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Botrytis cinerea'nın İn Vitro Miselyal Gelişmesi ve Sklerotial Çimlenmesi Üzerine Potasyum Bikarbonatın Etkisinin Çoklu Regresyon Analizi

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Özet

Bu çalışmada, Botrytis cinerea' nın miselyal gelişmesi ve sklerotial çimlenmesi üzerine potasyum bikarbonat ($KHCO_3$)'ın artan dozlarının ve zamanın etkisi çoklu regresyon analizi ile matematiksel olarak değerlendirilmiştir. B. cinerea'nın miselyal gelişmesi ve sklerotial çimlenmesi için üretilen tüm eşitliklerin $KHCO_3$'nun konsantrasyonu ve zamanın etkilerinden türetilmiştir. ANOVA ve çoklu regresyon analizi sonuçlarına göre, B. cinerea'nın miselyal gelişmesi ve sklerotial çimlenmesinin gerçek ve tahmin edilen değerleri arasında yakın bir ilişki olduğu bulunmuştur. Mevcut çalışmada üretilen tahmini modelde $MG= (a) – (b \times T^2) + [c \times (D \times T)]$, MG B. cinerea'nın miselyal gelişmesi; $SG = (a)+(b \times T)–[c \times (D \times T)]+[d \times (D^2 \times T)]$, SG B. cinerea'nın sklerotial çimlenmesini göstermektedir. Formülde a, b, c ve d çoklu regresyon analizinden elde edilen katsayılardır. Zaman ve Doz sırasıyla T ve D ile gösterilmiştir. Miselyal gelişme ve sklerotial çimlenme için R^2 değerleri sırasıyla 0.83 ve 0.81 ve standart hatalar $p<0.001$ seviyesinde önemli bulunmuştur.

Anhtar Kelimeler: *Botrytis cinerea, Miselyal Gelişme Oranı, Sklerotial Çimlenme, Potasyum Bikarbonat, Çoklu Regresyon Analizi*

Multi Regression Analysis of The Effect of Potassium Bicarbonate on In Vitro The Mycelial Growth and Sclerotial Germination of Botrytis cinerea

Abstract

In this study, the effect of time and increased doses of potassium bicarbonate ($KHCO_3$) on mycelial growth and sclerotial germination of Botrytis cinerea was evaluated by mathematical modeling with multi regression analysis. All equations produced for mycelial growth and sclerotial germination of B. cinerea were derived as affected by doses and times. As a result of ANOVA and multi-regression analysis, it was found that there was close relationship between actual and predicted mycelial growth and sclerotial germination of B. cinerea. The produced prediction models in the present study are $MG= (a) – (b \times T^2) + [c \times (D \times T)]$ where MG is mycelial growth of B. cinerea; $SG = (a)+(b \times T)–[c \times (D \times T)]+[d \times (D^2 \times T)]$ where SG is sclerotial germination of B. cinerea. In this formula a, b, c and d symbolizes the co-efficient obtained as a result of multi regression analysis. Time and Dose are represented as T and D, respectively. R^2 values of mycelial growth and sclerotial germination were 0.83 and 0.81 respectively and standard errors were found to be significant at the $p<0.001$ significance level.

Key words: *Botrytis cinerea, Mycelial Growth Rate, Sclerotial Germination, Potassium Bicarbonate, Multi-Regression Analysis*

Introduction

Botrytis cinerea Pers.:Fr. causes pre and post-harvest diseases in many plant species such as grapes, apples, kiwifruit, strawberries, tomatoes, cucumbers, peppers, bulb flowers, and ornamental plants (Agrios, 2005; Elad et al., 2007). *B. cinerea* attacks flowers, leaves, stems, fruit and other parts of many plants including kiwifruit. The pathogen was reported from Italy (Bisiach et al., 1984), Japan (Ieki, 1993), USA, New Zealand (Michailides and Elmer, 2000) and Turkey (Karakaya and Bayraktar, 2009) on kiwifruit. It causes stem end rot of kiwifruit which commonly occur during post-harvest in storage facilities and on harvested fruit during cold storage (Brook, 1991; Michailides and Elmer, 2000).

