Antifungal Activity of Hexanal As Dependent on Its Vapor Pressure

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Hexanal was taken as a model molecule to study the relationship between the antifungal activity of a volatile compound and its vapor pressure. A Central Composite Design (3 variables, 5 levels) was developed in order to modulate the hexanal vapor pressure and evaluate the effect of water activity (a_w), temperature and hexanal dose on the inhibition of the growth of *Aspergillus niger* as well as the changes of the hexanal vapor pressure during incubation in model systems. The different indices taken into consideration evidenced the importance of the hexanal vapor pressure for its bioactivity against the mold. Moreover, the temperature increase enhanced the hexanal antifungal activity, due to its effect on vapor pressure. This indirect effect of temperature sustained the assumption that the volatile molecule effectiveness depends on the vapor pressure.

Keywords: *Vapor pressure; hexanal; antifungal activity; temperature; water activity*

INTRODUCTION

There is considerable interest in the possible use of natural alternatives as food additives to prevent bacterial or fungal growth and to extend the shelf life of foods. Many naturally occurring compounds such as phenols, aldehydes, and organic acids, present in spices and herb extracts, showed antimicrobial activity (Wilson and Wisniewski, 1989; Song et al., 1996). However, the mechanisms at the basis of their antimicrobial activity have been poorly explored.

In a preliminary work concerning the antifungal activity of some esters, aldehydes, alcohols, and terpenes, characteristic of apple flavor, Caccioni et al. (1997) proposed that the effectiveness against *Penicillium expansum* and *Botrytis cinerea* of the considered molecules, and in particular of hexanal, benzyl acetate, and estragole, was dependent on their actual vapor pressure rather than on their whole concentration in the system. The vapor pressure of a molecule, at a given concentration, depends on temperature and on its interaction with the other solutes and provides a measure of the tendency of a molecule to enter into the vapor phase.

The ability of a potentially active molecule to interact with the hydrophobic cell membranes can be regarded as the result both of its intrinsic hydrophobicity, which increases with the hydrocarbon chain length (Guerzoni et al., 1994a), and of its "actual hydrophobicity", which is inversely related to its capacity to form links with the water molecule cloud surrounding its polar groups (Guerzoni et al., 1994b, 1997). The vapor pressure of a specific volatile molecule in a given system and at a given temperature can be assumed as an indirect measure of this "actual hydrophobicity" because the tendency of the molecules to pass in the vapor phase is related to the extent of its interaction with water and the other solutes.

Six-carbon aldehydes are dominant compounds released by plant material through the lipoxygenase pathway after tissue damage (Hildebrand et al., 1988). The production of these molecules is related to their toxic action toward the mold which could attack the wounded area causing the decay of fruits. Moreover they are important precursors of six-carbon alcohols and esters which are among the most abundant volatile compounds contributing to fruit odors (Paillard, 1986, 1990). In particular, the potential of hexanal as a metabolizable fungicide for minimally processed apples has been explored by Song et al. (1996), who showed how the aroma production was enhanced by the interconversion of hexanal vapor to other aroma volatiles.

In this work, the hexanal was taken as a model molecule to analyze the dependence of the antifungal activity on its vapor pressure in the system. In order to evaluate the individual and interactive effects of the temperature, a_w, and hexanal dose on *Aspergillus niger* as test organism and on the distribution of hexanal in the phases of the systems, evaluated as vapor pressure, a Central Composite Design (CCD) at 3 variables and 5 levels was developed.

MATERIALS AND METHODS

Microbial Strain. The *A. niger* 101 strain used was obtained from the collection of the Dipartimento di Protezione e Valorizzazione Agroalimentare of the University of Bologna.

Experimental Design. The influence of various temperatures, a_w, and micromoles of hexanal on the fungal activity and on the presence of hexanal in the vapor phase was analyzed by using a five-level Central Composite Design (CCD) (Box et al., 1978). Table 1 shows the levels of the three factors in the 17 runs of CCD. The different values of a_w were obtained by adding NaCl to the culture medium. The values of a_w were measured by means of an Aqua Lab model CX2 water activity measurement instrument (Decagon Devices, Inc., Pullman, WA). The effect of a_w on the dependent variables cannot be satisfactory fitted by means of a quadratic function as already observed by Gibson et al. (1994) who proposed the following transformation:

$$b_w = \sqrt{1 - a_w} \quad (1)$$

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Table 1. Central Composite Design of 17 Runs (Each Repeated Three Times) for the Study of the Three Variables Modulated at Five Levels

run	temperature (°C)	NaCl (a_w)[a]	hexanal (μmol)
1	20	2 (0.992)	0.814
2	20	2 (0.992)	2.442
3	20	6 (0.975)	0.814
4	20	6 (0.975)	2.442
5	30	2 (0.992)	0.814
6	30	2 (0.992)	2.442
7	30	6 (0.975)	0.814
8	30	6 (0.975)	2.442
9	25	4 (0.988)	1.628
10	25	4 (0.988)	1.628
11	25	4 (0.988)	0.000
12	25	4 (0.988)	3.256
13	25	0 (0.998)	1.628
14	25	8 (0.964)	1.628
15	15	4 (0.988)	1.628
16	35	4 (0.988)	1.628
17	25	4 (0.988)	1.628

[a] For the variable NaCl the percentage of salt added is reported and, within brackets, the relative value of a_w instrumentally measured

This reparametrization of the variable a_w was used to obtain the final models.

Evaluation of Antimicrobial Activity of the Substances in the Vapor Phase. Vials (10 mL capacity) containing 5 mL of medium (Sabouraud dextrose agar, Difco, Detroit, MI) were superficially inoculated with 0.1 mL of *A. niger* spore suspension (5×10^5 spores mL^{-1}). A tiny steel hook, with a 6 mm diameter filter paper disk (Antibiotica-Testblättchen, Schleicher & Schull, Dassel, Germany) fastened to its end, was applied to the internal part of the butyl septa on the vials. The filter was soaked with the desired doses of hexanal diluted in 9 μL of methanol (Table 1), and 9 μL of methanol was added to the samples in which the hexanal was absent. The compound and the methanol were previously sterilized through filtration (Millex-GS, Millipore, 0.22 μm, Molsheim, France). The vials were then sealed and incubated at the temperatures indicated in the CCD (15, 20, 25, 30, 35 °C). The head spaces of vials were automatically sampled at intervals of 5 h, up to stationary phase of growth, for the gas chromatographic analysis of hexanal and of the CO_2 produced by the fungus.

Carbon Dioxide Determination. *A. niger* growth was monitored through the metabolic CO_2 produced by the fungi in equilibrium in the head space (Guerzoni et al., 1985, 1990; Gardini et al., 1988). The detection of the CO_2 in the head space of the vials was performed according to Caccioni et al. (1997). Each analysis required 3.5 min. The gas chromatograph was connected with a model HS250 head space autosampler (Carlo Erba Instruments, Milan, Italy) equipped with a Hamilton Gastight gas syringe (Hamilton, Bonaduz, Switzerland). This automatic gas sampling is not destructive and allows several analyses of the same vials over time (Gardini et al., 1997). During the sampling, all the vials were maintained in the autosampler at the temperatures established according to the CCD, and for each analysis 0.05 mL of head space was sampled. Three repetitions for each run were analyzed over time.

The CO_2 percentage (v/v) in the head space was analyzed over time according to the Gompertz equation as modified by Zwietering et al. (1990):

$$y = A \exp\left\{-\exp\left[\left(\frac{\mu_{max} e}{A}\right)(\lambda - t) + 1\right]\right\} \quad (2)$$

where y is the CO_2 percentage at time t, A represents the maximum percentage of CO_2 production, μ_{max} is the maximum CO_2 production rate (as Δ% CO_2/h), and λ is the lag time (h) for CO_2 production.

The CO_2 data were fitted with the modified Gompertz equation for each run of the CCD and for the reference samples incubated at the different temperatures of the experimental design in the absence of hexanal and NaCl.

To evaluate the influence of the three variables on the fungal growth two different indices were taken into consideration:

1. The extension of the lag phase, λ_R, for each run of the CCD.
2. The ratio λ_R/λ_C, where λ_C is the lag phase for each control at the same temperature in the absence of hexanal and NaCl.

Determination of Hexanal in the Head Space. The gas chromatographic analysis of hexanal was performed according to the method of Caccioni et al. (1997). The gas chromatograph was connected to a model HS250 head space autosampler (Carlo Erba Instruments) equipped with a Gastight gas syringe (Hamilton). For each analysis 0.1 mL of the head space was sampled. Three repetions for each run were analyzed.

