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Some Factors Affecting Growth of Fruit Decay Fungi in Vitro

Mokhtar M. Abdel-Kader, N.S. El-Mougy, N.G El-Gammal

Abstract *- The pathogenic fungi Geotricum candidum, Penicillium digitatum, Penicillium italicum and Botrytis cenerea were isolated from citrus (orange, mandarin) and strawberry fruits showing decay symptoms. The effect of some fungicides alternatives, i.e. antagonistic yeast, chemical preservatives and some essential oils on the linear growth of decay fungi were evaluated In vitro. The efficacy of yeast isolates against the growth of decay fungi showed that C. tenuis has higher inhibitory effect on the growth of the pathogenic tested fungi followed the yeast isolate S. cerevisiae. Reduction in the growth of B. cinerea, G. candidum, P. italicum and P. digitatum was recorded as 51.1, 45.5, 37.7 and 35.5%, respectively, when the yeast P. digitatum was inoculated in the growth medium. Meanwhile, S. cerevisiae showed lower inhibitory effect on fungal growth which recorded as 25.5, 24.4, 15.5 and 20.0% in respective order. On the other hand, all tested concentrations of salts significantly reduced the linear growth of all tested pathogenic fungi. It was also noticed that the reduction in growth were correlated to the gradual increase in either sodium benzoate or potassium sorbate concentrations. High inhibition was observed in the linear growth of all tested fungi when exposed to tested organic salts at concentrations of 4% and 2% of either potassium sorbate or sodium benzoate, respectively. The essential oils evaluated in this work have a great variety of phytochemicals that could be considered as responsible for a larger or smaller antifungal activity. Fungal mycelia growth decreased significantly as the concentrations of essential oils were increased, to reach the fungal growth's minimum at the highest concentration used. Thyme oil showed more inhibitor effect against the growth of decay fungi than cinnamon oil. Also, the maximum growth inhibition was observed for the growth of B. cinerea and G. candidum followed by P. italicum and P. digitatum, respectively.*

Index: **Antagonistic yeast, decay fruits, essential oils, food preservatives**

I. INTRODUCTION

Postharvest diseases affect a wide variety of crops particularly in developing countries which lack sophisticated postharvest storage facilities [1]. Losses caused by postharvest diseases are greater than generally realized because the value of fresh fruits and vegetables increases several-fold while passing from the field to the consumer [2]. Postharvest losses are estimated to range from 10 to 30% per year despite the use of modern storage facilities and techniques [3]. Postharvest decay of citrus fruits caused by *Geotricum candidum* Link. (Sour rot incident); *Penicillium digitatum* Pers. Sacc. (Green mould incident) and *P. italicum* When. (Blue mould incident) are the most important diseases affecting harvested citrus fruits during handling, transportation, exportation and storage [4,5,6]. Strawberry (*Fragaria* × *ananassa*) is among the most perishable fruits and is vulnerable to physical injuries and fungal invasion. Gray mold caused by *Botrytis cinerea* Pers. ex. Fr. is the most economically important postharvest disease of strawberry fruits that causes losses before or after harvest [7,8,9]. Strawberries deteriorate during storage as a result of decay along with physical senescence and dehydration. *Botrytis cinerea* is the most frequently reported as decay fungus responsible for microbial deterioration of strawberries [10]. Postharvest diseases caused by pathogenic fungi resulting in major losses of fruits and vegetables, and synthetic chemical fungicides are the primary means to application at present [11]. However, synthetic chemical fungicides are potentially harmful on human health and the emergence of pathogens which are resistant to these chemicals [12]. Moreover, public concern over the indiscriminate use of synthetic fungicides has been growing. Thus, it is significant to develop new alternatives such as food preservatives for disease control measures [13]. Essential oils are also considered a promising alternative with many having antifungal properties. However, very high concentration is needed when applied to real food systems [14,15]. Application of essential oil is a very attractive method for controlling postharvest diseases. Essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use [16]. Moreover, Biological control has been advanced as an alternative to synthetic fungicides and considerable success in laboratory and pilot scale tests has been realized utilizing antagonistic microorganisms to control postharvest diseases. Several antagonistic yeasts and bacteria have been isolated and shown to have a broad spectrum of activity against a number of postharvest pathogens on a variety of fruit [17]. The objectives of this study were to evaluate the effectiveness of some food preservatives, essential oils and antagonistic yeast isolates to inhibit the mycelia growth of decay fungi, *Botrytis cinerea*, *Geotricum candidum* , *Penicillium digitatum* and *P. italicum* under *in vitro* conditions.

