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A Box-Behnken design for predicting the combined effects of relative humidity and temperature on antagonistic yeast population density at the surface of apples

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Abstract

The objective of this work was to develop models predicting the combined effects of relative humidity (RH, 75–98%), temperature (5–25 °C), and initial applied yeast concentration (10^4–10^8 CFU/ml) on the apple-surface population densities of two biocontrol agents fused against postharvest diseases; the antagonistic yeasts *Pichia anomala* strain K and *Candida oleophila* strain O. Experiments were carried out according to a Box-Behnken matrix. Multiple regression analyses showed that both models yielded a good prediction of yeast density. The effect of relative humidity appeared greater than that of temperature. The number of yeast colony-forming units per square centimeter of apple fruit surface increased with increasing relative humidity, temperature, and initial applied yeast concentration. The models predict that under optimal growth conditions (25 °C, 98%), strains O and K should reach a density of 10^4 CFU/cm^2 when applied initially at 2×10^7 (strain O) or 10^7 CFU/ml (strain K). The model results suggest that rainfall was likely the principal cause of the variability of yeast efficacy reported for previous preharvest orchard trials spanning two successive years. Temperature may also contribute to this variation. The models developed here are important tools for predicting population densities of both strains on the apple surface within the experimental limits. The use of these results should contribute to achieving yeast densities of 10^4 CFU/cm^2 on apples by controlling yeast application and environmental factors such as relative humidity and temperature. The results of this study also confirm our previous *in vitro* findings that water activity has a greater effect than temperature on yeast population density.

Keywords: Population density; *Pichia anomala* strain K; *Candida oleophila* strain O; Relative humidity; Temperature and Box-Behnken design

1. Introduction

Biological control of postharvest diseases of fruits appears as a realistic alternative to fungicide application, as only the fruits need to be treated, environmental factors are defined and stabilized in storage rooms, so that harvested commodities will have high value (Fokkema, 1991; Wilson and Wisniewski, 1994; Jijakli et al., 1999). Biocontrol of postharvest fruit decays is achievable by postharvest application of antagonists and by preharvest spraying of biocontrol agents in the field (Benbow and Sugar 1999; Korsten et al., 1997; Leibinger et al., 1997; Teixidõ et al., 1998a). In the latter practice, the antagonist is applied just before harvest so that it can colonize the fruit surface and any wounds inflicted during harvest before the arrival of wound pathogens (Ippolito and Nigro, 2000). Yet authors highlight the very real practical problem of promoting the effective establishment of prospective antagonists in a natural environment. This can be crucial, limiting the consistency of biocontrol under field conditions and the widespread commercialization of biocontrol agents.

The fluctuation of abiotic factors such as temperature, water availability, relative humidity and UV radiation has the greatest impact on the growth and biological proprieties of prospective biocontrol agents (Magan, 2001; Teixidõ et al., 1999). Tolerance to such abiotic fluctuations is a prerequisite to successful application of ecologically competent biocontrol agents under field conditions (Elad, 1990). Such tolerance will

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make it possible to establish a high biocontrol agent (BCA) population density. Physiological manipulation of antagonists has focused on improving their ecological fitness, which is particularly important in orchard applications where environmental conditions fluctuate widely (Janisiewicz and Korsten, 2002). Unfortunately, the study of the ecological fitness of antagonistic agents to be applied in the field has received little attention as compared with other topics such as mechanisms of action, genetic manipulation, and combination with chemical treatments. Nevertheless, some elegant studies on BCA desiccation tolerance have been published (Magan, 2001). Recent investigations carried out *in vitro* with the antagonistic yeast *Candida sake* (Teixidõ et al., 1998a) have shown that its *in vitro* growth is affected by environmental factors such as temperature, water activity, and pH. The water activity of the culture medium used to produce the biocontrol agent inoculum before preharvest application to apple fruits has been found to influence preharvest population density but not the agent's efficacy against blue mold. (Teixidõ et al., 1998b,c).

To our knowledge, the effects of relative humidity and temperature on yeast population density at the fruit surface before storage has never been studied, although these factors are crucial to cell population development. McGuire (2000) reports that BCA cells must be applied at a sufficient density to be immediately available to colonize the fruit surface and superficial wounds. Generally, the antagonist much reach a surface density of at least 10^4 CFU/cm^2 for effective biological control (Andrews, 1992), and this density must be maintained during the first 2 weeks of cold storage in order to control effectively the decay resulting from injuries during postharvest processing (McGuire, 1994).

Pichia anomala (strain K) and *Candida oleophila* (strain O), two antagonistic yeasts, have been isolated from apples and selected for their biocontrol activities against postharvest diseases of apples, pears, and citrus fruits (Jijakli et al., 1999, 2004; Lahlali et al., 2004; Lahlali et al., 2005a). Efficacy trials carried out with these antagonistic yeasts on apple fruits under field conditions have shown that strain K or strain O population density has to reach the threshold of 10^4 CFU/cm^2 apple-fruit surface just after harvest in order to effectively control postharvest diseases (De Clercq et al., 2003).