The conversion of the gas chromatographic measurements into vapor pressure was performed according to the following equation derived from the Raoult's law:

$$p_m = X_m \gamma_m P^* \quad (3)$$

where p_m is the vapor pressure of the molecule, X_m is the molecule molar fraction, γ_m is the activity coefficient of molecule, and P^* is the pure molecule vapor pressure expressed as kPa. The vapor pressure of pure hexanal was calculated, on the basis of the data reported in literature (*Beilsteins Handbuch der Organischen Chemie*, 1959), by means of the Clausius−Clapeyron equation. In fact, according to Kolb (1980), for a nonideal system under equilibrium condition, it is possible to write

$$A = C X_m \gamma_m P^* \quad (4)$$

where A is the gas chromatographic molecule peak area and C is the response factor. Thus combining the two equations (3 and 4):

$$p_m = A/C \quad (5)$$

Data Analysis. Polynomial equations describing the effect of the independent variables (temperature, a_w, and hexanal dose) as the individual or quadratic terms, and of their interactive effects on the dependent variables were obtained. The goodness of fit of the models obtained was evaluated using R (multiple determination coefficient) (Pike, 1986), the Fisher F-test (and the derived p-values), and the standard error of estimate (SE).

The response surfaces, as a function of two independent variables holding the other independent variable constant at fixed levels (the intermediate value), are helpful for understanding both the main and the interactive effects of the variables.

The data were processed using the statistical package Statistica for Windows (Statsoft Inc., Tulsa, OK).

RESULTS

Vapor Pressure and Partition of the Hexanal in the Phases of the Model Systems. Under the experimental conditions used, the hexanal migrated from its source to the head space and later it diffused in the aqueous phase, i.e., the agarized culture medium, of the system. The dynamics of the distribution and the partition of hexanal between the solid and the gas phases depended on the temperature, on its affinity for the various phases, on the diffusion coefficient, etc.

Under these experimental conditions, the partition and the physical state of hexanal were analyzed in terms of vapor pressure. The following empirical equation describes the changes of hexanal vapor pressure after 4 h of conditioning at the various temperatures:

$$P_H = \frac{a + bt}{ct} \quad (6)$$

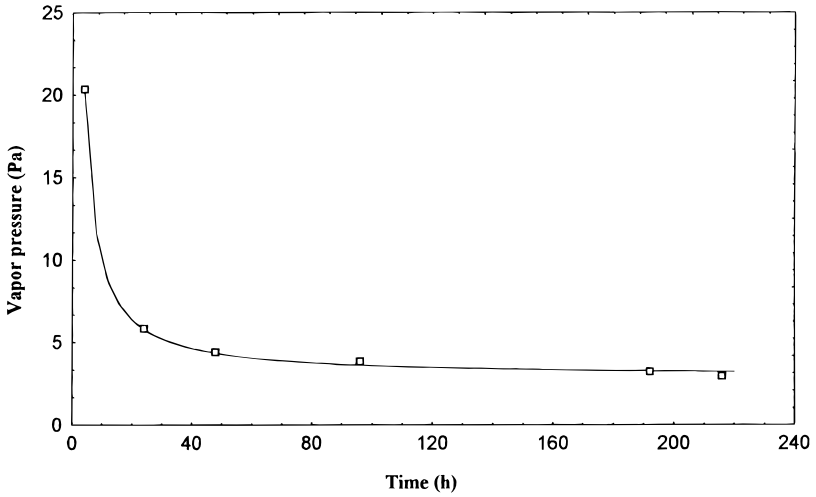

Figure 1. Changes of hexanal vapor pressure, over time, in the head space of run 2 of the CCD.

Table 2. Final Model Obtained for the Different Parameters Concerning the Vapor Pressure of Hexanal and the Lag Phase of *A. niger*[a]

eq	parameter	equation	R	SE	F	p
a	P_{Hi}	$0.645[H][T]$	0.909	13.99	76.01	<0.0000
b	P_{Hf}	$-0.00229[T]^2 + 0.0767[H][T] + 0.316[b_w][T]$	0.987	0.55	169.52	<0.0000
c	λ_R	$715.94 - 48.89[T] + 0.84[T]^2 + 201.84[H][b_w]$	0.906	24.96	19.86	<0.0000
d	λ_R/λ_C	$13.13[b_w] + 0.0183[H][T]$	0.977	0.56	156.16	<0.0000

