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BRIEF REPORT

Growth modeling to control (*in vitro*) *Fusarium verticillioides* and *Rhizopus stolonifer* with thymol and carvacrol

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KEYWORDS
Thymol;
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Abstract The aim of this study was to evaluate the antifungal activity (*in vitro*) of thymol and carvacrol alone or in mixtures against *Fusarium verticillioides* and *Rhizopus stolonifer*, and to obtain primary growth models. Minimal inhibitory concentration (MIC) was evaluated with fungal radial growth with thymol or carvacrol concentrations (0–1600 mg/l). Mixtures were evaluated using concentrations below MIC values. Radial growth curves were described by the modified Gompertz equation. MIC values of carvacrol were 200 mg/l for both fungi. Meanwhile, MIC values of thymol were between 500 and 400 mg/l for *F. verticillioides* and *R. stolonifer*, respectively. A synergistic effect below MIC concentrations for carvacrol (100 mg/l) and thymol (100–375 mg/l) was observed. Significant differences ($p < 0.05$) between the Gompertz parameters for the antimicrobial concentrations and their tested mixtures established an inverse relationship between antimicrobial concentration and mycelial development of both fungi. Modified Gompertz parameters can be useful to determine fungistatic concentrations.

PALABRAS CLAVE
Timol;
Carvacrol;
Sinergismo;
Crecimiento fúngico

Modelización del crecimiento *in vitro* para controlar *Fusarium verticillioides* y *Rhizopus stolonifer* con timol y carvacrol

Resumen El objetivo de este trabajo fue evaluar la actividad antifúngica *in vitro* del timol y del carvacrol, solos o en mezclas, contra *Fusarium verticillioides* y *Rhizopus stolonifer*, y

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obtener modelos primarios de crecimiento. Se evaluó la concentración inhibitoria mínima (CIM) con el crecimiento radial, se ensayaron concentraciones de timol y carvacrol de 0 a 1.600 mg/l. Las mezclas se evaluaron utilizando concentraciones por debajo de los valores de CIM. Las curvas de crecimiento radial fueron descritas por la ecuación de Gompertz modificada. Se obtuvieron los siguientes valores de CIM: carvacrol, 200 mg/l para las 2 especies; timol, 500 mg/l y 400 mg/l para *F. verticillioides* y *R. stolonifer*, respectivamente. Se observó un efecto sinérgico a concentraciones inferiores a las CIM para el carvacrol (100 mg/l) y el timol (100-375 mg/l). Hubo diferencias significativas ($p < 0,05$) entre los parámetros de crecimiento de Gompertz; se estableció que existe una relación inversa entre la concentración de los antimicrobianos y el desarrollo del micelio de ambos hongos.

Fusarium spp. and *Rhizopus* spp. are widespread and ubiquitous in the environment. They can contaminate food before harvest or under post-harvest conditions and are considered food spoilers. Some *Fusarium* species can produce mycotoxins, decreasing the commercial value of the affected products[1].

Fungal contamination, colonization and infection of plants are initiated by contact of the host with conidia (spores) and subsequent conidial germination. Germination and initiation of infections involve biochemical activities with an increase in metabolism and induction of morphological changes. Fungal survival and growth in food may lead to spoilage and toxin formation. These metabolites are structurally diverse compounds and represent an important category of natural toxins that can affect humans and result in economic losses worldwide[12].

For years, synthetic chemical additives were efficient to control food fungal growth; however, these products represent a potential hazard to human health. Currently, a lot of studies on food preservation by natural compounds are being carried out[8]. Many antimicrobial compounds from plants have been identified; several publications reported the antifungal activity of some phenolic components of essential oils such as thymol and carvacrol. Natural isopropyl cresols, carvacrol (5-isopropyl-2-methylphenol), and thymol (2-isopropyl-5-methylphenol) are the major components of oregano (*Origanum* spp.) and thyme (*Thymus* spp.) essential oils. Some researchers have pointed out the antimicrobial activity against bacteria, molds, and yeast[11] of these natural extracts. Carvacrol and thymol are Generally Recognized as Safe (GRAS) food additives, and are used as flavoring agents in baked goods, sweets, beverages, and chewing gum[3].