The objective of the present research was to study the combined effects of temperature, relative humidity, and initial applied yeast concentration on the population density of both strains after a 48-h incubation under various combinations of these factors. For this purpose, response surface modeling is a valuable tool (Myers and Montgomery, 2002), as it can be used to determine the experimental-factor settings giving maximum (or minimum) response values. This methodology can also be used to determine the environmental conditions required to reach a yeast population density of 10^4 CFU/cm^2 or more. To reduce the number of experiments, a Box-Behnken design was used. This design minimizes the number of factor combinations required to evaluate the effects of three factors on a response. The combined effects are represented by means of a quadratic polynomial model for each antagonistic strain.

2. Materials and methods

2.1. Yeast strains

P. anomala strain K and *C. oleophila* strain O were grown on potato dextrose agar (PDA, Merck, Darmstadt, Germany) at 25 °C for three successive subcultures under the same conditions with an interval of 24 h. Before application to the apples, yeast colonies were flooded with sterile distilled water and scraped from plates. The concentration of the strain O or strain K suspension was adjusted according to optical density measurements with an UltrospecII spectrophotometer (LKB Biochron Ltd., Uppsala, Sweden) at 595 nm (Jijakli and Lepoivre, 1998).

2.2. Controlling chamber humidity

Small growth chambers (desiccators) with a maximal capacity of 1 l of water were used in these experiments. The approximate value of equilibrium relative humidity (98 ± 1, 86.5 ± 1 and 75 ± 1%) inside the desiccators was controlled by means of saturated salt solutions: K_2SO_4 (98%), KCL (86.5%), and NaCl (75%) (Xu et al., 2001). These pure salts were mixed in 1 l of distilled water and stirred until a saturated salt solution was obtained. The resulting relative humidity varied slightly and gradually with temperature (Winstoon and Bates, 1960). Desiccators with different humidities were incubated for 48 h at the experimental temperature before introduction of the apples treated with the yeast *P. anomala* (strain K) or *C. oleophila* (strain O). The relative humidity in each desiccator was monitored by means a thermohygrometer.

2.3. Application of antagonistic yeasts to apple fruits

'Golden delicious' apples were disinfected by soaking for 2 min in sodium hypochlorite solution (10%), then rinsed twice in sterile distilled water. After drying for 1 h, the fruits were treated with various concentrations of *C. oleophila* (strain O) or *P. anomala* (strain K) (10^4, 5×10^7 and 10^8 CFU/ml) by dipping in 400 ml suspension for 2 min.

2.4. Incubation conditions and recovery of yeast cells

The treated fruits were placed in desiccators at relative humidities (four apples per desiccator) and then incubated at various temperatures (5, 15 and 25 °C).

After 48 h of incubation, yeast cell recovery from intact fruit surfaces was performed as previously described (Massart et al., 2005). Briefly, apple fruits were introduced into 3000-ml plastic bags. Each bag contained 4 apples and 1000 ml KBPT washing buffer [6.8 g KH_2PO_4 (0.05 M), 8.7 g K_2HPO_4 (0.05 M), and 500 µl Tween 80] (one plastic bag per treatment). The plastic bags were centrifuged for 20 min at 120 rounds per minute. After shaking, the KBPT buffer wash was serially diluted and plated in triplicate on semi-selective HST-PDA medium. Colony-forming units (CFUs) were counted out after a 72-h incubation at 25 °C. The mean surface area of the apples was

evaluated as previously described (De Clercq et al., 2003; Massart et al., 2005) according to the following linear regression curve: [Surface (in cm^2)=0.488×volume displaced water (ml)+66.1 (r=0.99)]. Three trials were carried out over time and each treatment contained 3 replicates per trial.

2.5. Experimental design

To determine the effect of temperature, relative humidity, and initial concentration on the number of CFUs per square centimeter of apple surface, response surface methodology was applied with a Box and Behnken (1960) experimental design (Table 1). This design led to studying the effects of three factors in a single block of 15 sets of test conditions and 3 central points. The order of the experiments was fully randomized. Three levels were attributed to each factor, coded as −1, 0, +1 (Table 1). Statistical analysis was performed with the software package 'DESIGN-EXPERT® version 6.0.' (Stat-Ease, Inc., Minneapolis, USA). A quadratic polynomial model was defined to fit the response:

$$Y = \beta_0 + \beta_1 X_T + \beta_2 X_{RH} + \beta_3 X_{CON} + \beta_{11}(X_T)^2 + \beta_{22}(X_{RH})^2 + \beta_{33}(X_{CON})^2 + \beta_{12} X_T X_{RH} + \beta_{13} X_T X_{CON} + \beta_{23} X_{RH} X_{CON}$$

where Y is the response expressed as $\log_{10}$ (CFU/cm^2 fruit surface) and β_0 is a constant coefficient of the model. The regression coefficients (β_1, β_2 and β_3), (β_{11}, β_{22} and β_{33}) and (β_{12}, β_{13} and β_{23}) respectively represent linear, quadratic, and interaction effects of the model, estimated by multiple regression analysis. X_T (temperature), X_{RH} (relative humidity), and X_{CON} (initial concentration of yeast application) are coded variables ranging from −1 to +1. Interpretation of the data was based on the signs (positive or negative effect on the response) and statistical significance of coefficients (P<0.05). Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient).

