% Tween 80) did not inhibit spore germination under the same experimental conditions. The *Melaleuca alternifolia* and *Zingiber officinale* essential oils inhibited 100 percent germination of *Botrytis cinerea* spores. The essential oils of *Psidium guazava* showed significantly lower potential for controlling the germination of *Botrytis cinerea* spores, since the observed inhibition was 90.5% (Fig 2).

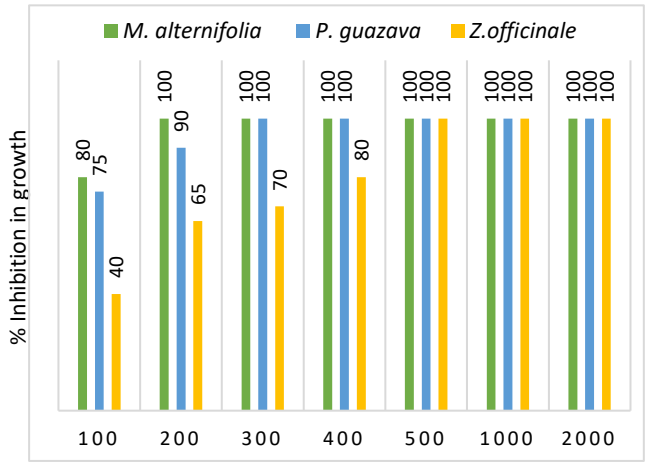

Fig 1 Inhibition in radial growth of *B. cinerea*

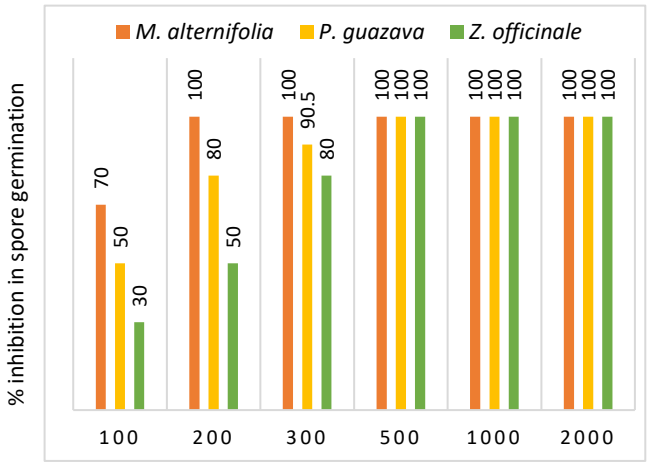

Fig 2 Inhibition in spore germination of *B. cinerea*

Table 2 Efficacy of essential oils by dip method under *in vivo* condition against *B. cinerea*

Essential oils	Initiation in rotting of Chilli fruits inoculated with *B. cinerea*	Enhancement of storage life (in days)
Control	9	
M. alternifolia	16	7
P. guazava	13	4
Z. officinale	17	8

In vivo efficacy of M. alternifolia, P. guajava and Z. officinale essential oils on Chilli fruits

Dip method

The efficacy of essential oils of *Melaleuca alternifolia*, *Psidium guazava* and *Zingiber officinale* was evaluated by dip method at the concentration of 0.02 %, 0.03% and 0.05%. It was found that the *Botrytis cinerea* inoculated chilli fruits when given dip treatment in aqueous suspension of *M. alternifolia, P.*

guajava and *Zingiber officinale* separately, showed enhancement of 7and 4 and 8 days respectively (Table 2).

Fumigation method

The efficiency of the vapor-phase of the essential oils of *Melaleuca alternifolia* and *Psidium guazava* and *Zingiber officinale* in chilli fruits artificially inoculated with *B. cinerea* is given in (Table 3). The treatment with essential oils of *M. alternifolia, P. guazava* and *Z. officinale* effectively reduced the incidence of disease on chillies at 27±1°C during seven days of storage. The inhibitory effect of essential oils against the deterioration of *B. cinerea* was observed at 0.02%, 0.03% and 0.05% respectively and enhancement of shelf life of 5 days, 4 days and 6 days with the treatment of respective oils separately was observed.