Food preservation trends indicate the use of new chemical preservatives simultaneously in mixtures to keep food safety and quality at lower antimicrobial doses[1]. These mixtures provide a wider range of increasing activity against different pathogenic microorganisms, or act on several points inside cells, which can enable a better control compared to individual agents[13]. Moreover, in order to evaluate antimicrobial action, predictive microbiology models are valuable. Different primary models describe either germination or mycelium growth kinetics of various fungal species on food products[15]. The modified Gompertz equation is a suitable predictive tool applied in nonlinear growth curves that describe quantitative parameters such as growth, lag phase and fungal growth rate[12].

Therefore, the aim of this study was to evaluate the antifungal activity (*in vitro*) of thymol and carvacrol alone or in mixtures against *Fusarium verticillioides* and *Rhizopus stolonifer* species, and to obtain predictive models of growth.

Microorganisms and preparation of cultures: *F. verticillioides* and *R. stolonifer* were obtained from the Facultad de Ciencias Químicas (Benemérita Universidad Autónoma de Puebla) collection. The microorganisms were maintained on Petri dishes containing sterilized potato-dextrose agar (PDA Merk, Mexico City, Mexico) and incubated in a dark environment at 28 °C for 5–6 days until fungal growth was observed. Fungal structures (conidia and mycelia) were observed using a Zeiss Primo Star microscope (Carl Zeiss AG, Göttingen, Germany) and identified according to taxonomic keys[4].

Minimum Inhibitory Concentration (MIC): thymol or carvacrol (Sigma–Adrich, Milwaukee, WI, USA) were mixed (using a vortex shaker) with sterilized PDA medium to achieve final concentrations of 100, 150, 200, 400, 800 and 1600 mg/l (concentrations were selected according to reference[1]). Agar solutions were poured into sterile petri dishes. Fungal spores were obtained by pouring 9 ml of sterile physiological water (0.90% w/v of NaCl) on the agar plate surface previously inoculated with each mold, followed by gentle scraping using a sterile rake to remove the maximum quantity of spores. Spore suspensions were transferred into sterile tubes. The number of spores present in the suspension was determined using a hemocytometer and an optical microscope (Zeiss Primo Star, Göttingen, Germany), and expressed as number of spores per milliliter (spores/ml). Suspensions were serially diluted to approximately 1000 spores/ml. Finally, plates were inoculated with 10 μl of spore suspension in the center of the plate and were incubated at 28 °C; radial growth was measured every 12 h during 84–96 h. A growth control was prepared in parallel to ensure that viable organisms were present. Every test was performed in triplicate. MIC values were determined as the lowest concentration at which no growth occurred[3].

Antimicrobial mixtures: Mixtures were prepared using concentrations below the MIC value; the evaluation was conducted in accordance with the procedure for the individual antimicrobial assay. In order to evaluate the effects of antimicrobial mixtures a checkerboard array was used; the MIC values for individual antimicrobial or their mixtures were defined as the minimal concentration required to inhibits fungal growth. The MIC data were transformed to fractional inhibitory concentration (FIC) (Eqs. (1) and (2)):

$$FIC_A = \frac{\left(MIC_{\text{compound A in the presence of B}}\right)}{\left(MIC_{\text{compound A individually}}\right)} \quad (1)$$

$$FIC_B = \frac{\left(MIC_{\text{compound A in the presence of B}}\right)}{\left(MIC_{\text{compound B individually}}\right)} \quad (2)$$

The FIC_{Index} (Eq. (3)) for mixtures was calculated with the FIC for individual antimicrobials[7], a FIC_{Index} near 1 indicates additive effect, whereas <1 indicates synergism and >1 indicates antagonism:

$$FIC_{Index} = FICA_A + FICA_B \quad (3)$$

Fungal growth modeling: All growth curves were fitted using the modified Gompertz model (Eq. (4))[5]

$$In\left(\frac{D_t}{D_O}\right) = A\exp\left\{-\exp\left[[(v)]_{max}\cdot\frac{e}{A}(\lambda - t) + 1\right]\right\} \quad (4)$$

where D_t is the average diameter at time t (h), D_o is the average diameter at initial time (0.02 cm), A is the maximum fungal growth achieved during the stationary phase, υ_{max} is the maximum specific growth rate (1/h), λ is the lag time (h), and $e = \exp(1)$; nonlinear regression was performed using the KaleidaGraph program (3.21 Synergy Software, Reading, PA., U.S.A.).