Internal validation of prediction accuracy of the Box-Behnken models was based on statistical evaluation of the following tests: Root–Mean–Square Error (RMSE), bias index and accuracy factor, and the lack-of-fit test (Ross 1996; Samapundo et al., 2005; Lahlali et al., 2007). Practical evaluation of the two models was based on a comparison of the responses observed in orchard trials spanning 2 years with the responses predicted on the basis of the models and the measured variables.

$$\mathrm{RMSE} = \sqrt{\frac{\mathrm{RSS}}{n}} = \sqrt{\frac{\sum\left(\mu_{\mathrm{observed}} - \mu_{\mathrm{predicted}}\right)^2}{n}}$$

$$\text{Bias factor} = 10^{\left[\sum \log\left(\mu_{\mathrm{observed}}/\mu_{\mathrm{predicted}}\right)/n\right]}$$

$$\text{Accuracy factor} = 10^{\left[\sum |\log\left(\mu_{\mathrm{observed}}/\mu_{\mathrm{predicted}}\right)|/n\right]}$$

3. Results

3.1. Observed effects of temperature, relative humidity, and initial applied concentration on yeast density at the apple surface

The experimental values obtained for the yeast population density at the apple surface under the various conditions tested are shown in Table 1, columns 5 (strain O) and 7 (strain K). With neither of the strains was it possible to achieve the dersirable threshold density of 10^4 CFU/cm^2 when both the temperature and the relative humidity were low (5 or 15 °C; relative humidity 75%). At higher temperature and/or relative humidity, this threshold was sometimes reached (see E2, E3,

Table 1
Experimental and predicted values of population densities, expressed in $\log_{10}$ (CFU/cm^2 apple fruit surface)

Experiments	Temperature (°C)	RH (%)	Applied concentration (CFU/ml)	Yeast population density ($\log_{10}$ CFU/cm^2) Strain K ($\log_{10}$ CFU/cm^2)			
				Strain O		Strain K	
				Observed values	Predicted values	Observed values	Predicted values
E1	5	86.5	1×10^8	3.83±0.017	3.54	3.31±0.066	3.28
E2	15	86.5	5×10^7	4.02±0.07	3.90	3.58±0.066	3.57
E3	25	75.0	5×10^7	4.15±0.05	4.18	3.54±0.035	3.33
E4	5	75.0	5×10^7	0.82±1.42	1.25	2.74±0.046	2.63
E5	5	86.5	1×10^4	0.21±0.37	0.11	0.55±0.58	0.61
E6	25	98.0	5×10^7	5.45±0.23	5.03	5.27±0.036	5.38
E7	15	86.5	5×10^7	3.50±015	3.90	3.57±0.115	3.57
E8	25	86.5	1×10^8	4.89±0.017	5.00	4.30±0.011	4.37
E9	5	98.0	5×10^7	4.50±0.14	4.47	3.15±0.242	3.46
E10	15	98.0	1×10^4	2.36±0.75	2.51	2.66±0.07	2.51
E11	25	86.5	1×10^4	1.83±0.15	2.13	2.02±0.164	2.13
E12	15	98.0	1×10^8	4.56±0.15	4.88	4.76±0.02	4.57
E13	15	75.0	1×10^8	3.76±0.158	3.62	3.42±0.05	3.53
E14	15	75.0	1×10^4	0.00±0.00	−0.31	0.73±0.84	0.67
E15	15	86.5	5×10^7	3.76±0.155	3.90	3.51±0.188	3.57

A Box-Behnken experimental design was applied with three controlled factors: temperature, relative humidity, and initial applied yeast concentration.