The control of grey mold disease caused by *B. cinerea* is mainly achieved by pre- and postharvest fungicide treatments (Bulit and Dubos, 1988). However, most of the classical fungicides embody the risk of residues on products and have negative effects on human health and environmental. Furthermore, *B. cinerea* may easily develop races with high resistance against many fungicides (Erkan et al., 1997; Palmer et al., 1997; Yıldırım and Yapıcı, 2007). Therefore, there is a need to the development of alternative strategies to control postharvest decay of different plants that are safe,

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effective, economical and compatible with commercial handling (Karabulut et al., 2005). One of the best ways to achieve this aim is the use of efficient natural substances such as bicarbonates compounds. Bicarbonates are widely utilized to regulate pH, to avoid undesirable fermentation processes, and to improve texture and taste in the food industry. They also have a wide-spectrum antimicrobial properties. Therefore, especially bicarbonates have been demonstrated to effectively control a wide range of fungi, including food spoilage organisms and plant pathogens (Ricker and Punja, 1991; Palmer et al., 1997; Gabler and Smilanick, 2001; Zhang and Swingle, 2003; Karabulut et al., 2003; Smilanick et al., 2006; Yıldırım and Yapıcı, 2007).

Models are commonly explored using computational or simulation techniques (Odabas et al., 2009). The simulation software may be general-purpose, intended to capture a variety of developmental processes depending on the input files, or special-purpose, intended to capture a specific phenomenon. Input data range from a few parameters in models capturing a fundamental mechanism to thousands of measurements calibrated descriptive models of specific plants (species). Standard numerical outputs (i.e. numbers or plots) may be complemented by computer-generated images and animations (Caliskan et al., 2010).

Fungi are generally very much difficult to study in their natural habitats by experimental methods alone and therefore it is a important need to models that can be analysed in far more detail. There are applied models relevant to epidemiological analyses of fungal plant pathogens such as pre-and-post-harvest fungal pathogens. Quantifying the relationships and effects of fungi, plants, and environmental factors such as temperature, pH and water activity (a_w) on disease development by means of quantitative models can help in the design and efficient use of management strategies for the pathogens. Mathematical modeling is an efficient tool for assessing how individual or combined environmental factors affect microorganisms that degrade processed foods. Different models have been developed in predictive microbiology for fitting growth curves and estimating biological parameters of food-borne and storage pathogens (McMeekin et al., 2002; Marín et al., 1996; Cuppers et al., 1997; Sautour et al., 2002; Lahlali et al., 2007). Cuppers et al. (1997) modelled mold growth on a solid culture medium at various temperatures and NaCl concentrations by using five common food spoilage molds. In other study, Lahlali et al. (2007) examinated to develop validated models predicting the in vitro effect of a_w and temperature on the radial growth of *B. cinerea*. The growth rate of this pathogen was calculated at three incubation temperatures and six water activities. It was determined that all models proved to be good predictors of the growth rates of *B. cinerea* within the limits of experiments. Additionally, Judet-Correia et al. (2010) evaluated to develop and to validate a model for predicting the combined effect of temperature and a_w on the radial growth rate (μ) of *B. cinerea* and *Penicillium expansum* on grape berries.

To our knowledge, there is no available literature about the mathematical modelling for mycelial growth and sclerotial germination of *B. cinerea* exposured to potassium bicarbonate ($KHCO_3$). The objective of this study was developed model of estimating the mycelial growth and sclerotial germination of *B. cinerea in vitro* exposure to increased doses of $KHCO_3$ at different times by multi linear regression.

Materials and methods

Fungal culture

B. cinerea RK-5 isolate was originally obtained from kiwifruit growing areas in Eastern Black Sea Region of Turkey during routine disease surveys in 2010. *B. cinerea* RK-5 isolate was isolated from kiwifruit leaves showing leaf blight symptoms and identified according to its cultural features. Culture of *B. cinerea* RK-5 isolate was maintained on potato dextrose agar (PDA: Oxoid). The PDA slants were stored at 4ºC and served as stock cultures for further use.

Assesment of mycelial growth

Different potassium bicarbonate concentrations (dose) on the following basis: 0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 mM ($KHCO_3$; Carlo Erba Reagenti (Milan, Italy) were added to autoclaved and cooled PDA medium at 50ºC for *B. cinerea*. The pH of each $KHCO_3$ concentration was 6.0, 7.4, 7.8, 7.9, 8.0, 8.1, 8.1, 8.1, 8.2, and 8.2 respectively. The medium was dispensed aseptically into 9-cm-diameter petri plates. A mycelial disc (5 mm diameter) taken from 7-day-old culture that was grown on PDA, was placed in the center of each potassium bicarbonate-amended PDA. The plates were then sealed with parafilm and incubated at 25 ºC, for 6 days. The growth of each fungal colony was measured daily (Ordonez et al., 2009). All experiments were repeated twice.