Table 3 Efficacy of essential oils under *in vivo* condition against *B. cinerea using* fumigation method

Essential oils	Initiation of rotting of Chilli fruits inoculated with *B. cinerea*	Enhancement of storage life (in days)
Control	9	
M. alternifolia	14	5
P. guazava	13	4
Z. officinale	15	6

Essential oils can inhibit the growth of fungi in the vapor phase, which is an important property for preserving fruits and vegetables and other food commodities [20]. Several *in vitro* studies have suggested that the use of essential oils will control fungal spoilage, of fruits and vegetables, very few studies have been conducted to control post-harvest *Botrytis* infection of chillies during storage. In the present investigation the essential oils of *M. alternifolia*, *P. guajava* and *Z. officinale* have been tested *in vitro* and *in vivo* to correlate the efficacy of the essential oils in both the conditions. The concentrations that were found to be effective in the present investigation were 200µl/l for *M. alternifolia*, 300µl/l for *P. guazava* and 500 µl/l for *Z. officinale*.

In the control of phytopathogens, the inhibition of spore germination by the bioactive compound is crucial, since spores are the starting point of fungal infection. Therefore, for the efficiency of natural antifungal compounds, it is necessary that they also inhibit spore germination, in addition to inhibiting mycelial growth. In the present study all the essential oils inhibited the spore germination of phytopathogen *B. cinerea*. Essential oils of *M. alternifolia* and *Z. officinale* inhibited 100 percent spore germination at 0.02 and 0.05% oil concentrations respectively, while *P. guazava* showed 90.5 percent inhibition of spore germination at 0.03% oil concentration. The results found in this study corroborate those described by Lorenzetti *et al*. [21], which showed that *B. cinerea* conidia were completely inhibited by the essential oil of palmarosa (*Cymbopogon martinii*) at the concentration of 0.1%. According to Tomazoni *et al.* [22], *C. camphora's* essential oil was effective in germinating *A. solani* spores with a spore germination rate below 34.33%, similar to what was observed in this study (23.00%). The essential oil of *M. piperita* inhibited 78% the germination of *Phakopsora euvitis* at the concentration of 0.1% [23].

The alternative fungicide treatments are needed for the management of postharvest diseases of fruits [24-25]. The potential of using essential oils by spraying or dipping fruits for controlling postharvest diseases has been reported by several investigators [26]. Essential oils have several components which have insecticidal and fungicidal activities against some important plant pathogens [26]. Allelopathic effect of eucalyptus (*Eucalypttus globulus*) against plant pathogens was reported by several investigators [27]. The essential oil of thyme (*Thymus vulgaris* L.) and its major component, thymol had antifungal activity against plant pathogenic fungi [28-29] as well as plant diseases of several fruits and vegetables [30-31]. Essential oil extracted from lemon grass (*Cymbopogon citratus* Stapf.) has antifungal activity against several plant pathogens [32]. In the present study the essential oils of *M. alternifolia*, *P. guajava* and *Z. officinale* at 0.02%, 0.03% and 0.05% respective concentrations have shown enhancement of shelf life of 5 days, 4 days and 6 days respectively when fumigated with the respective oils separately.

The combination of two or more natural products as edible coating on fruits has been used in postharvest control of anthracnose. It has been proposed that, because anthracnose is a latent infection and starts from the germination of conidia, which produce appressoria, cinnamon oil which contain cinnamaldehyde penetrates into the appressoria of fungus to inhibit its growth thereby slowing down the anthracnose [33]. Citral aldehyde or citral present in lemongrass oil is attributed to cause irreversible cell membrane disruption through cross-linkage reaction leading to leakage of electrolytes and subsequent depletion of amino acids and sugars, whiles others may selectively be inserted into the lipid rich portion of the cell membrane, thereby disturbing membrane function [34]. Also, the presence of monoterperne hydrocarbons such as limonene and camphone are reported to exert fungitoxicity by diffusing into cell membrane and causing deformation [35]. Hyldgaard *et al*. [36], reported that in experiments evaluating the antifungal activity of essential oils *in vivo*, interactions between foods and essential oils may require higher concentrations for the inhibition of the target fungus to be achieved. The results of this study corroborate those reported by Aguilar-gonzález, Palou and López-malo [37] in which the application of mustard and clove essential oils in vapor-phase was effective in inhibiting the growth of *B. cinerea* in strawberries.

CONCLUSION

It can be concluded that the essential oils being natural and of plant origin can be safely exploited to preserve the chillies during storage. Experiments are needed to confirm the exact dose of oils and complete phytochemistry should be done to know the exact active principle of the oils which will be essential for making formulations in the form of botanical pesticides. Some pharmacological tastings will be required before recommending these oils as natural pesticides. Further, studies are needed to elucidate the possible mechanisms of action of the essential oils in post-harvest management.

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