[a] Only terms with $p < 0.05$ are kept in the final model. The statistical indices reported are the coefficient of multiple correlation (R), the standard error of estimate (SE), and the F-value (F) of the overall model and the relative p.

where P_H is the vapor pressure (in Pa), t is the time (h), and a, b, and c are the equation coefficients. The changes of P_H modeled with such an empirical equation gave curves similar to that of Figure 1 corresponding to the run 2 of the CCD. For each combination of the CCD the partition of hexanal in the head space was analyzed by using two different indices, all expressed as vapor pressure (Pa):

1. The hexanal in the head space after a 4 h incubation assumed as initial vapor pressure (P_{Hi}), calculated by interpolating the equation describing P_H for the time 4 h.

2. The hexanal in the head space of the system at the equilibrium assumed as final vapor pressure (P_{Hf}), defined as the asymptotic values (a/c) characterizing the curve describing P_H. The curves obtained for the various runs presented differences concerning both the values of P_{Hi} and P_{Hf}. The calculated values of P_{Hi} and of P_{Hf} for each run of the CCD were analyzed to obtain polynomial equations describing the individual and interactive effects of the temperature, b_w and hexanal doses on the two indices. The equations obtained are reported in Table 2 (eqs a and b). According to eq a, P_{Hi}, i.e. the initial value of vapor pressure, depended only on the interaction between the temperature ([T]) and the dose of hexanal ([H]). Figure 2 represents the response surface relative to the interaction [T] × [H]: maximum values of P_{Hi} are generated when both the factors are at their highest levels. In fact, in this initial stage, the hexanal was partitioned between the paper disk and the head space and its physical state were not yet affected by the solid medium composition.

The interactions affecting the values of P_{Hf} at the equilibrium are more complex. In fact, according to the eq b, P_{Hf} depends not only on the interaction [T] × [H] but also on $[T]^2$ and on the interaction [T] × [b_w]. Figure 3a (relative to the interaction [H] × [b_w]) shows only a light increase of the asymptotic vapor pressure with the

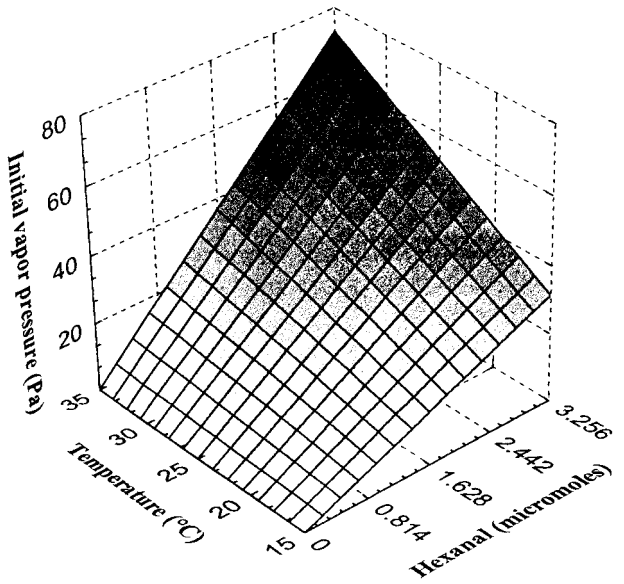

Figure 2. Three-dimensional plot relative to the interaction [T] × [H] on initial vapor pressure of hexanal (P_{Hi}).

NaCl content of the system. This can be attributed to the low water solubility of hexanal. According to the Figure 3b, which represents the interaction of [T] × [H], the asymptotic P_{Hf} showed maximum values when both factors were at their highest levels. However the negative sign of the coefficient of $[T]^2$ could account for the increase of diffusion coefficient and solubility of hexanal in the aqeous phase or for its decomposition favored by temperature increase.