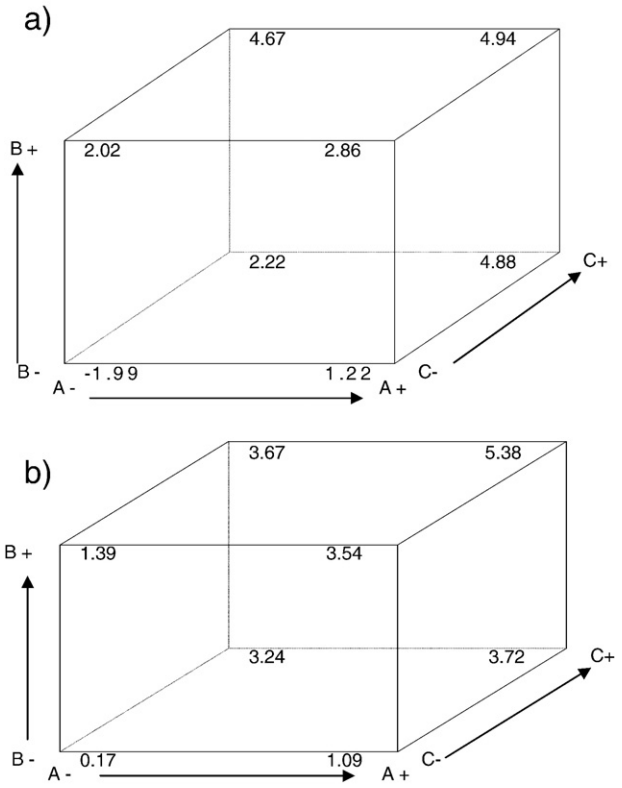

Fig. 1. Cubic presentation of the Box-Behnken (1960) experimental design for the yeast strains *C. oleophilea* strain O (a) and *P. anomala* strain K (b): temperature (°C); B: relative humidity (%); C: initial applied yeast concentration (CFU/ml).

E6, E8, E9, E12 for strain O; E6, E8, E12 for strain K). For each strain the highest density observed corresponded to 98% relative humidity, 25 °C, and an initial applied concentration of 5×10^7 CFU/ml.

3.2. Modeling the effects

Fig. 1 shows the maximal and minimal limits of the Box-Behnken experimental design adapted to evaluating the combined effects of initial concentration of yeast application, relative humidity, and temperature on the population density of *C. oleophila* strain O and *P. anomala* strain K (expressed in $\log_{10}$ (CFU/cm^2)) at the apple surface. This population density could best be predicted by the following equations

(1) $$Y_1 = 3.90 + 0.87X_T + 1.02X_{RH} + 1.58X_{CON} - 0.072(X_T)^2 - 0.092(X_{RH})^2 - 1.13(X_{CON})^2 - 0.02X_T \times X_{RH} - 0.23X_T \times X_{CON} - 0.30X_{RH} \times X_{CON}.$$ (for *C. oleophila* strain O)

(2) $$Y_1 = 3.57 + 0.65X_T + 0.72X_{RH} + 1.23X_{CON} - 0.046(X_T)^2 + 0.18(X_{RH})^2 - 0.92(X_{CON})^2 - 0.31X_T \times X_{RH} - 0.11X_T \times X_{CON} - 0.20X_{RH} \times X_{CON}.$$ (for *P. anomala* strain K)

where Y_1 and Y_2 are respectively the strain O and strain K densities on the apple surface ($\log_{10}$ (CFU/cm^2)) and X is the coded value (between −1 and +1) of the factor indicated by the attached subscript (T: temperature, RH: relative humidity, and CON: initial concentration).

The average predicted densities obtained with these yeast models under various conditions are summarized in Table 1, columns 6 and 8. With both studied strains, differences were slight between the predicted and observed values. Both models predict a low yeast density on the fruit surface for the antagonist strain applied at a concentration of 10^4 CFU/ml and incubated at a relative humidity ranging from 75 to 86.5% and an incubation temperature between 5 and 15 °C. At 98% relative humidity, the initial applied concentration required to enable the yeast population to reach the threshold value of 10^4 CFU/cm^2 should be 2.8 10^7 CFU/ml (strain K) or 2.7×10^7 CFU/ml (strain O) at 15 °C and 2×10^7 CFU/ml (strain O) or 10^7 CFU/ml (strain K) at 25 °C. At 98% relative humidity and 5 °C, only strain O should be able to reach the threshold density (when applied at about 3.6×10^7 CFU/ml).

For each model the R^2 (coefficient of determination) was calculated. This coefficient, ranging from 0 to 1, represents the part of the response variation that is attributable to variations of the factors studied in the model and their interactions. The closer the R^2 value is to 1 the higher the predictive power of the model. In this case the values for *C. oleophila* strain O and *P. anomala* strain K were respectively 0.936 (93.6%) and 0.958 (95.8%) (Table 3). This means that respectively only 6.4% and 4.2% of the total response variation remained unexplained by the model (Box and Draper, 1987). The fit is thus good between the quadratic model and the experimental data. The predicted and adjusted R^2 values were also calculated. The adjusted R^2 corrects the R^2 according to the sample size and the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be strikingly smaller than the R^2, and this should be a warning that the model may contain too many terms (Haaland, 1989). For strains O and K respectively, the predicted R^2 values were 0.88 and 0.92 and the adjusted R^2 vales were 0.92 and 0.94 (Table 3). These values are very similar and very close to the corresponding R^2 values. There is thus good agreement between the experimental and predicted values of the yeast population density on the apple fruit surface.