Assesment of sclerotial germination

Different potassium bicarbonate concentrations above mentioned (0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 mM) were added to autoclaved and cooled PDA medium at 50ºC. The medium was dispensed aseptically into 9-cm-diameter petri plates. Then, sclerotia were obtained from pure cultures of *B. cinerea* which were grown on PDA. These sclerotia were surface disinfested to avoid proliferation of bacterial contaminants by placing them in 70% ethanol for 40 s, and rinsed in sterile-distilled water (SDW), adding a solution of streptomycine (200 μg/ml), and keeping them at 4°C overnight and blotted dry on sterile paper towels to eliminate excess

antibiotic solution. Then, once dried, ten sclerotia were placed on 9-cm diameter petri dishes containing PDA with the concentrations of $KHCO_3$ (Ordonez et al., 2009). The Petri dishes were incubated at 25 °C for 6 days, and the number of germinated sclerotia was daily recorded for each treatment. All experiments were conducted twice.

Experimental design and data analyses

All experiments were conducted in a complete randomized designs with ten treatments and three replications. Analysis of variance was implemented using the program *Minitab* (version 12, "Minitab", USA) and Duncan at 0.05 significance level was used to compare treatment means.

Model Construction

Multiple regression analysis of the data obtained from *in vitro* study was executed for the mycelial growth and sclerotial germination of *B. cinerea*. The general purpose of multiple regressions is to learn more about the relationship between several independent or predictor variables and a dependent or criterion variable.

Given a data set $\{y_i, x_{i1}, \ldots, x_{ip}\}_{i=1}^{n}$ of n statistical units, a linear regression model assumes that the relationship between the dependent variable y_i and the p-vector of regressors x_i is linear. This relationship is modeled through a so-called "disturbance term" ε_i — an unobserved random variable that adds noise to the linear relationship between the dependent variable and regressors. Thus the model takes form

$$y_i = \beta_1 x_{i1} + \cdots + \beta_p x_{ip} + \varepsilon_i = x_i'\beta + \varepsilon_i, \qquad i = 1, \ldots, n,$$

where $'$ denotes the transpose, so that $x_i'\beta$ is the inner product between vectors x_i and β. Often these n equations are stacked together and written in vector form as $y = X\beta + \varepsilon$, where

$$y = \begin{pmatrix} y_1 \\ y_2 \\ \vdots \\ y_n \end{pmatrix}, x = \begin{pmatrix} x_1' \\ x_2' \\ \vdots \\ x_n' \end{pmatrix} = \begin{pmatrix} x_{11} \cdots x_{1p} \\ x_{21} \cdots x_{2p} \\ \vdots \ddots \vdots \\ x_{n1} \cdots x_{np} \end{pmatrix}, \beta = \begin{pmatrix} \beta_1 \\ \vdots \\ \beta_p \end{pmatrix}, \varepsilon = \begin{pmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \vdots \\ \varepsilon_n \end{pmatrix}$$

Some remarks on terminology and general use: y_i is called *dependent variable*. The decision as to which variable in a data set is modeled as the dependent variable and which are modeled as the independent variables may be based on a presumption that the value of one of the variables is caused by, or directly influenced by the other variables. Alternatively, there may be an operational reason to model one of the variables in terms of the others, in which case there need be no presumption of causality. x_i are *independent variables*. Usually a constant is included as one of the regressors. For example we can take $x_{i1} = 1$ for $i = 1, \ldots, n$. The corresponding element of β is called the *intercept*. Many statistical inference procedures for linear models require an intercept to be present, so it is often included even if theoretical considerations suggest that its value should be zero. Sometimes one of the regressors can be a non-linear function of another regressor or of the data, as in polynomial regression. The model remains linear as long as it is linear in the parameter vector β. The regressors x_i may be viewed either as random variables, which we simply observe, or they can be considered as predetermined fixed values which we can choose. Both interpretations may be appropriate in different cases, and they generally lead to the same estimation procedures; however different approaches to asymptotic analysis are used in these two situations. β is a p-dimensional *parameter vector*. Its elements are also called *effects*, or *regression coefficients*. Statistical estimation and inference in linear regression focuses on β. ε_i is called the *error term*, *disturbance term*, or *noise*. This variable captures all other factors which influence the dependent variable y_i other than the regressors x_i. The relationship between the error term and the regressors, for example whether they are correlated is a crucial step in formulating a linear regression model, as it will determine the method to use for estimation (Weisberg 2005).