Growth of *A. niger*. The instrumental method used to analyze the *A. niger* activity in the various experimental conditions permitted the accumulation of an appropriate number of data. It was based on the gas chromatographic determination of the metabolic CO_2, which is well correlated to the microbial growth (Guer-

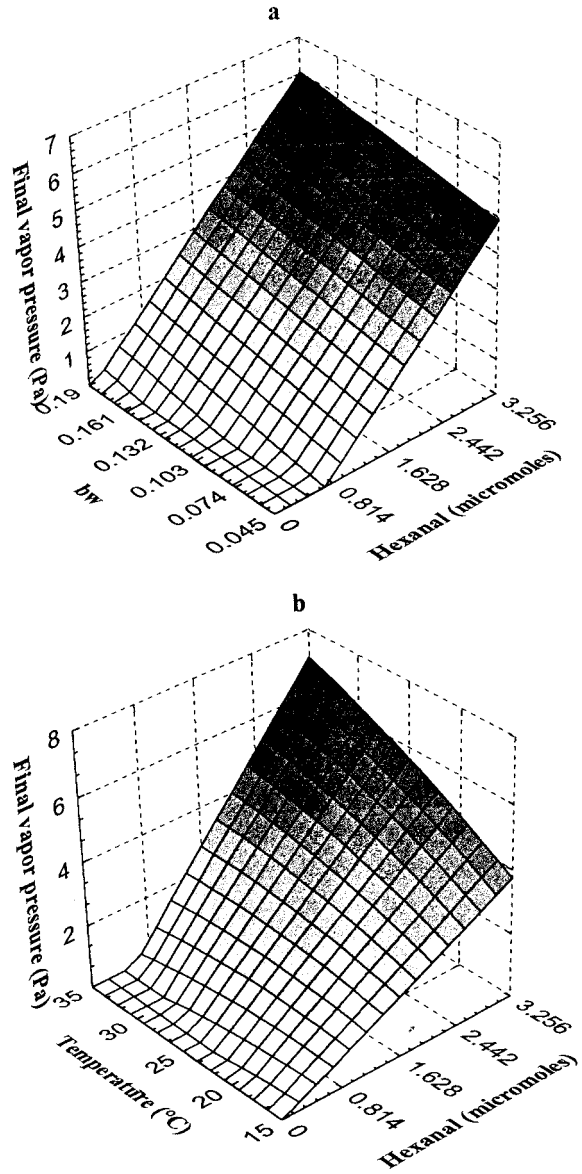

Figure 3. Three-dimensional plot relative to the interaction $[b_w] \times [H]$ (3a), where $b_w = \sqrt{1-a_w}$, and to the interaction $[T] \times [H]$ (3b) on final vapor pressure of hexanal (P_{Hf}).

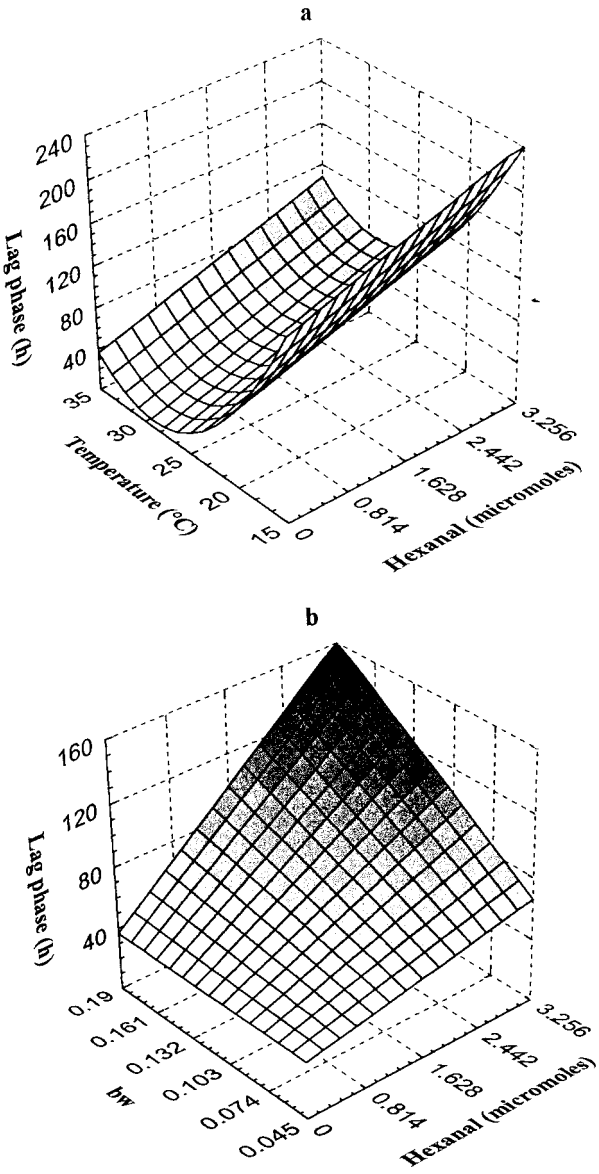

Figure 4. Three-dimensional plot relative to the interaction $[H] \times [T]$ (4a) and to the interaction $[H] \times [b_w]$ (4b) on extension of the lag phase (λ_S) of *A. niger*.