Statistical analysis: Growth parameters were analyzed by ANOVA. Significant values were subjected to mean analysis by the Tukey's test, using a confidence level of 95%. Statistical analyses were performed using Minitab15 (LEAD Technologies Inc., NJ, U.S.A.).

MIC values of thymol and carvacrol and mixtures of these compounds: thymol and carvacrol, which are isomer molecules, showed antimicrobial activity against both fungi. The MIC values for *F. verticillioides* for carvacrol was 150 mg/l and for thymol 400 mg/l. The MIC values for *R. stolonifer* for carvacrol was 200 mg/l and for thymol 800 mg/l. Mixtures were effective in 7 of 9 combinations for *F. verticillioides* and in 6 of 9 combinations for *R. stolonifer* (Tables 1 and 2). Only one of the seven inhibitory mixtures showed synergistic action (100 mg/l of thymol and 100 mg/l of carvacrol) against *F. verticillioides* (FIC_{Index} 0.92) while four of the six inhibitory mixtures showed synergistic action against *R. stolonifer* (FIC_{Index} average 0.85 ± 0.11). Moreover, the mixture containing 375 mg/l of thymol and 50 mg/l of carvacrol showed the lowest FIC_{Index} value (0.72).

Fungal growth modeling: Although the modified Gompertz equation is a primary predictive model whose parameters adequately described fungal growth (mean coefficient of determination 0.991 ± 0.04), the statistical analysis of modified Gompertz parameters showed differences ($p < 0.05$) in maximum fungal growth (A) and lag phase (λ). Moreover, the increase of both antimicrobial concentrations increases λ values. The modified Gompertz parameters (A and λ) for *F. verticillioides* showed significant differences ($p < 0.05$) with thymol and carvacrol individually and in mixtures (100/50 and 200/50 thymol/carvacrol mg/l). Some combinations under MIC values affect radial growth and increase the lag phase (Fig. 1A). For *R. stolonifer* three thymol/carvacrol combinations (125/50, 250/50 and 125/100 mg/l) showed the same effect in growth parameters (Table 2), especially in the lag phase ($p > 0.05$) with a fungistatic effect, as single compound applications of 200 mg/l of thymol and 100 mg/l of carvacrol (Fig. 1B). For both fungi an inverse relationship between antimicrobial concentration and mycelial development is established; carvacrol can extend the lag phase up to 5 and 60 h (for *F. verticillioides and R. stolonifer* respectively) compared to control.

Table 1 Modified Gompertz model parameters[+] (mean ± standard deviation) for *F. verticillioides* growth curves subjected to selected concentrations of thymol and carvacrol

	A	υ_{max} (1/h)	λ (h)
Control	5.50 ± 0.04^{a}	0.30 ± 0.00^{a}	12.10 ± 0.15^{a}
Thymol (mg/l)			
100	5.03 ± 0.03^{b}	0.28 ± 0.01^{a}	53.11 ± 0.04^{d}
150	5.09 ± 0.12^{b}	$0.22 \pm 0.08^{a,b}$	57.72 ± 0.19^{b}
200	4.90 ± 0.02^{c}	0.15 ± 0.02^{b}	61.54 ± 0.23^{b}
400	–	–	$>84^{c}$
Carvacrol (mg/l)			
100	4.92 ± 0.05^{b}	0.24 ± 0.05^{a}	60.42 ± 0.07^{b}
150	–	–	$>84^{c}$
Thymol/Carvacrol (mg/l)			
100/50	4.21 ± 0.02^{d}	0.88 ± 0.01^{c}	68.35 ± 0.02^{e}
200/50	3.98 ± 0.02^{e}	0.56 ± 0.00^{d}	77.38 ± 0.01^{f}
100/100	–	–	$>84^{c}$
100/150	–	–	$>84^{c}$
200/100	–	–	$>84^{c}$
200/150	–	–	$>84^{c}$
300/50	–	–	$>84^{c}$
300/100	–	–	$>84^{c}$
300/150	–	–	$>84^{c}$

+ A: maximum fungal growth in the stationary phase; υ_{max}: maximum specific growth rate; λ: lag phase.
– No growth. Means followed by a different superscript letter within a column for each are significantly different ($p < 0.05$).