A search for the best model to predict the mycelial growth and sclerotial germination were conducted with various subsets of the independent variables, namely, dose (mM) and time. The best estimating equation for the mycelial growth of *B. cinerea* was determined with the R-program and formulized as MG= (a) – (b x T^2) + [c x (D x T)] where MG is mycelial growth of *B. cinerea*; SG = (a)+(b x T)–[c x (D x T)]+[d x (D^2 x T)] where SG is sclerotial germination of *B. cinerea*, a, b and c are co-efficiencies. Time is time of sclerotial germination and D is dose. In this formula a, b, c and d symbolizes the co-efficient obtained as a result of multi regression analysis (Table 1 and Table 2). Multiple regression analysis was carried out until the least sum of square (R^2) was obtained. 3-D graphics were performed by *Slidewrite* program.

Results and discussion

Two experiments were performed with mathematical modeling for both the mycelial growth and sclerotial germination of *B. cinerea*.

Mycelial Growth of B. cinerea

The mycelial growth of *B. cinerea* started growing after 24 h. But, its growth significantly reduced with the increasing concentrations of $KHCO_3$. At 96 h, *B.*

cinerea covered the petri dishes completely at 0 mM concentrations of $KHCO_3$, while it covered the petri dishes after 120 h at 10 to 20 mM $KHCO_3$ concentrations. Also, mycelial growth of the fungus was inhibitited especially at concentrations greater than 50 mM. Especially, 90 mM concentration of the salt totally inhibited the mycelial growth (Fig 1). Our findings were supported by the previous studies having similar results on the use of $KHCO_3$ in the control of gray mold caused by *B. cinerea* (Palmer *et al.* 1997; Gabler and Smilanick 2001). As a result of the analysis the effects of doses and times on mycelial growth of *B. cinerea* have been found significant and an equation has been formed.

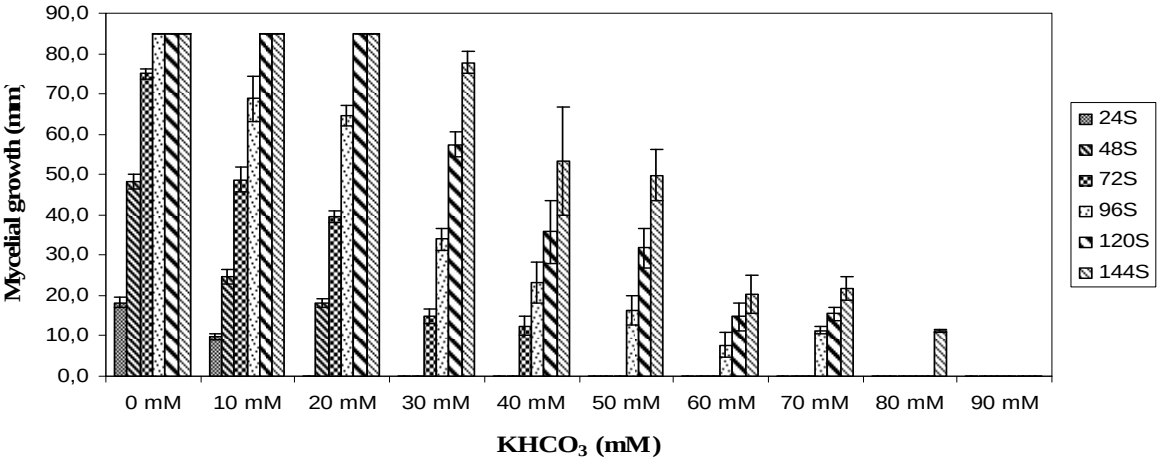

Figure 1. Mycelial growth of Botrytis cinerea on petri dishes exposure to increasing concentrations of potassium bicarbonate ($KHCO_3$) at different hours