zoni et al., 1990). The CO_2 data relative to the three replications of each run of the CCD were analyzed according to the modified Gompertz equation giving rise to the growth parameters λ, μ_{max}, and A. The λ parameter presented the most remarkable differences, with respect to the μ_{max} and A values, within the various runs. So this growth parameter was taken as a measure of the hexanal antifungal effectiveness. The calculated values of λ_R were analyzed in order to obtain polynomial equations describing the effects of the independent variables. Equation c (Table 2) indicates that the lag phase of *A. niger* decreases with the temperature (as indicated by the negative cofficient of this term) but, over a certain threshold, it increases, as indicated by the positive sign of the quadratic term (Figure 4a). In the considered range, the temperature increase can have two opposite effects: it increases the P_{Hi} or the P_{Hf} and, on the other hand, it reduces the lag phase of *A. niger* at least up to its optimum temperature. Moreover, the λ_R is positively affected by the interaction $[H] \times [b_w]$: these two variables showed a positive interaction in the prolongation of the mold's lag phase (Figure 4b).

The ratios between the lag phase of the samples with hexanal (λ_R) and those of the controls at the same temperature but without hexanal and NaCl (λ_C) have been considered in order to distinguish the indirect effect of the temperature on hexanal vapor pressure (and, consequently on its toxicity on the mold) from the direct effect of temperature on fungal growth. A polynomial equation describing the individual and interactive effects of the three variables on this dependant variable has been obtained (eq d, Table 2). According to this equation, the value of this ratio increases with the NaCl content and is positively affected by the interaction $[T] \times [H]$. Figure 5a shows that the extension of the lag phase, with respect to the control incubated at the same temperature, is maximum when both temperature and hexanal doses are at their highest values. This accounts for an indirect effect of the temperature on the hexanal antifungal effect and sustains the hypothesis that its effectiveness depends on the vapor pressure of the molecule, which is, in turn, dependent on temperature. The dependence of this ratio on b_w further supports the importance of the physical state of hexanal for its bioactivity. In fact, in

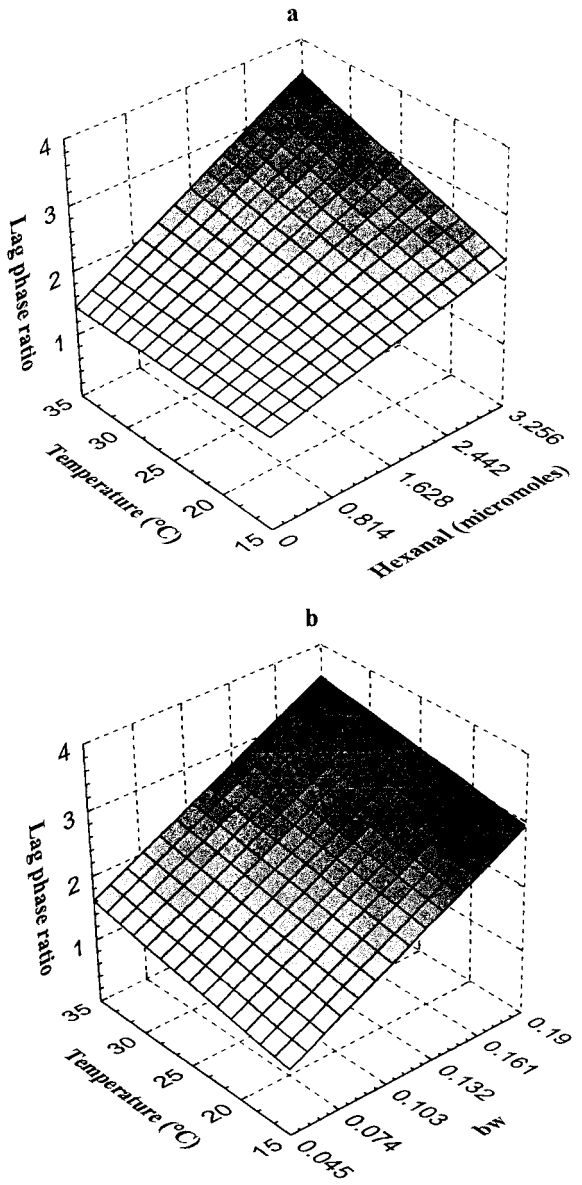

Figure 5. Three-dimensional plot relative to the interaction [H] × [*T*] (5a) and to the interaction [*T*] × [b_w] (5b) on lag phase ratio (λ_R/λ_C) for *A. niger*.