The purpose of statistical analysis is to determine which experimental factors generate signals that are large in comparison to the 'noise' (Haaland, 1989). 'Adequate precision' measures the signal-to-noise ratio. A ratio superior to 4 is generally desirable. For the strain K and strain O models the adequate precision was respectively 34.04 and 23.18. This indicates an adequate signal (Table 3). The results of the multiple regression analysis from which the model coefficients were derived are listed in Table 2. The higher the absolute value of a linear coefficient (β_1, β_2 or β_3), the greater the influence of the corresponding factor (Box and Draper, 1987) on the predicted density. With both strains, the applied initial yeast concentration had the greatest effect, followed by the relative humidity and finally by the temperature. In both models, all coefficients appeared significant, except for coefficients β_{11} (quadratic effect of temperature), β_{22} (quadratic effect of relative humidity), and

Table 2
Significance of the coefficients used in the Box and Behnken (1960) experimental design adopted for estimating population densities of strain O or strain K on the apple surface, obtained after multiple regression analysis (T: temperature; RH: relative humidity and Con: initial applied concentration)

		Antagonistic yeast	
		Strain O	Strain K
Response mean	β_0	3.90**	3.57**
T	β_1	0.87*	0.65**
RH	β_2	1.02**	0.72**
CON	β_3	1.58**	1.23**
T^2	β_{11}	-0.072^{ns}	-0.046^{ns}
RH^2	β_{22}	-0.092^{ns}	0.18^{ns}
CON^2	β_{33}	−1.13**	−0.92**
$T\times RH$	β_{12}	−0.02**	0.31**
$T\times CON$	β_{13}	-0.23^{ns}	-0.11^{ns}
RH × Con	β_{23}	−0.30**	−0.20*

* Significant.
** Highly significant.

β_{13} (effect of the interaction between temperature and initial applied yeast concentration). In the strain O model, all coefficients except the linear ones have negative effects. In the strain K model, all coefficients have positive effects except the quadratic effects of temperature and relative humidity and the interactions (temperature × relative humidity) and (relative humidity × initial concentration of application).

3.3. Statistical validation of the models

The tests used to validate our predictive models are listed in Table 3. The values of the RMSE parameter show that both models produced predictions close to the observed data. The results of the F test indicate that the predicted and observed values for both models are not significantly different.

For both strains, the bias index was close to 1.00. This result implies that, for this experiment, both models are good predictors of yeast density on the apple surface. The accuracy factors show that the prediction differs from the observation by 11% for strain O and 12% for strain K. The lack of fit is insignificant for both established models, suggesting that both quadratic models adequately approximate the true surfaces.

To determine the optimal conditions for high-density colonization of the apple surface by these strains, response surfaces showing the predicted effects of applied initial yeast concentration and relative humidity were drawn for three incubation temperatures from the equations for strain O (Fig. 2) and strain K (Fig. 3). The response surfaces show that the population density of both strains is strongly influenced by all three studied factors. At constant relative humidity and temperature, a higher initial yeast concentration leads to a higher final surface density. The highest yeast population densities are seen at the highest relative humidity values. Very low population densities are calculated for the lowest relative humidity level, especially at lower temperatures. As suggested by the experimental results, the conditions predicted to be optimal for both strains were 25 °C, 98% relative humidity, and an initial applied concentration of about 5×10^7 CFU/ml.

3.4. Practical validation of the models

In preharvest orchard trials carried out previously (De Clercq et al., 2003), contradictory results were obtained with strain O or strain K applied initially at 10^7 CFU/ml. In the first trial year, the yeast population density on the apple surface reached approximately 10^4 CFU/cm^2 and a high level of protection against *B. cinerea* and *P. expansum* was observed. The second trial year, the density achieved was much lower. The average temperature was 25 °C the first year and 19 °C the second year. The relative humidity was almost stable, ranging between 98 and 100% during both campaigns.

These data were incorporated into the models described above, and the results are shown in Table 4. For the first year, the strain K model predicted fairly accurately the strain K density on the apple surface (predicted value: 1.13×10^4 CFU/cm^2; measured value: 1.6×10^4). The strain O model underestimated the strain O density by a factor of 10 (predicted value: 2.9×10^3 CFU/cm^2; measured value: 3×10^4). For the second trial year, both models predicted somewhat lower densities than for the first year, but the actual measured values (<10 CFU/cm^2) were much lower than the predicted ones ($\geq1.9\times10^3$ CFU/cm^2). This means that temperature and relative humidity cannot fully explain the observed variation. It is necessary to take into account an additional, unknown cause. The preharvest treatments were performed 2 days before harvest in year 1 and 3 days before harvest in year 2. Strong rains in year 2 may have been a major factor explaining the discrepancy.