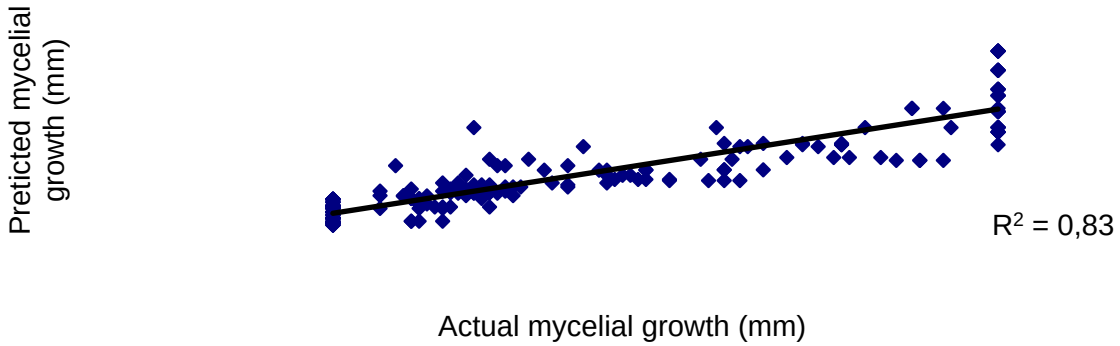

Figure 2. Relationship between actual and predicted the mycelial growth of Botrytis cinerea

According to the regression statistic which is about the mycelial growth of *B. cinerea*, it is observed that R^2 is 0.83. ANOVA significance F value has shown the validity of the model. As this value is below 1% in this study, the result of analysis has a significance of 1%. Following the determination of the importance level, mathematical equation has been obtained by using co-efficients and corresponding independent x variable (MG) and dependent y variables (dose and time).

Mathematical model which was developed by multi regression analysis, for mycelial growth of *B. cinerea* has been formed as MG= (a) – (b x T^2) + [c x (D x T)] where MG is mycelial growth of *B. cinerea*, a, b and c are co-efficiencies. T is time of mycelial growth and D is dose. In this formula a, b and c symbolizes the co-efficient obtained as a result of multi regression analysis. By taking into consideration the co-efficient in the regression statistics, mycelial growth model of *B. cinerea* has been formed.

Mycelial Growth (MG) = (18.185) – (2.581 x T^2) – [0.203 x (A x T)]

Standard Error (SE) = 1.363*** 0.079*** 0.007***

Regression Coefficient (R^2) = 0.83

The relation between the mycelial growth of *B. cinerea* corresponding to the real values and the approximate mycelial growth of *B. cinerea* obtained from mathematical equation has been shown in Fig 2.

The other points represent the mycelial growth of *B. cinerea* obtained from the model. R^2, also known as the co-efficient of determination is commonly used statistic to evaluate model fit. When the variability of the residual values around the regression line relative to the overall variability is small, the predictions from the regression equation are good. The regression line expresses the best prediction of the dependent variable (*Y*), given the independent variables (*X*). However, nature is rarely perfectly predictable, and usually there is substantial variation of the observed points around the fitted regression line. The closer these values are to reality, the higher R^2 value of the mathematical model. In this study R^2 value obtained (0.83) shows that a model with 83% close to the reality has been formed. The effects of doses and times on mycelial growth of *B. cinerea* are shown in Fig 3.