Our results demonstrate that mixtures of thymol and carvacrol inhibit the studied mold strains, with lower concentrations than those needed when the antimicrobial is utilized individually. The use of antimicrobial mixtures provides a wider range of activity, which can enable a better control of the evaluated molds under *in vitro* conditions, as it has been reported when results were compared with the use of an individual antimicrobial agent[8]. Similar results of MIC values for *F. verticilliodes* were obtained[6], which showed that thymol and carvacrol affect radial growth and conidial production and germination. Moreover, similar MIC values were obtained when thymol and carvacrol were used against *F. oxysporum* (350 mg/ml and 150 mg/ml) and *R. oryzae* (500 mg/ml and 250 mg/ml)[1]. There are no

Table 2 Modified Gompertz model parameters+ (mean ± standard deviation) for *R. stolonifer* growth curves subjected to selected concentrations of thymol and carvacrol

	A	υ_{max} (1/h)	λ (h)
Control	5.61 ± 0.00^{a}	0.31 ± 0.01^{a}	0.42 ± 0.11^{a}
Thymol (mg/l)			
100	5.26 ± 0.04^{d}	0.31 ± 0.04^{a}	6.35 ± 0.10^{e}
150	$5.16 \pm 0.07^{b,d}$	0.29 ± 0.02^{a}	6.60 ± 0.26^{e}
200	4.98 ± 0.05^{e}	0.29 ± 0.01^{a}	6.45 ± 0.15^{be}
400	4.47 ± 0.01^{f}	0.28 ± 0.03^{a}	12.58 ± 0.10^{b}
800	–	–	$>84^{d}$
Carvacrol (mg/l)			
100	5.10 ± 0.02^{b}	0.33 ± 0.03^{a}	13.06 ± 0.17^{b}
150	4.58 ± 0.01^{c}	0.30 ± 0.02^{a}	25.21 ± 0.07^{c}
200	–	–	$>84^{d}$
Thymol/Carvacrol (mg/l)			
125/50	4.94 ± 0.01^{e}	0.29 ± 0.03^{a}	13.27 ± 0.02^{b}
250/50	4.46 ± 0.03^{f}	0.21 ± 0.02^{b}	13.57 ± 0.02^{b}
25/100	4.84 ± 0.02^{e}	0.22 ± 0.01^{b}	13.35 ± 0.01^{b}
125/150	–	–	$>84^{d}$
250/100	–	–	$>84^{d}$
250/150	–	–	$>84^{d}$
375/50	–	–	$>84^{d}$
375/100	–	–	$>84^{d}$
375/150	–	–	$>84^{d}$

+ *A*: maximum fungal growth in the stationary phase; υ_{max}: maximum specific growth rate; λ: lag phase.
– No growth. Means followed by a different superscript letter within a column for each are significantly different ($p < 0.05$).