agreement with the fact that vapor pressure of a volatile solute increases with decreasing a_w (increasing b_w), the ratio presented its maximum value when the factors [*T*] and [b_w] were at their highest levels (Figure 5b).

DISCUSSION AND CONCLUSIONS

Plant secondary metabolites appear to be involved in plant fitness and in postharvest diseases of fruit and vegetables (Wilson and Wisniewski, 1989). Although plant pathologists have long recognized the importance of secondary metabolites in plant resistance, their antimicrobial potential has not been sufficiently exploited. Further opportunities associated with the application of such metabolites and particularly of aldehydes lie in the ability of the plant tissues to convert them into alcohols or esters that can enhance the fruit flavor and antimicrobial activity (Bartley et al., 1985; Song et al., 1996). However, though the potential of using a number of volatile molecules has been widely explored, the incomplete knowledge of their action mechanisms and of the effects of the environmental conditions has limited their practical application. The results of this investigation concerning hexanal as a model molecule provided an interpretation key based on the linkage between toxicity and vapor pressure.

A direct relationship between hexanal vapor pressure during the storage and the antifungal activity cannot be obtained within the framework of this experiment due to the fact that both vapor pressure and growth parameters are variables dependent on the 3 factors of the CCD. However indirect evidence has been provided by the significant effect of the interaction between temperature and hexanal dose on the ratios between the lag phases in the presence and in the absence of hexanal at the same temperature (λ_R/λ_C). This analysis focused on distinguishing the direct effects of temperature on *A. niger* growth from its indirect effects and namely of hexanal vapor pressure. The importance of the tendency to pass into the vapor phase, generically described as "volatility", in the expression of the toxicity against microorganisms has also been observed for hydrocarbons by Walker et al. (1975). Likewise the role of temperature in increasing the growth inhibition of the pyrene-utilizing *Rhodococcus* strain, observed by Walter et al. (1991), could allude to a toxic effect of pyrene resulting from an increase in its vapor pressure with a consequent increase of the partitioning of the molecule into the cytoplasmatic fungal membrane.

Direct evidence of the vapor pressure importance might be provided only through a deeper investigation of the hexanal mass transfer and partition into cytoplasmic fungal membrane. Nevertheless the practical applications could be interesting. In fact, one might take advantage of the increase of vapor pressure of the hexanal or other volatiles in order to compensate the negative shelf life effects of temperature fluctuations and the frequent cold chain interruptions in food distribution. In fact the optimum storage temperature is rarely attained or even sought for products being distributed within North America and Europe (Gill and Jones, 1992; Gill et al., 1991).

Moreover, other practical applications can be for packaged refrigerated foods and for the creation of special atmospheres in unrefrigerated or refrigerated rooms. The increase of the vapor pressure of the volatile molecules with temperature and the possibilty of controlling it with appropriate measures might create promising opportunities either in the mold control in unrefrigerated packaged foods or in the plant disease control in greenhouses.

Several commodities treated with hexanal or other volatiles could be stored in same cases under high humidity conditions. However, a high water vapor does not affect the vapor pressure of hexanal, due to its very poor water solubility. In fact, such systems can be regarded as ternary sistems, based on water, "third components" (i.e. sugar, salts, organic acids, etc.), and hexanal or other nonwater-soluble volatiles. In such systems, as reported by Lerici and Nicoli (1996), compounds such as NaCl, which are very soluble in water but not in hexanal, can enhance hexanal partition in the head space, while the water, due to its poor affinity with hexanal, does not affect its partition or, consequently, its vapor pressure. Moreover, compounds soluble in both water and hexanal, such as glycerol, reduce the latter vapor pressure.

A technological aspect to be investigated and solved at the practical level concerns a programmed molecule release in the head space or the definition of the

physicochemical conditions able to maintain over longer time spans a high vapor pressure of the chosen volatiles.

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