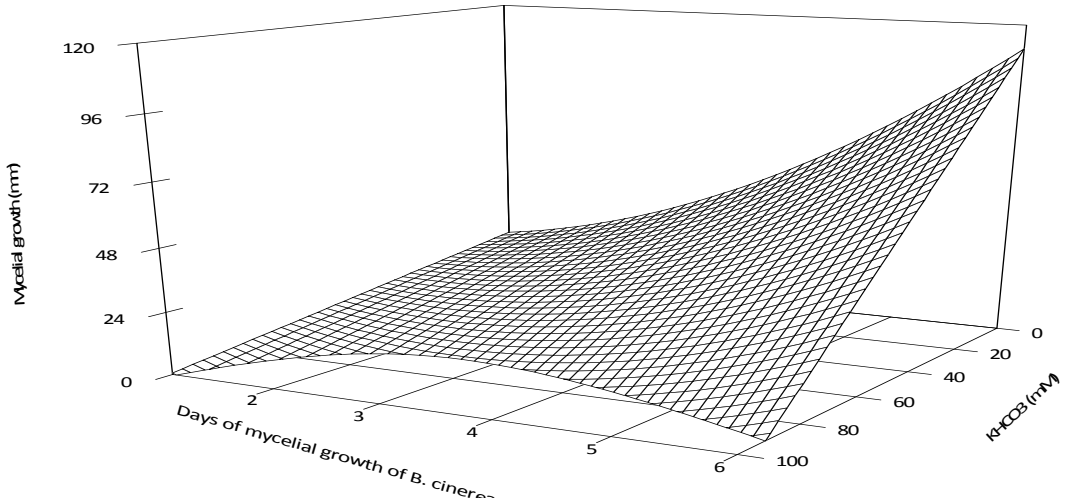

Figure 3. Mycelial growth of Botrytis cinerea on petri dishes exposure to increasing concentrations of potassium bicarbonate ($KHCO_3$) on different times

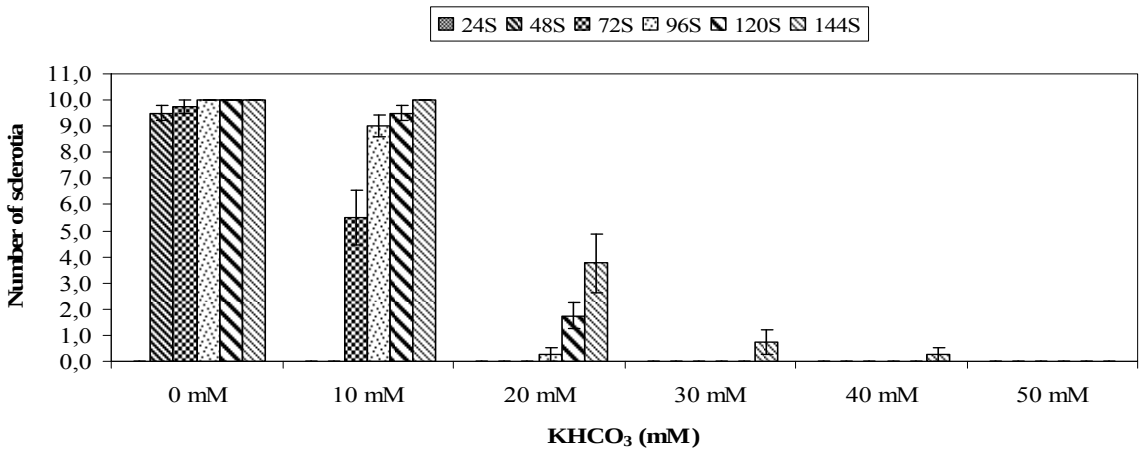

Figure 4. Number of sclerotia of Botrytis cinerea exposed to different concentrations of potassium bicarbonate ($KHCO_3$) after 144 h in vitro. Vertical lines represent standard errors of the means

Mathematical equation has been benefited while showing this change caused by doses and times on the mycelial growth of *B. cinerea*. In this graphic (Fig 3) mesh part shows the change in the mycelial growth throughout *B. cinerea* with times and doses of $KHCO_3$. It is drawn with the help of mathematical equation obtained and *Slitewrite* graphic program. The most important feature of this program is to draw 3

dimension graphic by using not only the values entered but also the mathematical equation obtained. Fig 3 shows that the mycelial growth of *B. cinerea* decreases and increases when the doses of potassium bicarbonate decrease and increase.