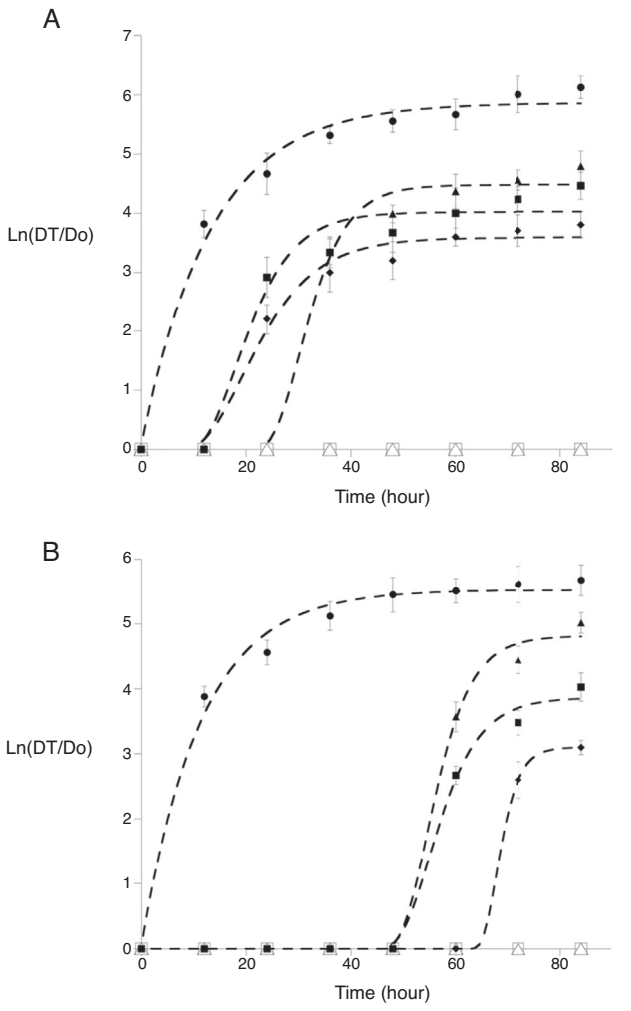

Figure 1 Effect of carvacrol, thymol, and his mixtures at selected concentrations [0 (●), 150 carvacrol (▲), 200 carvacrol (△), 400 thymol (■), 800 thymol (□), and 250/50 thymol/carvacrol (♦) mg/l] on *R. stolonifer* growth (A) and on *F. verticillioides* (B) [0 (●), 100 carvacrol (▲), 150 carvacrol (△), 200 thymol (■), 400 thymol (□), and 200/50 thymol/carvacrol (♦) mg/l] D_t is the average colony diameter at time *t* and D_o is the average colony diameter at initial time, fitted (—) with the modified Gompertz model.

studies on *R. stolonifer* inhibition with natural antimicrobial mixtures. Thymol and carvacrol have a hydroxyl group at different locations on the phenolic ring that increases the ability to dissolve the microbial membrane causing the loss of macromolecules of the cell inhibiting fungal conidial germination[10]. Moreover, they can change pH homeostasis, K+ gradient, resulting water imbalance, intracellular ATP depletion and cell death[2]. These isomers affect radial growth, production, and conidial germination and cause morphological changes[9]. Thymol and carvacrol inhibit ergosterol biosynthesis which consequently affects cell membrane integrity[1]. These cell modifications affect morphology, such as lack of sporulation, loss of pigmentation, irregular development of conidiophores and distortional hyphae[3,5].

Antimicrobial interaction mechanisms are less known. There are some hypotheses that mention that phenolic mixtures could increase the number, size or duration of membrane pores by binding phenolic compounds with different proteins or enzymes embedded in the cell membrane[14]. A synergistic effect would be achieved when two components probably disintegrate the lipid membrane and make it easier for the other component to enter the cytoplasm[13].

Primary models are suitable to predict fungal growth and may be useful for research purposes, although it is possible to be a routine measurable parameter on food analysis. Modified Gompertz parameters can be useful to determine fungistatic concentrations (2 of 9 mixtures for *F. verticillioides* and 3 of 9 for *R. stolonifer*). Fungal growth rate and lag time are parameters, which subsequently may be used for a secondary modeling if growth curves for different constant conditions are available[15]. There are few reports that describe the effect of natural antimicrobials using mathematical models; fungal growth parameters provide information that can be used to assess the response of other fungal strains as well as to evaluate antifungal agents in additional systems or foods.

Ethical disclosures

Protection of human and animal subjects. The authors state that no human or animal experiments have been performed for this research.

Confidentiality of data. The authors state that no patient data appears in this article.

Right to privacy and informed consent. The authors state that no patient data appears in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

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