II. MATERIALS AND METHODS

Pathogens and antagonists

One of each virulent pathogenic fungal isolates of *Botrytis cinerea*, *Geotricum candidum*, *Penicillium digitatum* and *P. italicum* and isolates of the antagonistic yeast, *i.e. Saccharomyces cerevisiae* [Meyen ex E.C.

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Hansen] and *Candida tenuis* [Berkh] were obtained from Plant Pathology Department of the National Research Centre, Giza, Egypt. These microorganisms were isolated from various healthy and decayed Citrus [18] and Strawberry [19] fruits, and their high pathogenic or antagonistic ability was examined during previous work at the same Department.

Growth media

Potato dextrose agar (Difco Laboratories, Detroit, MI) and NYDB [8 g of nutrient medium (Difco Laboratories, Detroit, MI), 5 g of yeast extract, and 10 g of dextrose in 1 liter of water] were used for growing fungal and yeast isolates tested in the present work. Fungal and yeast cultures were maintained on PDA and NYD agar slant media at 5±1 ^{o} C as stock cultures until use. All isolates were activated by growing at the optimum growth conditions at the beginning of the present experiments.

Tested materials

Food preservatives, i.e. sodium benzoate and potassium sorbate were purchased from El-Nasr Company for Chemical industries –Cairo Egypt. Meanwhile, Pure-grade of essential oils, i.e. Cinamon (Cinnamomum zeylanicum) and Thyme (Thymus vulgaris) oils were obtained from Cairo Company for oils and aromatic extractions CID, Egypt. The essential oils were stored in dark glass bottles at 4o C.

In vitro growth inhibition of tested decay pathogenic fungi

The inhibitory effect of antagonistic yeast, food preservatives and essential oils on the growth of decay fungi was evaluated in vitro.

The inhibitory effect of the antagonistic yeast, Saccharomyces cerevisiae and Candida tenuis against the linear growth of fruit decaying pathogenic fungi was evaluated using the modified dual culture technique [20]. Abundant fungal and bacterial growth was first prepared. Ten mL of each individual yeast isolate was grown for 48 h on NYDB broth medium and poured into flasks containing sterilized PDA medium. Before solidifying, each flask was rotated gently to ensure equal distribution of yeast growth, and then poured into 9-cm-diameter Petri dishes. Inoculated plates were incubated for 48 h at 28±1oC. For fungal growth, a 5-mm disk of each tested fungi was transferred to the centre of a PDA plate then incubated for ten days at 25±10C. In vitro antagonistic studies between biocontrol yeast and decay pathogenic fungi were performed on PDA medium in 9-cm-diameter Petri dishes. A 5-mm disk of each yeast growth culture was placed onto the PDA, 10mm from the edge of the Petri dish. Another disk of the same diameter of each decay pathogenic fungal growth culture was placed on the opposite side of the dish at the same distance. The control treatment was inoculated with a culture disk of either a pathogenic or antagonistic culture alone at the same conditions. Both experimental and control dishes were assigned to a completely randomized design, with five replicates per treatment. All inoculated Petri dishes were incubated at 25±1oC and the fungal growth diameter away from and towards the antagonist agent was measured after the pathogenic fungal growth in the control treatment had reached the edge of the Petri dish. This test was repeated three times and the inhibition was calculated as the percentage reduction in colony diameter growth compared with the control. The efficacy of food preservatives on linear growth of decay pathogenic fungi was evaluated. Sodium benzoate and Potassium sorbate at different concentrations of 0.5, 1.0, 2.0, and 4.0% were tested for their inhibitory effect on the linear growth of various decay pathogenic fungi. Water salts solutions were added to conical flasks containing sterilized PDA medium to obtain the proposed concentrations, then rotated gently and dispensed in sterilized Petri plates (10 cm Ø). The plates were individually inoculated at the center with equal disks (5 mm Ø) of 10-day old culture of the tested fungi. Five plates were used as replicates for each particular treatment. Inoculated plates were incubated at 25 ± 2 oC. The average linear growth of fungi tested was calculated after the tested fungi reach full growth in check treatment. Then, reduction in mycelial growth was calculated as percentage of fungal growth diameter in treatment relatively to the growth diameter in control. Essential oils i.e Cinamon and Thyme at concentrations of 0.5, 1.0, 2.0 and 4% were evaluated for their inhibitory effect against the growth of decay fungi. Emulsified stocks at high concentration of tested essential oils were prepared by dissolving in sterilized distilled water. Few drops of the emulsifier Tween 20 (Sigma Co.) were added to essential oil volumes to obtain emulsion feature. Different volumes of the essential oils emulsion were added to conical flasks containing 100 ml of sterilized PDA medium before its solidification to obtain the proposed concentrations. The supplemented media were poured into Petri-dishes (10 cm Ø) about 20 ml each. Control check treatment was PDA medium free of essential oils. Disks (5 mm Ø) of each pathogenic fungi taken from ten days-old cultures were placed on the centre of Petri-dishes. All plates were incubated at 25 ± 2 oC until the tested fungi reach full growth in check treatment. The linear growth in each treatment was measured and reduction in mycelial growth was calculated as percentage of fungal growth diameter in treatment relatively to the growth diameter in control.