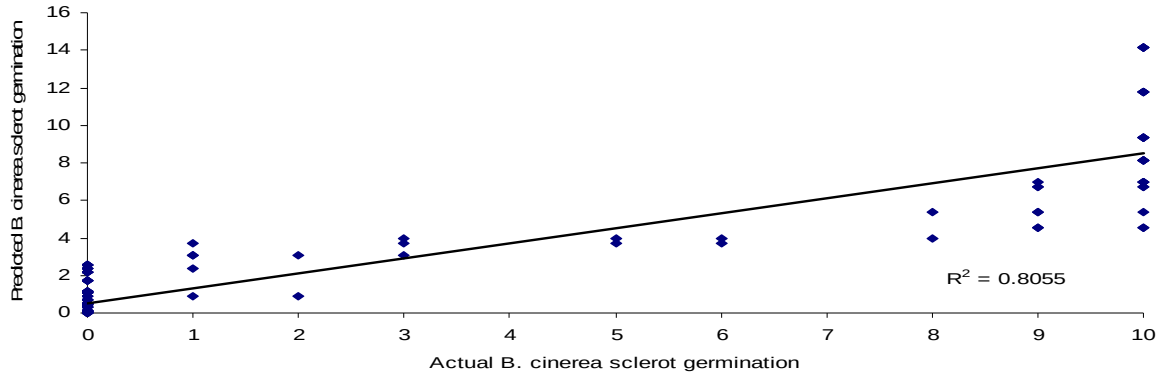

Figure 5. Relationship between actual and predicted the sclerotial germination of Botrytis cinerea

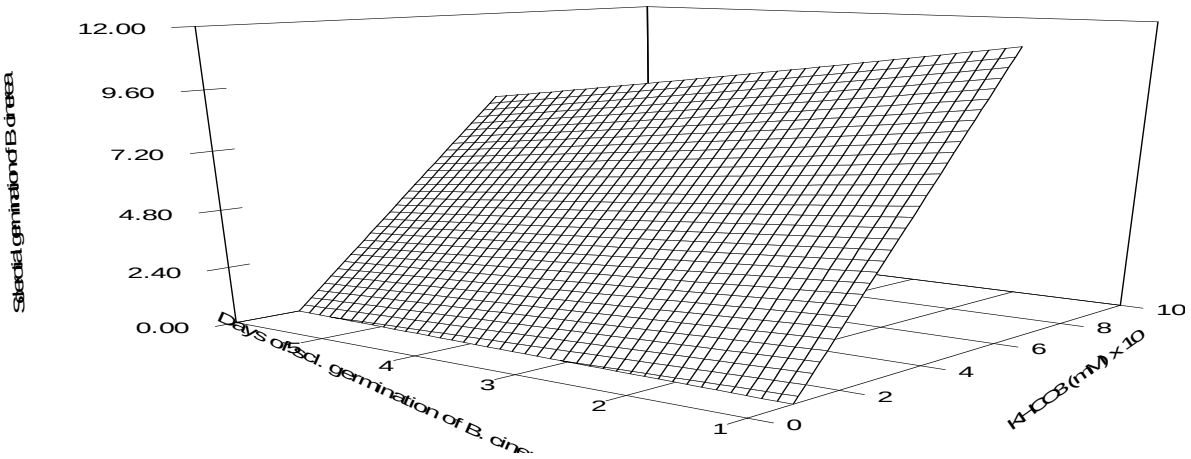

Figure 6. Sclerotial germination of Botrytis cinerea on petri dishes exposure to increasing concentrations of potassium bicarbonate ($KHCO_3$) on different times

Sclerotial germination of B. cinerea

The increasing concentrations of potassium bicarbonate applications affected sclerotial germination of *B. cinerea*. Firstly, at 48 h sclerotia germination only was determined at control (0mM). However, at the end of six days, no sclerotia germination was observed at concentrations over 40 mM (Fig 4). As a result of the analysis the effects of doses and times on germination of sclerotia of *B. cinerea* have been found significant and an equation has been formed. According to the regression statistic which is about the germination of sclerotia of *B. cinerea*, it is observed that R^2 is 0.81. ANOVA significance F value has shown the validity of the model. As this value is below 1% in this study, the result of analysis has a significance of 1%. Following the determination of the importance level, mathematical equation has been obtained by using co-efficients and corresponding independent x variable (SG) and dependent y variables (dose and time).

Mathematical model which was developed by multi regression analysis, for germination of sclerotia of *B. cinerea* has been formed as SG = (a)+(b x T)–[c x (D x T)]+[d x (D^2 x T)] where SG is sclerotial germination of *B. cinerea*, a, b and c are co-efficiencies. T is time of sclerotial germination and D is dose. In this formula a, b, c and d symbolizes the co-efficient obtained as a result of multi regression analysis. By taking into consideration the co-efficient in the regression statistics, sclerotial germination of *B. cinerea* has been formed.