4. Discussion

It should be possible to suppress postharvest diseases of fruits by field and/or postharvest application of a biocontrol agent. The combination of field and postharvest application has great potential for achieving effective control of postharvest decays (Janisiewicz and Korsten, 2002).

Most reports on biocontrol agents have focused on postharvest application (Spadaro and Gullino, 2004), but field application seems a promising way to achieve effective protection against some postharvest diseases (Benbow and Sugar, 1999), provided the BCA population density reaches a sufficient level to allow good colonization of inflicted wounds. Unpredictable climate changes can interfere with population growth, however, and thus affect the efficacy against postharvest

Table 3
ANOVA of quadratic response surface models for *C. oleophila* strain O and *P. anomala* strain K

	Strain O	Strain K
R^2	0.936	0.958
RMSE	0.49	0.30
Adjusted R^2	0.92	0.947
Predicted R^2	0.88	0.926
Adequacy precision	23.18	34.047
F-value model	57.33	88.93
Lack of fit F-value	2.39	2.87
Bias indice	1.006	0.9942
Accuracy indice	1.117	1.121

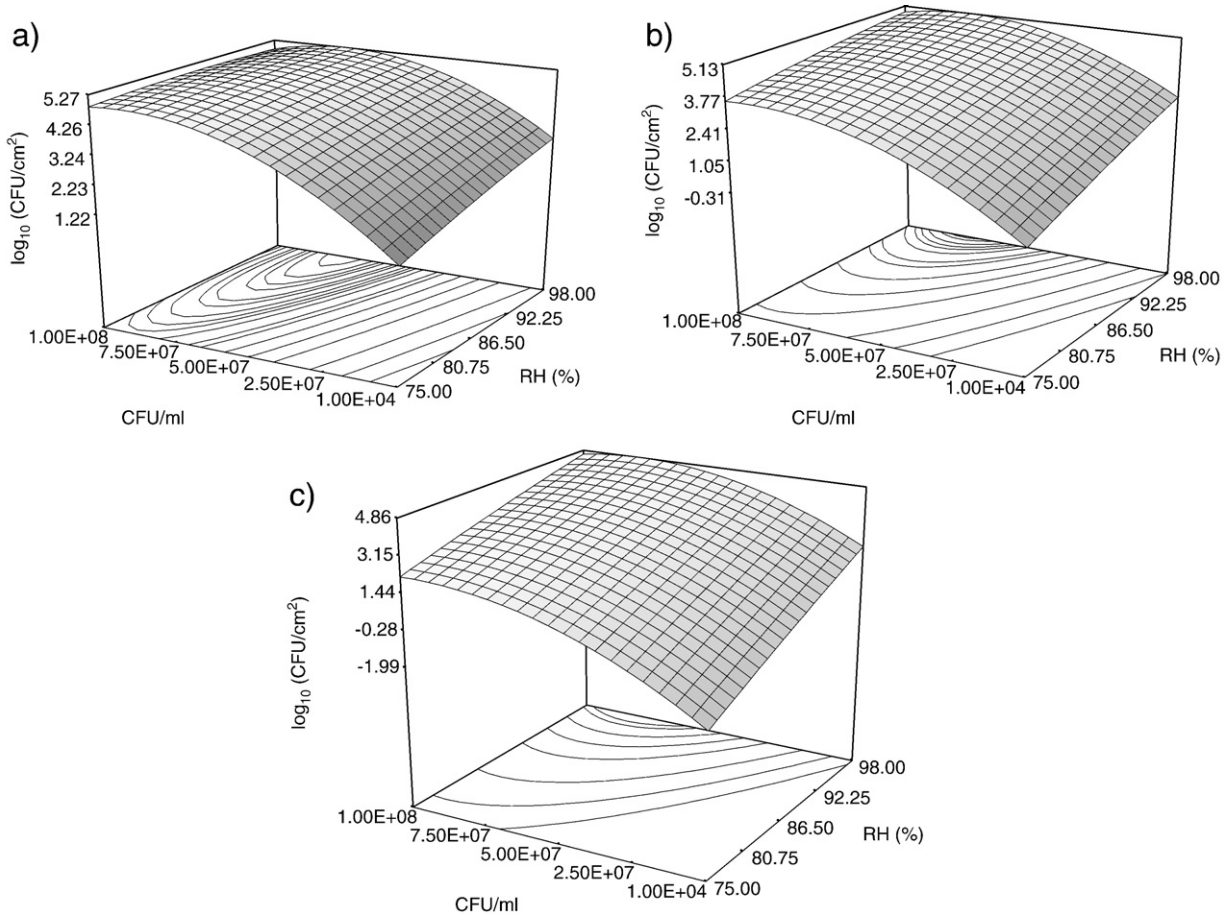

Fig. 2. Response surface curves showing the predicted effects of relative humidity and initial applied yeast concentration on the density of *C. oleophila* strain O at 25 (a), 15 (b) and 5 °C (c).

decay. Ippolito and Nigro (2000) stress that the efficacy of preharvest treatments depends on factors such as rain, wind, temperature, and relative humidity. There is thus an urgent need to evaluate and model the influence of environmental factors on BCA population density. With adequate models it should be possible to predict the BCA density on the apple surface before storage.