Statistical analysis

The obtained results were analyzed using Tukey test for multiple comparisons among means were utilized for analyzing the obtained results [21].

III. RESULTS AND DISCUSSION

Decay of harvested fruits is a major cause of losses during storage and marketing of citrus and strawberry

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which considered as one of the most serious and frequent postharvest and post-packaging diseases. Postharvest biological control is a relatively new approach and offers several advantages over conventional biological control [22,23]. Several biological control agents have been developed in recent years, and a few have actually been registered for use on fruit crops. Yeasts such as *Pichia guilliermondii* [24] and *Cryptoccocus laurentii*, yeast that occurs naturally on apple leaves, buds, and fruit [25] were the first to be applied for control of postharvest decay on fruit. The yeast, *Candida oleophilia* has been registered for control of postharvest decay on fruit crops. The yeasts, *Cryptococcus infirmo-minutus* and *Candida sake* successfully control brown rot and blue mold on sweet cherry [26], and three diseases of apple [27], respectively, and may be developed into commercial products. In the present study the efficacy of yeast isolates was evaluated against the growth of decay fungi *in vitro*. Results showed that *C. tenuis* has higher inhibitory effect on the growth of the pathogenic tested fungi followed the yeast isolate *S. cerevisiae* (Table 1). Reduction in the growth of *B. cinerea*, *G. candidum*, *P. italicum* and *P. digitatum* was recorded as 51.1, 45.5, 37.7 and 35.5%, respectively, when the yeast *P. digitatum* was inoculated in the growth medium (Fig. 1). Meanwhile, *S. cerevisiae* showed lower inhibitory effect on fungal growth which recorded as 25.5, 24.4, 15.5 and 20.0% in respective order. Although there is no doubt that biocontrols are effective, they do not always give consistent results. This could be because biocontrol efficacy is so directly affected by the amount of pathogen inoculums present or antagonistic ability of the bio agent itself [28].

Table (1) Mycelial linear growth of some decay pathogenic fungi affected with antagonistic yeast isolates

Tested Yeast isolates	Fungal linear growth (mm)			
	P. digitatum	P. italicum	G. candidum	B. cinerea
S. cerevisiae	72 b	76 b	68 c	67 c
C. tenuis	58 d	56 d	49 e	44 e
Control	90 a	90 a	90 a	90 a

Figures with the same letter are not significantly different ($P \leq 0.05$).