SG = (-0.21589) + (2.395704 x T) – [0.11392 x (D x T)] +[0.00134 x (D^2 x T)]

SE = 0.3439*** 0.117*** 0.008*** $1.55E^{-4}$***

R^2= 0.81

The relation between the SG of *B. cinerea* corresponding to the real values and the approximate SG of *B. cinerea* obtained from mathematical equation is shown in the Fig 5. The other points represent the germination of sclerotia of *B. cinerea* obtained from the model. R^2, also known as the co-efficient of determination is a commonly used statistic to evaluate model fit. When the variability of the residual values around the regression line relative to the overall variability is small, the predictions from the regression equation are good. The closer these values are to reality, the higher R^2 value of the mathematical model. In this study R^2 value shows that a model with 81% close to the reality has been formed.

The effects of doses and times on SG of *B. cinerea* are shown in Fig 6. Mathematical equation has been benefited while showing this change caused by doses and times on the SG of *B. cinerea*. In this graphic mesh part shows the change in the SG throughout *B. cinerea* with times and doses of $KHCO_3$. Fig. 6 shows that the sclerotial germination of *B. cinerea* increases as times raises. In contrast, when the doses increase germination of sclerotia decreases.

Mathematical modelling the epidemiological analyses of some plant pathogens such as pre-and-post-harvest pathogens were determined in the previous studies. This modelling is an efficient means for evaluation how individual or combined environmental factors affect microorganisms that degrade processed foods. Different models have been developed in predictive microbiology for fitting growth curves and estimating biological parameters of food-borne and storage pathogens (Marin *et al.* 1996; Cuppers *et al.* 1997; McMeekin *et al.* 2002; Sautour *et al.* 2002; Lahlali *et al.* 2007). It was reported that mold growth on a solid culture medium at various temperatures and NaCl concentrations by using five common food spoilage molds (*Penicillium roqueforti*, *Trichoderma harzianum*, *Paecilomyces variotii*, *Aspergillus niger*, and *Emericella nidulans*) were modelled (Cuppers *et al*. 1997*)*. In another studies, Lahlali *et al.* (2007) determinated to develop validated models predicting the *in vitro* effect of water activity (a_w) and temperature on the radial growth of *B. cinerea*. The growth rate (g, mm d^{-1}) of *B. cinerea* was calculated at three incubation temperatures and six water activities. Ultimately, all models proved to be good predictors of the growth rates of *B. cinerea* within the limits of experiments. In addition, the results from modelling confirmed the general finding that a_w had a greater influence on fungal growth than temperature. Similarly, Judet-Correia *et al.* (2010) investigated a model for predicting the combined effect of temperature and a_w on the radial growth rate (μ) of *B. cinerea* and *Penicillium expansum* on grape berries. This study demonstrated the usefulness of the gamma concept for validating predictive models in foods or agricultural products. Contrary to the main values, it was shown that the optimum growth rates depended strongly on the strain and the medium. Also in this study, grape analogues were used to validate the combined effects of temperature and water activity on growth, then the optimum growth rate was determined on grape berries once the model validated. This approach allowed validation of the model over a wide range of variation of temperature and water activity, but also the estimation of the optimal growth rate on grape berries under non optimal conditions. Up to date, the studies on mathematical modeling evaluated the effect of times and different doses of $KHCO_3$ on mycelial growth and sclerotial germination of *B. cinerea* by multi regression analysis are not available. In present study, models of estimating the mycelial growth and sclerotial germination of *B. cinerea in vitro* exposure to increased doses of $KHCO_3$ at different times by multi linear regression was developed. As a result of *Anova* and multi-regression analysis, it was found that there was close relationship between actual and predicted mycelial growth and sclerotial germination of *B. cinerea* (Fig 2, Fig 5).

Conclusion

Multi linear regression is an approach to modeling the relationship between dependent variable (Y) and independed variables denoted (X). In our study, dependent variable was mycelial growth and independent variables were time and doses. This study concluded that the model is used as the parameter in mycelial growth and sclerotial germination of *B. cinerea*. The regression co-efficients among the parameter are 0.83 and 0.81, respectively. Using multiple regression equations it is very much likely to predict the variation in mycelial growth and sclerotial germination of *B. cinerea* as related to doses and times with high probability. There are needed to apply models studies for epidemiological analyses of other important fungal pathogens. Additionally, quantifying the effects of environmental factors on fungal disease development by means of quantitative models can help in the design and efficient use of management strategies for fungal plant pathogens such as postharvest pathogens.

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