The main objectives of this study were to evaluate and model the influence of temperature, relative humidity, and applied initial yeast concentration on the population density of *P. anomala* strain K and *C. oleophila* strain O on the apple surface under controlled conditions. To our knowledge, this is the first attempt to model the influence of such factors on the establishment of biocontrol agents on the apple surface.

The modeling approach used was response surface methodology applied to the Box and Behnken (1960) experimental design. This design allows prediction of the combined effects of three controlled factors. In our experiment we examined the effects of temperature, relative humidity, and initial applied yeast concentration on the yeast density at the apple surface 48 h after treatment. The Box-Behnken design minimizes the number of factor combinations and maintains good precision of the predicted response. It has been widely used to predict the growth of food-borne pathogens in relation to at least three environment factors (Sautour et al., 2003).

In our models, the factors showing the greatest influence on yeast density at the apple surface are the initial yeast concentration and the relative humidity. Of the two environmental factors tested, relative humidity has a greater effect than temperature. This result is in agreement with the results of Artes et al. (1995) who, focusing on 'Satsuma' mandarins, found the density of an antagonistic yeast on the fruit surface to increase with relative humidity.

In our experiments and model predictions, the yeast population density was found to increase with increasing relative humidity and increasing incubation temperature. These results are in accordance with *in vitro* data showing a positive correlation between yeast growth and both the water activity of the medium and the incubation temperature (Lahlali, 2006). Our results are also in agreement with *in vitro* data showing that strain O reaches a higher concentration than strain K at 5 °C, whatever the water activity (Lahlali, 2006).

Previous studies in our laboratory have shown that a yeast density of at least 10^4 CFU/cm^2 must be reached with both strains in order to achieve good protection against *P. expansum* and *B. cinerea* (De Clercq et al., 2003). In previous orchard trials carried out over two successive years, we actually measured such densities and observed effective protection during two successive years of trials. It was therefore interesting to incorporate the meteorological data related to both years into