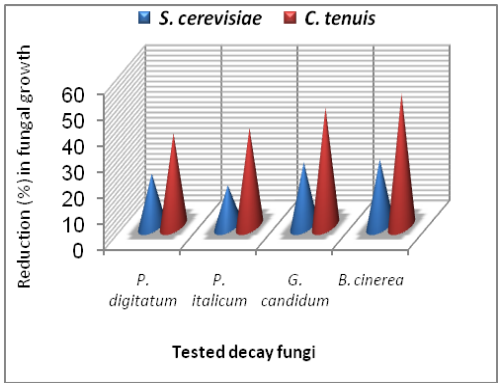

Fig. (1) Growth reduction (%) of some decay pathogenic fungi affected with antagonistic yeast isolates

On the other hand, four concentrations, *i.e.* 0.5, 1.0, 2.0, and 4.0% of sodium benzoate and potassium sorbate were tested for their inhibitory effect on linear growth of *B. cinerea, G. candidum, P. digitatum and P. italicum*. Results obtained are shown in Table (2) and Fig. (2). Presented data indicate that all tested concentrations of salts significantly reduced the linear growth of all tested pathogenic fungi. It was also noticed that the reduction in growth were correlated to the gradual increase in either sodium benzoate or potassium sorbate concentrations. High inhibition was observed in the linear growth of all tested fungi when exposed to tested organic salts at concentrations of 4% and 2% of either potassium sorbate or sodium benzoate, respectively.

Table (2) Mycelial linear growth of some decay pathogenic fungi affected with different concentrations of some food preservatives

Tested salts	Con.	Fungal linear growth (mm)			
		P. digitatum	P. italicum	G. candidum	B. cinerea
Sodium benzoate	0	90 a	90 a	90 a	90 a
	0.5	66 c	63 c	64 c	58 d
	1.0	55 d	52 d	55 d	50 d
	2.0	41 f	46 f	43 f	43 f
	4.0	33 g	37 g	36 g	32 g
Potassium sorbate	0	90 a	90 a	90 a	90 a
	0.5	54 d	58 d	59 d	58 d
	1.0	42 f	44 f	46 f	44 f
	2.0	33 g	35 g	34 g	40 f
	4.0	30 g	32 g	31 g	32 g

Figures with the same letter are not significantly different ($P \leq 0.05$).

On minimizing the tested concentrations of organic salts down to 2%, the tested fungi fluctuated in their response. The obtained results demonstrate that several salts can inhibit growth of *B. cinerea, G. candidum, P. digitatum* and *P. italicum in vitro* and reduce their spore production. These results are consistent with other studies which demonstrated that suppression of microbial strains varied in response to different organic and inorganic acids or salts [29,30,31,32,33]. Furthermore, organic and inorganic salts were effective in inhibiting growth and sporulation of *Helminthosporium solani* [34]. Also, the most commonly applied salt of sorbic acid, potassium sorbate, is reported to completely suppress growth of the banana pathogen *Colletotricum musae in vitro* [35]. It was found that inhibition of microorganisms by sorbic acid and its salts might be caused by alternation of cell-transport function, inhibition of enzymes involved in the glycolytic pathway or tricarboxylic acid cycle by inhibition of RNA, DNA, and protein synthesis, and by uncoupling of the oxidative phosphorylation in mitochondria [32, 36].

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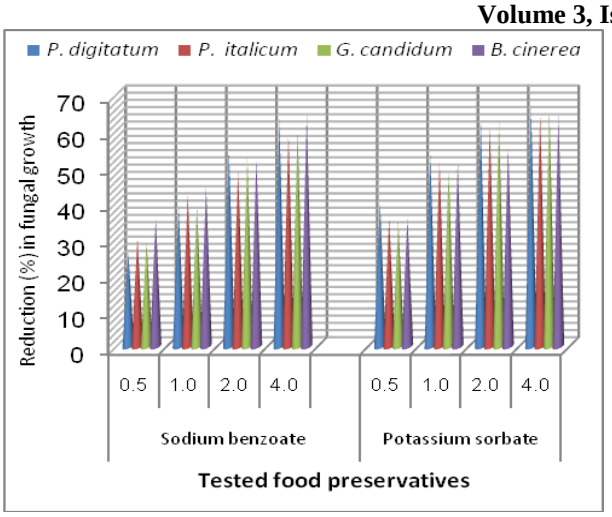

Fig. (2) Growth reduction (%) of some decay pathogenic fungi affected with different concentrations of some food preservatives

The essential oils evaluated in this work have a great variety of phyto-chemicals that could be considered as responsible for a larger or smaller antifungal activity. The inhibitory effect of cinnamon and thyme essential oils against the mycelia growth of decay pathogenic fungi are presented in Table (3) and Fig. (3). Fungal mycelia growth decreased significantly as the concentrations of essential oils were increased, to reach the fungal growth's minimum at the highest concentration used.

Table (3) Mycelial linear growth of some decay pathogenic fungi affected with different concentrations of some essential oils

Tested essential oil	Con.	Fungal linear growth (mm)			
		P. digitatum	P. italicum	G. candidum	B. cinerea
Cinnamon	0	90 a	90 a	90 a	90 a
	0.5	77 c	75 c	75 b	75 b
	1.0	72 c	64 c	66 c	67 c
	2.0	66 d	53 d	57 d	58 d
	4.0	50 f	46 f	43 f	46 f
Thyme	0	90 a	90 a	90 a	90 a
	0.5	76 c	71 c	69 c	63 d
	1.0	65 d	58 d	56 d	54 d
	2.0	48 f	47 f	47 f	43 f
	4.0	38 g	34 g	36 g	30 g

Figures with the same letter are not significantly different ($P \leq 0.05$).

Thyme oil showed more inhibitor effect against the growth of decay fungi than cinnamon oil. Also, the maximum growth inhibition was observed for the growth of *B. cinerea* and *G. candidum* followed by *P. italicum* and *P. digitatum*, respectively. It is well established that some plants contain compounds able to inhibit the microbial growth [37]. These plant compounds can be of different structures and different mode of action when compared with antimicrobials conventionally used to control the microbial growth and survival [38]. Potential antimicrobial properties of plants had been related to their ability to synthesize, by the secondary metabolism, several chemical compounds of relatively complex structures with antimicrobial activity, including alkaloids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, terpens, phenylpropannes, organic acids [39]. The aesthetic, medicinal and antimicrobial properties of plant essential oils have been known since ancient times.

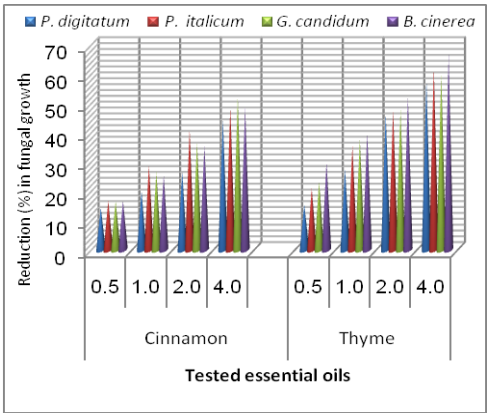

Fig. (3) Growth reduction (%) of some decay pathogenic fungi affected with different concentrations of some essential oils

Numerous studies on the fungicidal and fungistatic activities of essential oils have indicated that many of them have the power to inhibit fungal growth. Thyme oil proved to be extremely effective as a fumigant as well as a contact fungicide against a range of the economically significant fungi *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Erysiphe graminis* [40]. The information was found in the literature concerning mode of action of essential oils on/in the fungal cell in order to promote fungistatic or fungicide effect. In general, inhibitory action of natural products on moulds involves cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intercellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition [41]. Also, it is reported that plant lytic enzymes act in the fungal cell wall causing breakage of b-1,3 glycan, b-1,6 glycan and chitin polymers [42]. The mode by which microorganisms are inhibited by essential oils and their chemical compounds seem to involve different mechanisms. It has been hypothesized that the inhibition involves phenolic compounds, because these compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane causing increased permeability and unavailability of vital intracellular constituents [43]. Their reports indicated that essential oils containing carvacrol, eugenol and thymol (phenolic compounds) had the highest antibacterial performances. Hence, the objective of this study was to determine if natural antagonistic yeast, organic salts and essential oils could provide inhibitor effect against fruit decay pathogenic fungi. Considering their attribute and broad-spectrum activities, successful development of such compounds as antifungal would not only provide a potent tool for control of fruit decay, but also could promise success in multipurpose

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biorational alternatives to conventional fungicides for the management of postharvest diseases.

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AUTHOR BIOGRAPHY

First Author: Department of Plant Pathology, National Research Centre – Egypt, Email: mokh_nrc@yahoo.com.

Second Author: Department of Plant Pathology, National Research Centre – Egypt, Email: nehal_nrc@yahoo.com

Third Author: Department of Plant Pathology, National Research Centre – Egypt, Email: nadia_gamil2000@yahoo.com