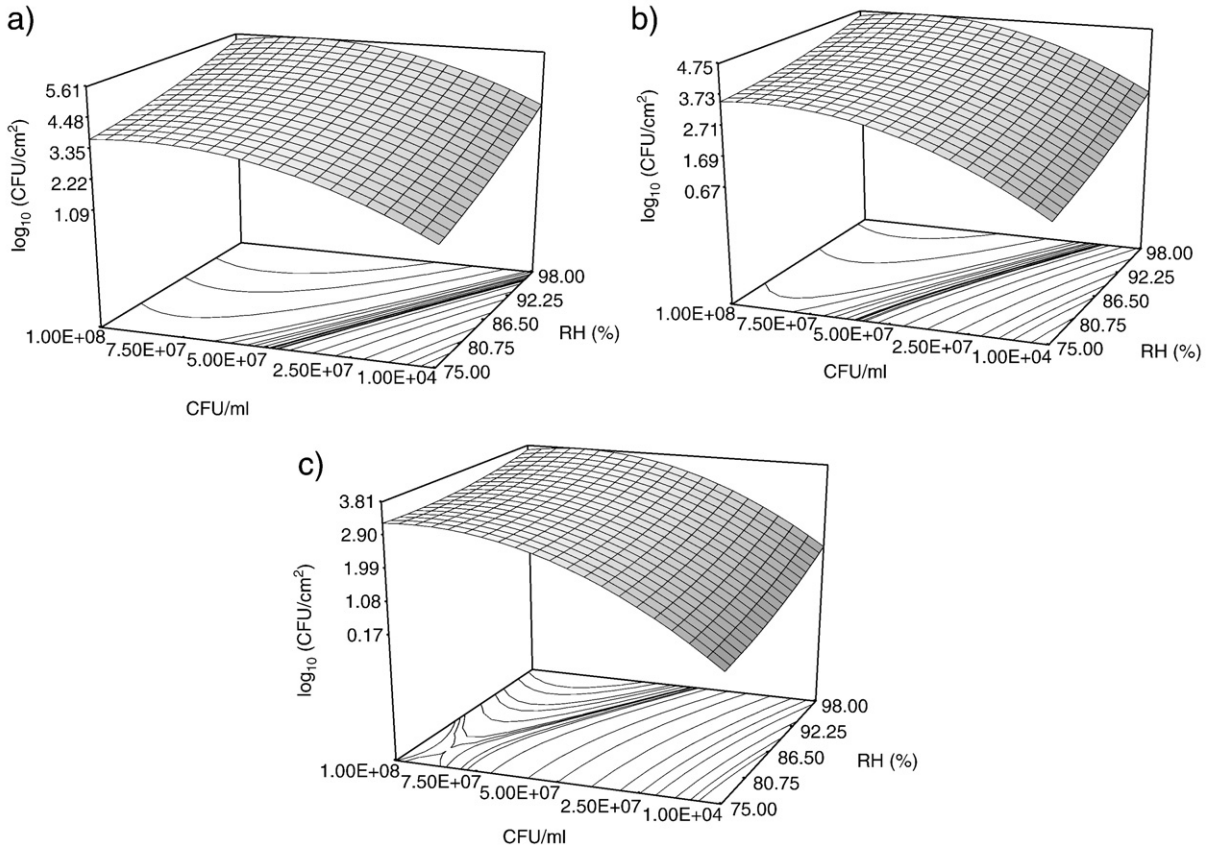

Fig. 3. Response surface curves showing the predicted effects of relative humidity and initial applied yeast concentration on the density of *P. anomala* strain K at 25 (a), 15 (b) and 5 °C (c).

our models, to see if these would predict big differences between the 2 years. It turns out that our models describe reasonably well what happened the first year (especially as regards strain K), but not the second. We suspect that preharvest rainfall may be the major factor contributing to the poor performance of our models for the second year (De Clercq et al., 2003). Teixidõ et al. (1999) reported that *C. sake* populations reached 48 h after biological treatment were much higher in the first season of their trial (25 °C) than in the second (32 °C).

Another aim of this work was to determine temperature, relative humidity, and initial concentration conditions making it possible to reach the threshold population density of 10^4 CFU/cm^2. Our models predict that this threshold should be reached by both strains under favorable conditions: 25 °C, 98% relative humidity, and an applied concentration of about 10^7 for strain K and 2×10^7 CFU/ml for strain O. These results are in agreement with those reported by Lahlali and Jijakli (2004), showing that under prevailing temperature conditions and at a relative humidity near 100%, it should take at least 10^7 to 10^8 CFU/ml strain K or strain O to reach this threshold density on the apple surface.

Mercier and Wilson (1995) have studied the effect of relative humidity on the growth of *C. oleophila* and *B. cinerea* on wounded apples. They report that the population density of both microorganisms increases fast when water is periodically applied to wounds on the fruit. These results raise the possibility that moisture, being a limiting factor for both the antagonist and the pathogen, might be involved in the mechanisms of biocontrol. So far, little is known about the antagonistic action of agents used in the biocontrol of postharvest diseases. Competition for moisture should be included as a possibility.

Our models show a positive correlation between the population density of antagonistic yeasts and both temperature and relative humidity, with a greater effect of the latter. Similar results have been obtained *in vitro* with the yeast *C. sake*, another biocontrol agent for postharvest diseases of apples. In this case, the effect of a_w was much more pronounced than that of temperature or pH (Teixidõ et al., 1998a,b).

Models based on multi-factor analyses like those described here are valid only for the specific strain/substrate combination studied and within the experimental domain (Delignette-Muller,

Table 4
Comparison of predicted and measured values for years 1 and 2 of a published orchard trial[1]

Year of trial	Predicted values (CFU/cm^2)		Measured values (CFU/cm^2)	
	Strain O	Strain K	Strain O	Strain K
Year 1[a]	2.9×10^3	$1.13\times10^{4*}$	3×10^4	$1.6\times10^{4*}$
Year 2[b]	1.9×10^3	2.89×10^3	<10	<10

[a]Preharvest trials performed 2 days before harvest (Low spray volume).
[b]Preharvest trials performed 3 days before harvest (Low spray volume).
*Predicted and practical values $\geq10^4$ CFU/cm^2.
[1]De Clercq et al., 2003.

1997; Lahlali et al., 2005b, 2007). Any extrapolation to other strains or growth substrates or beyond the tested ranges of the considered factors would be hazardous. Consequently, it is important to specify any conditions under which a model proves inadequate (Barayni et al., 1996). In our case, we have no basis for comparison, as we know of no other published models describing the combined effects of relative humidity, temperature and initial applied yeast concentration on yeast density at the apple surface. As mentioned above, preharvest rainfall would appear to reduce the predictive power of our models (De Clercq et al., 2003).

In conclusion, the present results may help in choosing the concentration of yeast suspensions to be applied in order to achieve on the apple surface a yeast density of at least 10^4 CFU/cm^2, required for protection against pathogens affecting wounded apples in storage. Our models may also provide guidance as to how to control this density by altering two key environmental factors, temperature and the relative humidity. Both models are capable of predicting the yeast population densities on the apple surface 48 h after field spraying of biocontrol agents, and they might be useful in deciding whether preharvest treatment is sufficient to allow fast colonization of wounds inflicted during harvest and packaging, prior to the arrival of wound pathogens, or whether it is wise to apply further postharvest treatment to increase the yeast population density and thus ensure better protection against postharvest apple decays arising in the storage room.

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